Coordinate control of virulence gene expression in Francisella tularensis

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Coordinate control of virulence gene expression in *Francisella tularensis*

A dissertation presented

by

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Coordinate control of virulence gene expression in *Francisella tularensis*

Abstract

Francisella tularensis is a Gram-negative, intracellular pathogen and the causative agent of tularemia. Due to its low infectious dose, ability to cause potentially fatal disease, and ability to be easily aerosolized, several countries have developed *F. tularensis* as a potential bioweapon. Three proteins, MglA, SspA, and PigR, and the small molecule guanosine tetraphosphate (ppGpp), are transcription factors critical for the virulence of this organism. These regulators function coordinately to positively regulate the expression of genes present on the *Francisella* pathogenicity island, as well as many other genes that are required for the virulence of this organism. MglA and SspA form a complex that associates with RNA polymerase (RNAP); the interaction between the MglA-SspA complex and RNAP is thought to be critical for MglA and SspA to regulate gene expression. PigR, a putative DNA-binding protein, associates with the RNAP-associated MglA-SspA complex and may stabilize the binding of RNAP at regulated promoters. The interaction between the MglA-SspA complex and PigR in *F. tularensis* has been shown to be promoted by ppGpp.

A direct interaction between the MglA-SspA complex and PigR had previously been found using a modified version of an *E. coli* two-hybrid assay, referred to as the

bridge-hybrid assay, that permits the detection of interactions between a protein of interest and a protein complex. However, the role of this direct interaction in controlling gene expression in *F. tularensis* had not been investigated. Conflicting reports in the literature over the ability of PigR to interact with the MglA-SspA complex led to differing models of how PigR regulates virulence gene expression in *F. tularensis*. To address the importance of the interaction between the MglA-SspA complex and PigR in regulating gene expression, we used a combination of genetic approaches to identify mutants of either MglA or SspA that are specifically defective for interaction with PigR. The identified mutants of MglA and SspA were unable to functionally substitute for MglA or SspA, respectively, and were unable to promote expression of MglA- and SspA- regulated genes in *F. tularensis*. These results indicate that the interaction between the MglA-SspA complex and PigR is critical for expression of virulence genes in *F. tularensis*. Our work also identified a surface on the MglA-SspA complex that is important for the interaction with PigR and which may constitute a binding site for PigR.

The small molecule ppGpp has previously been shown to promote the interaction between the MglA-SspA complex and PigR in *F. tularensis*. It is unknown if ppGpp directly or indirectly promotes this interaction. We determined that ppGpp is required to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay, indicating that ppGpp is either directly involved in promoting this interaction or works through an indirect mechanism that is conserved between *F. tularensis* and *E. coli*. One potential conserved mechanism through which ppGpp may be influencing the interaction between the MglA-SspA complex and PigR is through regulation of the levels of the molecule polyphosphate. However, we determined that

polyphosphate is not required in order for the MglA-SspA complex and PigR to detectably interact with one another in the *E. coli* bridge-hybrid assay. Furthermore, analysis of the role of polyphosphate in gene expression in *F. tularensis* revealed that polyphosphate is a negative regulator of virulence gene expression.

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To my parents,

Greg and Cindy Rohlfing,

for all your love and support

Chapter 1

Introduction

Introduction

Francisella tularensis, the causative agent of tularemia, is a Gram-negative, intracellular pathogen. The bacteria was first isolated in 1911 after an outbreak of a plague-like disease in rodents in Tulare County, Colorado (McCoy and Chapin, 1912). In addition to infecting rodents, *F. tularensis* is able to infect humans and has the ability to cause severe, potentially fatal disease. *F. tularensis* is also one of the most infectious bacterial pathogens known and as few as ten organisms can constitute an infectious dose (Tärnvik and Chu, 2007). Due to the low infectious dose of *F. tularensis*, its ability to be easily aerosolized, and its ability to cause severe disease, several countries have developed *F. tularensis* as a bioweapon. The United States, therefore, considers *F. tularensis* a potential bioterrorist threat and the Centers for Disease Control (CDC) have classified *F. tularensis* as a Category A select agent (Oyston et al., 2004).

Tularemia

F. tularensis is able to infect a person through many different routes. The severity and progression of the disease depends upon how an individual was exposed to the bacteria (Sjöstedt, 2007). *F. tularensis* is known to be carried by rodents, rabbits, hares, and other small mammals and contact with an infected animal carries the risk of infection. Infection can also occur through an arthropod vector, such as ticks and biting flies, or through ingestion of contaminated food or water, although this latter route of infection is generally more rare (Ellis et al., 2002).

The most common form of the disease, ulceroglandular tularemia, occurs from exposure to the bacteria through the skin, most often through a bite by a tick or other vector (Oyston et al., 2004). The onset of symptoms occurs rapidly (3-5 days) after exposure. Symptoms are flu-like and include, fever, chills, headache, malaise, and an ulcer at the site of infection (Tärnvik and Berglund, 2003). Bacteria are disseminated to regional draining lymph nodes and often cause swelling of these lymph nodes that resemble the bubos associated with bubonic plague (Evans, 1985). *F. tularensis* can also disseminate to other tissues, including the spleen, the liver, and the lungs from the lymph nodes. Although the ulceroglandular form of tularemia is rarely fatal, recovery from the disease can require a long period of time (Ellis et al., 2002).

A more severe form of the disease occurs after inhalation of the bacteria. The symptoms of pneumonic tularemia are similar to, but more severe than, those of the ulceroglandular form (without an ulcer) and may or may not include symptoms of respiratory disease (Oyston et al., 2004). Pneumonic tularemia can also result from progression of ulceroglandular tularemia (Ellis et al., 2002). Without antibiotic treatment, mortality rates of pneumonic tularemia are estimated to be around 30% (although some estimates put the mortality rate as high as 60%) (Dennis et al., 2001; Ellis et al., 2002). *F. tularensis* is naturally resistant to β-lactam antibiotics, but treatment with aminoglycosides, generally gentamicin, is able to clear the infection (Tärnvik and Chu, 2007).

Francisella tularensis subspecies

There are four known subspecies of *F. tularensis* that have different geographical distributions and different abilities to cause disease (Ellis et al., 2002). *F. tularensis* subsp. *tularensis* (also called Type A) is the most virulent subspecies and is the only subspecies of *F. tularensis* known to cause fatal respiratory infection (Evans, 1985) (Ellis et al., 2002). This strain has been found exclusively in North America (Johansson et al., 2004; Keim et al., 2007). The sequenced strain of *F. tularensis* subsp. *tularensis* SchuS4 is the strain most commonly used in laboratories to study this particular subspecies (Larsson et al., 2005).

F. tularensis subsp. *holarctica* (also called Type B) is found in North America, Asia, and throughout Europe (Oyston et al., 2004). Subspecies *holarctica* is less virulent than strains of subspecies *tularensis*. Although *F. tularensis* subsp. *holarctica* is able to cause pneumonic tularemia, it is not fatal (Ellis et al., 2002). A live vaccine against *F. tularensis* infection was developed from a strain of *F. tularensis* subsp. *holarctica* in the former USSR (Sjöstedt, 2007). It is not known why this strain is attenuated and does not cause disease in humans, although there are several candidate genes for attenuation that have mutations in the vaccine strain compared to *F. tularensis* subsp. *holarctica* (Rohmer et al., 2006). One of these genes, *pilA*, encodes a putative type IV pilus and has been shown to required for full virulence of the organism (Forslund et al., 2006; 2010). Deletions within the *pilA* locus are found in strains of LVS, but not other *F. tularensis* subsp. *holarctica* strains (Rohmer et al., 2006; Salomonsson et al., 2009). Restoration of pilus expression in LVS partially restores virulence of the organism (Salomonsson et al., 2009). This live vaccine strain (LVS) is used as a model organism in laboratories instead of the more virulent strains of *F. tularensis* (Oyston et al., 2004).

LVS is a useful model organism as it is attenuated for virulence in humans but still causes tularemia-like disease in mice (Eigelsbach and Downs, 1961). All of the studies described in this work were performed using LVS.

      F. tularensis subsp. *novicida* was previously thought to only be found in North America, however, a case of infection by *F. tularensis* subsp. *novicida* was recently found in Australia (Oyston et al., 2004; Whipp et al., 2003). *F. tularensis* subsp. *novicida* is essentially avirulent and rarely causes disease in immunocompetent individuals (Clarridge et al., 1996; Ellis et al., 2002; Hollis et al., 1989). *F. tularensis* subsp. *novicida* had previously been considered a separate species in the genus of *Francisella*, however, it has been reclassified as a subspecies of *F. tularensis* due to high sequence similarity between *F. tularensis* subsp. *novicida* and other subspecies of *F. tularensis* (Santic et al., 2006). For simplicity, *F. tularensis* subsp. *novicida* will be referred to as *F. novicida* throughout this work. Although *F. novicida* is avirulent in humans, it is able to cause tularemia-like disease in mice, making it useful as a model organism (Oyston and Griffiths, 2009). Many of the studies that have lead to a better understanding of *F. tularensis* biology and pathogenesis have been performed using either *F. novicida* or LVS (Ellis et al., 2002).

      The fourth subspecies of *F. tularensis*, subspecies *mediasiatica* is only found in a region of Central Asia and is not well studied (Oyston et al., 2004).

Intracellular lifestyle of *F. tularensis*

      As previously mentioned, *F. tularensis* is an intracellular pathogen and during infection replicates within host cells. Although *F. tularensis* is able to replicate in many

different cells types (including epithelial and hepatic cells) (Ellis et al., 2002), macrophages appear to be an important niche for *F. tularensis* during infection. It is thought that the ability of *F. tularensis* to infect and replicate within macrophages is critical for disease progression and eventual dissemination of the bacteria (Barel and Charbit, 2013; Chong and Celli, 2010; Gray et al., 2002; White et al., 1964). During infection, when *F. tularensis* encounters a macrophage within a host, it induces its own phagocytosis through looping pseudopods and *F. tularensis* becomes contained within a phagosome within the macrophage (Figure 1.1) (Clemens et al., 2005). Normally, bacteria-contining phagosomes merge with endosomes, becomes acidified, and eventually fuse with lysosomes, leading to the destruction of the bacteria within this compartment. *F. tularensis*, however, is able to inhibit fusion of a *F. tularensis*-containing phagosome with a lysosome and escape into the cytosol, potentially by degrading the phagosomal membrane (Oyston, 2008; Pechous et al., 2009; Santic et al., 2010a). Once in the host cell cytosol, *F. tularensis* replicates to high numbers and eventually triggers apoptosis. The death of the host cell releases *F. tularensis*, allowing the bacteria to infect other cells (Belhocine and Monack, 2012; Lai et al., 2001; Lai and Sjöstedt, 2003; Santic et al., 2010b).
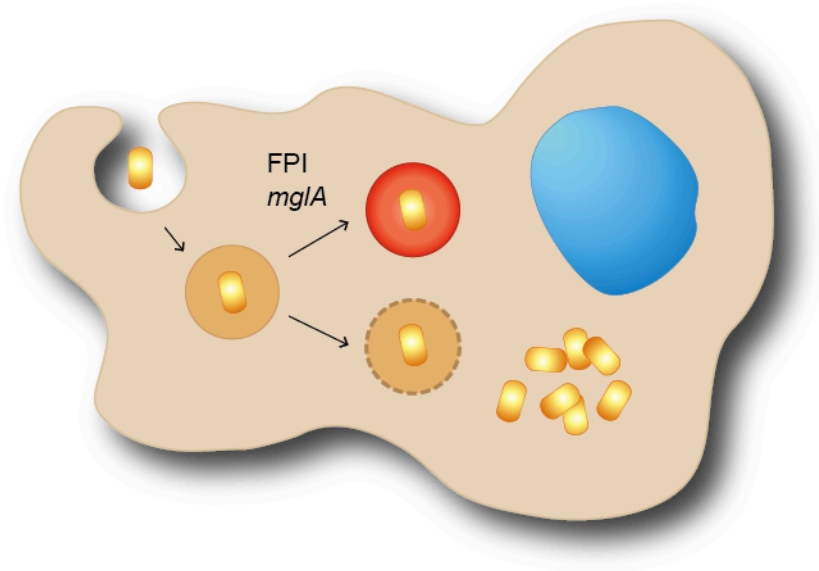
Figure 1.1 Intracellular lifestyle of _F. tularensis_ The diagram depicts the intramacrophage growth of _F. tularensis_. Bacteria are phagocytosed via looping pseudopods and are contained within a phagosome in the macrophage (circle with solid brown line). _F. tularensis_ is able to escape from the phagosome (circle with dashed brown line) to replicate within the cytosol and eventually trigger cell death. Bacteria with defects in the _Francisella_ pathogenicity island (FPI) or the gene _macrophage growth locus A_ (_mglA_) are unable to escape from the phagosome and are degraded (circle with solid red line).
Figure adapted from Pechous, et al., 2009.

Francisella **pathogenicity island**

The genes encoded on the *Francisella* pathogenicity island (FPI) are necessary

for intramacrophage growth and survival (Bröms et al., 2010). The FPI is a 25-30 kb

region of the genome that has been predicted to be composed of two operons (Figure

1.2) (Barker and Klose, 2007). The GC-content of the FPI is lower than the GC-content

than the rest of the genome (27% versus 33%), suggesting that this region may have

been horizontally acquired (Nano and Schmerk, 2007). A core set of 16 FPI genes is

present in all subspecies of *F. tularensis* although there are a few differences in FPI

organization between the subspecies (Bröms et al., 2010). Two extra genes, *pdpD* and

anmK, are present in the FPI in *F. novicida* and *F. tularensis* subsp. *tularensis* (although

the *anmK* genes in sequenced *F. tularensis* subsp. *tularensis* strains contain premature

stop codons) (Ludu et al., 2008). *F. tularensis* subsp. *tularensis* and subsp. *holarctica*

encode two copies of the FPI in the genome, while the less virulent *F. novicida* contains

only one copy of the FPI (Nano et al., 2004). Genes encoded on the FPI were originally

identified in *F. novicida* using a genetic screen to discover genes required for

intramacrophage growth of the organism (Gray et al., 2002). The use of *F. novicida* for

these studies was critical for the identification of the FPI as the FPI is present in single

copy in *F. novicida*, allowing for identification by inactivating mutagenesis (Nano et al.,

2004).

All of the genes present on the FPI, with the exception of *pdpE*, have been

shown to be important for virulence and/or intracellular growth of *F. tularensis* (Bröms et

al., 2010). One of the genes present on the FPI, *iglC*, encodes one of the most highly

upregulated proteins during intracellular growth in macrophages (Golovliov et al., 1997;

Lindgren et al., 2004). Several of the other genes encoded on the FPI show homology to genes encoding a type VI secretion system found in other pathogens, such as *Vibrio cholerae* and *Pseudomonas aeruginosa* (Barker and Klose, 2007; Barker et al., 2009; Broms et al., 2011; Brotcke et al., 2006; Clemens et al., 2015; de Bruin et al., 2011; 2007; Lindgren et al., 2013; Nano et al., 2004; Santic et al., 2007). Several factors encoded on the FPI, IglE, IglC, VgrG, IglI, PdpE, PdpA, IglJ, and IglF, are thought to be secreted by LVS into the macrophage cytosol during infection (Bröms et al., 2012b) and may constitute effectors that directly modify the host environment. Secretion of these potential effectors is dependent upon two conserved, core components of the type VI secretion system, VgrG and DotU, as well as IglG (Bröms et al., 2012b; 2012a). The functions of these potential effectors is still not well understood, however, it is thought that one of the main functions of the type VI secretion system is to aid in escape of *F. tularensis* from the phagosome (Clemens et al., 2015; Lindgren et al., 2004; Santic et al., 2005). Recently, a cryoEM structure of the type VI secretion system from *F. novicida* has been solved (Clemens et al., 2015). Two proteins encoded on the FPI, IglA and IglB, interact to form the sheath-like structure of the secretion system. This structure shows homology to both the contractile tail of bacteriophage T4 and a recent structure of the type VI secretion system sheath in *V. cholerae*. Furthermore, mutational analysis indicated that contraction of this sheath structure is necessary for secretion of VgrG and IglC as well as for phagosomal escape (Clemens et al., 2015).
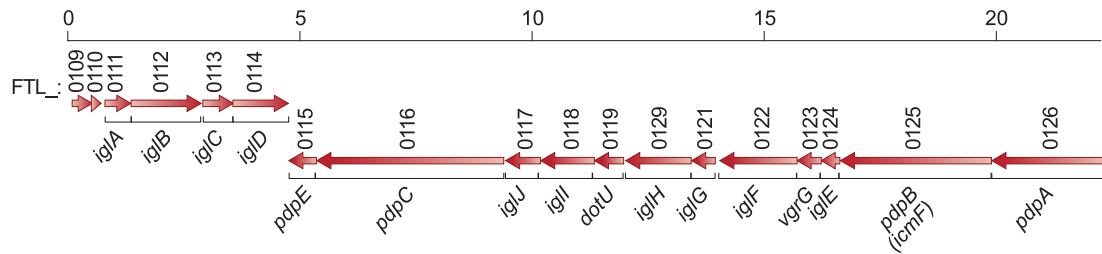
Figure 1.2 _Francisella_ pathogenicity island in LVS The FPI is predicted to be comprised of two operons. The _iglA_ operon, which includes _iglABCD_, and the _pdp_ operon, which includes the genes from _pdpA_ to _pdpE_ (Barker and Klose, 2007). The scale is indicated at the top in kb.

Regulators of virulence gene expression in *F. tularensis*

MglA and SspA

Several positive regulators of FPI gene expression have been indentified. One of these regulators, *mglA* (which stands for <u>m</u>acrophage <u>g</u>rowth <u>l</u>ocus A), was one of the first genes discovered to be required for the intracellular growth of *F. tularensis* (Baron and Nano, 1998). Subsequent work on the role of MglA during intramacrophage growth found that MglA positively regulates expression of several genes present on the FPI (Lauriano et al., 2004). MglA has since been determined to be a global regulator of gene regulation in *F. tularensis*. MglA regulates a set of about 100 genes, including all the gene present on the FPI, other virulence genes, and genes not thought to play a role in virulence (Brotcke et al., 2006; Charity et al., 2007; Guina et al., 2007) Although MglA is mostly a positive regulator of gene expression, there are a handful of genes negatively regulated by MglA (Brotcke et al., 2006; Charity et al., 2007; Guina et al., 2007).

MglA is a member of the stringent starvation protein A (SspA) protein family; MglA in *F. tularensis* and SspA in *E. coli* share 21% identity and 34% similarity at the amino acid level (Baron and Nano, 1998; Charity et al., 2007). SspA is a RNA polymerase (RNAP)-associated protein that was originally identified in *E. coli* as the predominant protein expressed in cells during conditions of amino acid starvation in what is referred to as the stringent response (Ishihama and Saitoh, 1979; Reeh et al., 1976). In *E. coli*, SspA expression is inversely correlated with growth rate and was found to increase during several different starvation conditions, including amino acid, carbohydrate, nitrogen, or phosphate limitation (Williams et al., 1994). SspA is required

for survival during stationary phase and conditions of prolonged nutrient limitation (Hansen et al., 2005b; Williams et al., 1994), most likely due to its ability to positively regulate expression of genes required for acid resistance (Hansen et al., 2005b). The regulation of acid tolerance in *E. coli* is not thought to be due to a direct effect of SspA transcription of genes involved in acid resistance, but rather through an effect on the global regulator H-NS. Through an unknown post-transcriptional mechanism, SspA reduces the overall protein levels of H-NS, leading to increased expression of genes repressed by H-NS, including the genes required for acid resistance (Hansen et al., 2005b). However, SspA, which is required for the lytic development of P1 phage in *E. coli*, has been shown to have a direct effect on transcription of P1 late genes (Hansen et al., 2003; Williams et al., 1991). *E. coli* SspA is required, along with the P1 protein late promoter activator protein (Lpa), for transcription of P1 late genes in an *in vitro* transcription assay and data from elecrophoretic gel mobility shift assays and DNase I footprinting analysis indicate that both P1 and SspA facilitate binding of RNAP to the promoters of P1 late genes (Hansen et al., 2003).

SspA homologs are conserved throughout Gram-negative bacteria and are found in many pathogens (Hansen et al., 2005b; 2005a). SspA homologs in other pathogens, including *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and enterohemorrhagic *E. coli*, have also been shown to be involved in regulating virulence gene expression (Badger and Miller, 1998; De Reuse and Taha, 1997; Hansen and Jin, 2012; Merrell et al., 2002; Yin et al., 2013). The mechanism by which SspA functions in these pathogens is not well understood. In the cases of *V. cholerae*, *N. gonorrhoeae*, and *Y. enterocolitica*, it is only known that SspA regulates

virulence or virulence gene expression (Badger and Miller, 1998; De Reuse and Taha, 1997; Merrell et al., 2002). In enterohemorrhagic *E. coli* (EHEC), SspA was shown to positively regulate the expression of virulence genes located both within and without a pathogenicity island that encodes a Type III secretion system. The upregulation of pathogenicity island genes appears to be dependent upon the ability of SspA to reduce H-NS protein levels (Hansen and Jin, 2012; Hansen et al., 2005b). In *P. aeruginosa*, SspA seems to regulate expression of genes required for the mucoid phenotype, a phenotype associated with virulence of the organism in the lung, by influencing the ability of different sigma factors to associate with RNAP (Yin et al., 2013). More work needs to be done to determine if SspA homologs are indeed working through different mechanisms in these different organisms.

A structure of the SspA homolog from *Yersinia pestis* has provided some additional insights as to how SspA homologs may be functioning (Hansen et al., 2005a). SspA was found to have a protein fold characteristic of Glutathione S-Transferase (GST) enzymes, although SspA has no measurable GST activity and does not seem to bind glutathione. *Y. pestis* SspA crystallized as a homodimer and mutational analysis indicated that *E. coli* SspA most likely functions as a homodimer (Hansen et al., 2005a). The structure of *Y. pestis* SspA, along with amino acid sequence alignments of SspA orthologs from other bacteria, revealed a conserved, surface-exposed region on the SspA homodimer. Residues in this region were found to be critical for the ability of *E. coli* SspA to promote acid tolerance and to support transcription of P1 phage late genes. It was proposed that this surface constitutes the binding site for RNAP on the SspA

homodimer, although whether or not these residues contribute RNAP binding was not explicitly tested (Hansen et al., 2005a).

F. tularensis actually encodes two homologs of SspA, MglA and another that is called SspA. It is believed that MglA and SspA function as a heteromeric complex in *F. tularensis* (Charity et al., 2007). MglA and SspA regulate the expression of the same set of genes, including the genes present on the FPI, and both MglA and SspA associate with RNAP in *F. tularensis* (Charity et al., 2007). As with *E. coli* SspA, it is believed that the ability of MglA and SspA to associate with RNAP is critical for the function of these two proteins (Charity et al., 2007; Hansen et al., 2005a). However, MglA was no longer able to associate with RNAP in a *ΔsspA* strain of LVS, indicating that MglA requires SspA to associate with RNAP. MglA and SspA were also shown to directly interact with one another in a bacterial two-hybrid assay and in a biochemical assay using purified proteins (Charity et al., 2007). Taken together, these data suggest that rather than functioning as a homomeric complex, as in *E. coli*, MglA and SspA likely form a heteromeric compex (likely a heterodimer) that associates with RNAP in *F. tularensis* (Charity et al., 2007).

PigR

Another key regulator of virulence gene expression in *F. tularensis* was identified in genetic screens for additional positive regulators of MglA- and SspA-regulated genes in *F. novicida* and LVS (Brotcke and Monack, 2008; Charity et al., 2009). In *F. novicida*, a protein which was named <u>F</u>rancisella <u>e</u>ffector of <u>v</u>irulence <u>r</u>egulation (or FevR) was identified in a screen for positive regulators of *pepO*, one of the most differentially

expressed genes between wild-type and *ΔmglA* strains of *F. novicida* (Brotcke and Monack, 2008). A screen in LVS for positive regulators of the gene *iglA*, which is present on the pathogenicity island, identified the LVS homolog of FevR; this protein was named PigR for p̲athogenicity i̲sland gene r̲egulator (Charity et al., 2009). PigR/FevR, similar to MglA, was found to be necessary for intramacrophage growth and virulence of LVS and *F. novicida,* respectively, in a mouse model of infection (Brotcke and Monack, 2008; Charity et al., 2009) Global analysis of the role of PigR/FevR in regulating gene expression in LVS and *F. novicida* found that PigR/FevR regulates the same set of genes as MglA and that PigR does not affect the expression of MglA or SspA (Brotcke and Monack, 2008; Charity et al., 2009). To determine where PigR/FevR functions in the regulatory hierarchy compared to the MglA-SspA complex, the ability of PigR/FevR to complement a *ΔmglA* strain of either LVS or *F. novicida* and restore expression of a MglA- and SspA-regulated gene was tested. PigR/FevR was unable to complement a *ΔmglA* strain, indicating that PigR/FevR functions in parallel with the MglA-SspA complex to regulate gene expression (Brotcke and Monack, 2008; Charity et al., 2009). For the remainder of this work, we will refer to this protein as PigR, unless specifically referring to the function of the protein in *F. novicida*.

PigR has limited homology to members of the MerR family of transcription regulators (Brotcke and Monack, 2008; Charity et al., 2009). MerR family members are mostly positive regulators of gene expression that respond to metal ions and antibiotics in the environment. Each regulator is specific for one co-factor (Brown et al., 2003). For example, the archetype of this family, MerR, responds to mercury ions and regulates expression of genes responsible for mercury tolerance. MerR binds directly to the

promoter of regulated genes in both the presence and absence of mercury. Upon binding of mercury to MerR, a protein conformation shift occurs which leads to activation of MerR regulated genes (Summers, 2009). These MerR family regulators are distinguished by a similar N-terminal helix-turn-helix DNA-binding domain and a C-terminal effector-binding domain. The C-terminal effector-binding domains are variable and specific to the effector the family member responds to (Brown et al., 2003; Hobman, 2007). A region of PigR shows homology to the DNA-binding helix-turn-helix region of MerR family members, indicating it may be able to directly bind DNA, however PigR appears to lack an effector binding domain (Brotcke and Monack, 2008; Charity et al., 2009).

To better understand how PigR regulates virulence gene expression in concert with the MglA-SspA complex, the ability of PigR to interact with the MglA-SspA complex was tested. PigR co-purified with the MglA-SspA complex and RNAP in *F. tularensis*, but only after formaldehyde was used to cross-link proteins together (Charity et al., 2009). To determine if the interaction between PigR and the MglA-SspA complex is direct, a bacterial two-hybrid assay was modified to allow for detection of an interaction between a protein of interest and a protein complex. Using this assay, referred to as the bacterial bridge-hybrid assay, it was determined that PigR is able to directly interact with the MglA-SspA complex (Charity et al., 2009). However, the physiological relevance of this direct interaction was not tested.

Conflicting reports in the literature on the ability of the MglA-SspA complex to interact with PigR have led to confusing models for how virulence gene expression is regulated in *F. tularensis*. In the study that originally identified FevR in *F. novicida*, the

authors attempted to co-immunoprecipitate epitope-tagged versions of MglA, SspA, and FevR from *F. novicida*. While MglA and SspA were found to co-precipitate with one another (as previously reported (Charity et al., 2007)), FevR was unable to co-precipitate with MglA or SspA, leading the authors to speculate that FevR most likely does not function through an interaction with the MglA-SspA complex (Brotcke and Monack, 2008).

Furthermore, another study in *F. novicida* suggested that an orphan response regulator, PmrA, is working in concert with the MglA-SspA complex to positively regulate expression of genes present on the FPI (Bell et al., 2010). PmrA has been found to be important for intramacrophage growth of *F. tularensis* and virulence of the organism in mice and to regulate a subset of MglA-, SspA-, and PigR-regulated genes, including *pigR* itself (Mohapatra et al., 2007; Sammons-Jackson et al., 2008). Subsequent work found that PmrA is able to bind to its own promoter as well as the promoter of the FPI gene *pdpD*, which is present in *F. tularensis* subsp. *tularensis* and *F. novicida*, but not *F. tularensis* subsp. *holarctica* (Bell et al., 2010). PmrA was also able to co-precipitate with MglA and SspA from *F. novicida* cell lysates (Bell et al., 2010). Due to the ability of PmrA to bind DNA and to interact with the MglA-SspA complex in cell lysates, the authors proposed a model for virulence gene expression in which DNA-bound PmrA interacts with the MglA-SspA complex to recruit RNAP to regulated promoters (Bell et al., 2010).

The work presented in Chapter 2 of this dissertation aims to better understand how the MglA-SspA complex and PigR work in concert to regulate a common set of

genes by determining the physiological role of the interaction between the MglA-SspA complex and PigR in *F. tularensis*.

ppGpp

The small molecule guanosine 5'-diphosphate-3'-disphosphate (also referred to as guanosine tetraphosphate or ppGpp) is another positive regulator of the genes present on the FPI and other virulence genes in *F. tularensis* (Charity et al., 2009). ppGpp and its precursor guanosine 5'-triphoshpate-3'-diphoshpate (guanosine pentaphosphate or pppGpp) are generated in bacterial cells in response to amino acid starvation (Cashel and Gallant, 1969; Cashel and Kalbacher, 1970). The physiological effects of ppGpp and pppGpp (collectively referred to as (p)ppGpp) are numerous but include decreased transcription from rRNA promoters, increased expression of amino acid biosynthesis genes, inhibition of protein synthesis, inhibition of DNA replication, and slowed growth (Potrykus and Cashel, 2008). This overall cellular response to amino acid starvation, particularly the inhibition of rRNA transcription, is referred to as the stringent response (Potrykus and Cashel, 2008). The role of (p)ppGpp as a signal of nutrient limitation has led to it being referred to as an alarmone (Dalebroux et al., 2010a).

Cashel and Gallant first visualized ppGpp and pppGpp as two "magic spots" that appeared during thin-layer chromatography of radiolabeled nucleotides from amino-acid starved *E. coli* cells (Cashel and Gallant, 1969). The addition of a pyrophosphate group from ATP to the ribose 3' carbon to either GDP or GTP generates ppGpp or pppGpp, respectively (Srivatsan and Wang, 2008). The two enzymes that are responsible for the

synthesis of (p)ppGpp in *E. coli* are RelA and SpoT. RelA is a monofunctional enzyme and is only able to synthesize (p)ppGpp, while SpoT is a bifunctional enzyme that is both able to synthesize and degrade (p)ppGpp (Potrykus and Cashel, 2008). The ability of SpoT to degrade (p)ppGpp is required for cell viability, presumably because it prevents uncontrolled synthesis of (p)ppGpp (Dalebroux et al., 2010a). Some bacterial species, including most gammaproteobacteria, encode both RelA and SpoT homologs, while other bacteria encode one or more bifunctional enzymes referred to as Rel Spo homologs (RSHs) (Dalebroux et al., 2010a; Potrykus and Cashel, 2008). *F. tularensis* encodes both RelA and SpoT and these two proteins are thought to be the only (p)ppGpp synthethase enzymes in *F. tularensis*. Both of these enzymes are thought to contribute to (p)ppGpp synthesis in *F. tularensis,* although RelA appears to be the major (p)ppGpp synthethase enzyme in the conditions tested (Charity et al., 2009; Dean et al., 2009).

In addition to differing from one another in the ability to degrade (p)ppGpp, RelA and SpoT also differ in the signals they respond to. In *E. coli,* it is known that RelA synthesizes (p)ppGpp in response to amino-acid starvation (Potrykus and Cashel, 2008). During conditions of amino acid starvation, uncharged tRNAs accumulate within a cell. When an uncharged tRNA enters the A site of a translating ribosome, the ribosome becomes stalled and is unable to continue translation until the uncharged tRNA is removed. RelA binds directly to translating ribosomes and generates (p)ppGpp in response to stalling of a ribosome (Haseltine and Block, 1973; Haseltine et al., 1972). SpoT responds to a variety of stimuli, including conditions of carbon, phosphate, iron, and fatty acid limitation that switch the balance of synthesis and hydrolysis activities

toward synthesis. The ability of SpoT to respond to the state of fatty acid metabolism in the cell is most well understood (Potrykus and Cashel, 2008). SpoT from *E. coli* and *P. aeruginosa* has been found to directly interact with Acyl carrier protein (ACP), a highly conserved protein involved in carrying acyl intermediates during fatty acid synthesis (Battesti and Bouveret, 2006; 2009). A conserved region in the N-terminal region of SpoT, the region responsible for regulation of RSH protein activity, is involved in binding ACP. Mutations in *E. coli* SpoT which influenced the ability of SpoT to interact with ACP led to misregulation of (p)ppGpp levels (Battesti and Bouveret, 2006). Thus, it appears that SpoT is able to sense the state of fatty acid metabolism through an interaction with ACP and switch between the opposing enzymatic functions of degradation and synthesis (Battesti and Bouveret, 2006; 2009). The ability of SpoT to bind ACP and respond to fatty acid starvation appears to be specific to SpoT homologs in bacteria that encode both RelA and SpoT homologs; RelA homologs and other RSH proteins have not been found to respond to fatty acid starvation (Battesti and Bouveret, 2009).

(p)ppGpp is able to both directly and indirectly control transcription during the stringent response. Genes for stable RNAs (tRNAs and rRNAs) are directly repressed by (p)ppGpp while genes involved in amino-acid biosynthesis are directly activated by (p)ppGpp (Potrykus and Cashel, 2008). The strong inhibition of transcription of rRNA genes is the hallmark of the stringent response (Potrykus and Cashel, 2008). The ability of (p)ppGpp to directly control the transcription of a gene, either positively or negatively, depends on the kinetic properties of the promoters involved. Promoters directly repressed by (p)ppGpp tend to be GC-rich in the region between the promoter -10 element and the transcription start site, while promoters directly activated by (p)ppGpp

tend to be AT-rich in this region (Srivatsan and Wang, 2008). In fast growing cells, rRNA is synthesized from strong promoters and employs the majority of RNAP in the cell. Upon induction of (p)ppGpp during the stringent response, transcription from promoters for rRNA genes is rapidly inhibited (Jin et al., 2012). (p)ppGpp affects transcription by decreasing the half-life of promoter open-complexes (Barker et al., 2001). RNAP forms intrinsically unstable open complexes at rRNA promoters; addition of (p)ppGpp further destabilizes the open complex at rRNA promoters, leading to a decrease in transcription initiation (Barker et al., 2001; Gourse, 1988). Transcription is increased from promoters, such as promoters for amino acid biosynthetic genes, that form intrinsically stable open complexes with RNAP. In the case of these promoters, (p)ppGpp promotes transcription initiation (Srivatsan and Wang, 2008).

(p)ppGpp is able to interact directly with RNAP in *E. coli* and several attempts had been made to identify the binding site of (p)ppGpp on RNAP (Artsimovitch et al., 2004; Chatterji et al., 1998; Toulokhonov et al., 2001). Recently, a binding site at the interaface of the ω and β' subunits on *E. coli* RNAP was mapped using a cross-linkable version of (p)ppGpp and protease mapping (Ross et al., 2013). Mutational analysis indicated that binding of (p)ppGpp to this site is necessary for the ability of (p)ppGpp to inhibit transcription from rRNA promoters. This was the first study that was able to show that direct binding of (p)ppGpp to RNAP is required for (p)ppGpp to exert its effects on transcription (Ross et al., 2013).

The effects of (p)ppGpp on transcription in many bacteria are potentiated by the small protein DksA. DksA is required for (p)ppGpp to both directly repress and directly activate transcription in *E. coli* (Paul et al., 2004; 2005). Although both (p)ppGpp and

DksA on their own are able to repress transcription of *rrn* promoters in *in vitro*

transcription assays, the full effects of inhibition are only seen when both (p)ppGpp and

DksA are included in the *in vitro* transcription assays (Paul et al., 2004). The

mechanism by which DksA modulates the effects of (p)ppGpp is not well understood.

However, it is known that in *E. coli*, DksA binds to RNAP near the entry point of

nucleotide triphosphates into RNAP, called the secondary channel, during transcription

(Haugen et al., 2008; Potrykus and Cashel, 2008) .

   The available evidence suggests that (p)ppGpp is likely to be important for

virulence in many diverse pathogens, including Enterohaemorrhagic *E. coli* (EHEC),

Pseudomonas aeruginosa, *Legionella pneumophila*, *Vibrio cholerae*, *Mycobacterium*

tuberculosis, *Salmonella enterica,* and *Enterococcus faecalis* (Erickson et al., 2004;

Gaca et al., 2012; Hammer and Swanson, 1999; Haralalka et al., 2003; Nakanishi et al.,

2006; Nowicki et al., 2013; 2014; Pizarro-Cerdá and Tedin, 2004; Primm et al., 2000;

Thompson et al., 2006).

The mechanism by which (p)ppGpp influences virulence of these organisms is often not

well understood. It appears that (p)ppGpp may function through a variety of pathways in

these organisms to regulate virulence and gene expression (reviewed in (Dalebroux et

al., 2010a)). In EHEC, for example, (p)ppGpp, together with DksA, directly activates the

expression of genes encoded on a pathogenicity island required for virulence

(Nakanishi et al., 2006). However, the role of (p)ppGpp in EHEC virulence appears to

be more complicated, as (p)ppGpp also inhibits induction of prophages within the EHEC

genome that encode the virulence factor Shiga toxin (Nowicki et al., 2013; 2014). Thus

pathogenicity island and phage encoded virulence factors are differentially regulated by

(p)ppGpp in EHEC and (p)ppGpp production may need to be tightly regulated for virulence of the organism (Nakanishi et al., 2006; Nowicki et al., 2013; 2014).

The intracellular pathogens, *Legionella pneumophila* and *Salmonella enterica*, which both replicate within macrophages during infection, also both require (p)ppGpp for virulence (Dalebroux et al., 2010b). *Legionella pneumophila* senses perturbations in fatty acid metabolism through SpoT to increase (p)ppGpp production and increase expression of a set of genes which facilitate the switch of the bacteria from a replicative state to a transmissive state (Edwards et al., 2009). *Salmonella enterica* uses (p)ppGpp to control two separate virulence systems required for survival of *S. enterica* during different points of infection (Dalebroux et al., 2010b). During early infection, (p)ppGpp promotes the expression of factors required for expression of genes encoded on the *Salmonella* pathogenicity island 1 (SPI-1) which are required to induce uptake of the bacteria within epithelial cells of the small intestine (Dalebroux et al., 2010a; Pizarro-Cerdá and Tedin, 2004; Song et al., 2004). (p)ppGpp is also required for virulence gene expression at later times during *S. enterica* infection to activate the expression of genes on the *Salmonella* pathogenicity island 2 (SPI-2). The regulator SlyA is expressed after phagocytosis of *S. enterica* by a macrophage. (p)ppGpp promotes the dimerization of SlyA which allows SlyA to bind DNA and activate transcription of genes present on SPI-2 (Dalebroux et al., 2010b; Zhao et al., 2008).

(p)ppGpp is also required for the virulence of *F. tularensis*. A *ΔrelA ΔspoT* mutant strain of LVS is defective for intramacrophage growth, similar to *ΔmglA* and *ΔsspA* mutant strains of LVS, and is avirulent in a mouse model of infection (Charity et al., 2009). A *ΔrelA* mutant strain of LVS, however, is able to replicate in macrophages

nearly as well as wild-type LVS and is virulent in mice, indicating that SpoT can produce

sufficient (p)ppGpp for full virulence of the organism (Charity et al., 2009). It was

determined that (p)ppGpp is a positive regulator of virulence gene expression in *F.*

tularensis. In fact, (p)ppGpp regulates expression of essentially the same set of genes

as MglA, SspA, and PigR. And while (p)ppGpp does control expression of *pigR*, this

does not fully explain how (p)ppGpp regulates gene expression as ectopic expression of

pigR in a *ΔrelA ΔspoT* mutant strain of LVS only partially restores the expression of

virulence genes (Charity et al., 2009). It was suggested that (p)ppGpp may be

regulating virulence gene expression in *F. tularensis* through modulation of the

interaction between PigR and the MglA-SspA complex. In a wild-type strain of LVS,

PigR co-purifies with the MglA-SspA complex and RNAP after cross-linking of proteins

with formaldehyde. However, PigR can no longer co-purify with the MglA-SspA complex

after cross-linking from a *ΔrelA ΔspoT* mutant strain of LVS. Thus, (p)ppGpp may

regulate virulence gene expression in *F. tularensis* by promoting the interaction between

the MglA-SspA complex and PigR (Charity et al., 2009).

Model for coordinate control of virulence gene expression in *F. tularensis*

The previous work described in this chapter about MglA, SspA, PigR, and

(p)ppGpp has led to our current model for how these regulators coordinately control the

expression of certain virulence genes in *F. tularensis*, including all of those on the FPI

(Figure 1.3). In this model, the putative DNA-binding protein PigR interacts directly with

the RNAP-associated MglA-SspA complex. This interaction between the MglA-SspA

complex and PigR is promoted by (p)ppGpp. The contacts between the DNA and

RNAP, made by PigR and the MglA-SspA complex, respectively, would stabilize the

binding of RNAP to promoters and lead to an increase in transcription from regulated

promoters.
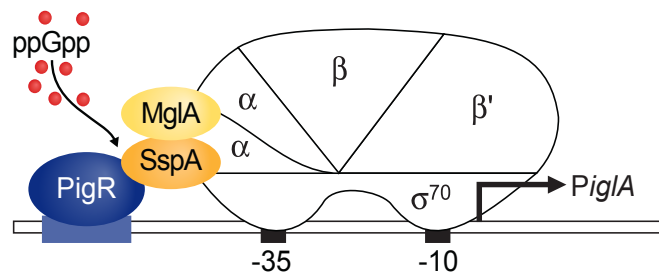
Figure 1.3 Model for coordinate control of virulence gene expression in *F. tularensis* by MglA, SspA, PigR, and (p)ppGpp In this model, the RNAP-associated MglA-SspA complex directly interacts with DNA-bound PigR to positively regulate expression of genes on the FPI. ppGpp promotes, either directly or indirectly, the interaction between the MglA-SspA complex and PigR. Figure adapted from Charity et al., 2009.

A potential role for polyphosphate in regulating virulence gene expression in *F. tularensis*

In addition to its role in regulating gene expression, (p)ppGpp regulates the production of polyphosphate, another molecule involved in the stringent response. The accumulation of (p)ppGpp in a cell also leads to the accumulation of polyphosphate (Rao et al., 2009). Therefore, it is possible that some effects of (p)ppGpp on cellular physiology may actually be due to polyphosphate. In Chapter 3 of this work, we investigate whether the effects of (p)ppGpp on gene expression in *F. tularensis* might be mediated by polyphosphate.

Polyphosphate is a chain of inorganic phosphate molecules linked by high-energy phosphoanhydride bonds. Molecules of polyphosphate are of variable length and may contain tens to hundreds of phosphate molecules in a single chain (Kornberg et al., 1999). It has been hypothesized that polyphosphate was present in the prebiotic world. Organisms in all domains of life have been found to contain polyphosphate, making it a widely conserved signaling molecule (Brown and Kornberg, 2004).

Polyphosphate is synthesized by the enzyme polyphosphate kinase (PPK) (Brown and Kornberg, 2008). There are two conserved classes of PPK enzymes, referred to as PPK1 and PPK2. Some bacteria encode either a PPK1 homolog or a PPK2 homolog while others encode both a PPK1 homolog and a PPK2 homolog (Rao et al., 2009). PPK1 enzymes utilize ATP to add phosphates to polyphosphate chains while PPK2 enzymes utilize either GTP or ATP to synthesize polyphosphate. PPK2 enzymes can also catalyze the reverse reaction and generate GTP from GDP and polyphosphate (Brown and Kornberg, 2008). The PPK2 homolog from *P. aeruginosa*

(an organism that encodes both a PPK1 and a PPK2), was shown to favor catalysis of the reverse reaction *in vitro* to synthesize GTP from GDP and polyphosphate (Zhang et al., 2002). However, a PPK2 homolog from *Corynebacterium glutamicum*, which encodes two PPK2 homologs, was found to favor polyphosphate synthesis over degradation (Rao et al., 2009). Thus, it appears the function of PPK2 may differ in bacteria that contain PPK1 and PPK2 homologs versus bacteria that contain only PPK2 homologs.

Polyphosphate is degraded by several enzymes, including polyphosphate-AMP-phosphotransferase, exo-polyphosphatase, and endo-polyphosphatase. Endo-polyphosphatase enzymes are found in eukaryotes and Polyphosphate-AMP-phosphotransferase enzymes have only been identified in a handful of bacteria (Brown and Kornberg, 2008). Exo-polyphosphatase (PPX) enzymes are found in bacteria and degrade polyphosphate by releasing the terminal inorganic phosphate molecule from the polyphosphate chain. The activity of PPXs is directly inhibited by (p)ppGpp during the stringent response (Rao et al., 2009).

In *E. coli,* inhibition of PPX during the stringent response by (p)ppGpp leads to an accumulation of polyphosphate (Kornberg et al., 1999). One of the roles of polyphosphate during the stringent response appears to be activation of Lon protease. During the stringent response, activation of Lon protease leads to degradation of ribosomal proteins, freeing up amino acids which can be used to synthesize amino acid biosynthetic enzymes and other required proteins (Rao et al., 2009). Polyphosphate is able to bind directly to Lon protease *in vitro* and activate degradation of ribosomal proteins (Kuroda et al., 2001). Polyphosphate has also been shown to be involved in

survival in several other stress conditions, including oxidative stress, heat shock, and desiccation (Brown and Kornberg, 2008). Polyphosphate induces expression of *rpoS*, the sigma factor responsible for expression of genes involved in survival during stationary phase, in *E. coli*. The induction of *rpoS* by polyphosphate may contribute to the influence of polyphosphate on survival during conditions of stress (Rao et al., 2009). In *Helicobacter pylori*, polyphosphate binds to the principal housekeeping sigma factor during the starvation response, suggesting that polyphosphate may be able to directly control transcription in response to conditions of starvation (Yang et al., 2010). Polyphosphate has also recently been shown to bind to proteins and function as a protein chaperone, reducing protein damage during conditions of oxidative stress (Gray et al., 2014). Thus, a major role of polyphosphate during the stress response may be its ability to function as a chaperone.

Polyphosphate has also been shown to positively influence the production of virulence factors in a few pathogens. Motility and surface attachment, two key virulence factors of *V. cholerae*, are decreased in a *Δppk* mutant strain of the organism (Ogawa et al., 2000). In *P. aeruginosa*, polyphosphate influences biofilm formation and twitching, swimming, and swarming motility, although cells of a *Δppk* mutant are still flagellated (Rashid and Kornberg, 2000; Rashid et al., 2000a; 2000b). Interestingly, PPX is also required for biofilm formation and swimming motility of *P. aeruginosa*, suggesting that polyphosphate production must be properly regulated for virulence of the organism (Gallarato et al., 2014). Polyphosphate has also been shown to influence virulence of *M. tuberculosis*. *M. tuberculosis* encodes both a PPK1 and PPK2 homolog and both homologs have been implicated in virulence (Chuang et al., 2013; Singh et al., 2013;

Sureka et al., 2007). PPK1 in *M. tuberculosis* is responsible for polyphosphate

production and loss of polyphosphate in a *Δppk1* mutant strain increases antibiotic

susceptibility and decreases virulence in a guinea pig model of infection (Singh et al.,

2013). Polyphosphate may influence gene expression in *M. tuberculosis* through

regulation of alternative sigma factors, however there conflicting results in the literature

about which sigma factors are influenced by polyphosphate (Sanyal et al., 2013; Singh

et al., 2013; Sureka et al., 2007). PPK1 appears to be the major polyphosphate kinase

for *M. tuberculosis* (Singh et al., 2013), while the PPK2 homolog seems to function as a

polyphosphatase, generating GTP from polyphosphate (Chuang et al., 2013).

     F. tularensis encodes a functional PPK2 homolog that has been implicated in

virulence (Richards et al., 2008). *ppk* was identified in *F. novicida* with a screen

designed to detect genes expressed during intramacrophage growth of the organism

(Richards et al., 2008). *Δppk* mutant strains of both *F. novicida* and *F. tularensis* subsp.

tularensis were found to be avirulent in a mouse model of infection, suggesting that

polyphosphate is required for virulence of the organism. However, polyphosphate was

found to have little to no effect on intramacrophage replication of *F. novicida* and only a

modest effect on intramacrophage replication of *F. tularensis* subsp. *tularensis*,

indicating that polyphosphate is necessary for full virulence of the organism, but has

only a modest effect on intramacrophage growth (Richards et al., 2008). The effects of

polyphosphate on gene expression in either *F. novicida* or *F. tularensis* subsp.

tularensis were not determined.

Summary

Francisella tularensis is an intracellular pathogen capable of causing severe disease. In order to survive within the hostile intracellular environment, the bacteria must be able to sense and respond to changes in environment. Virulence gene expression in *F. tularensis* is regulated by two homologs of the stringent starvation protein A, MglA and SspA, a putative DNA-binding protein, PigR, and the small molecule, (p)ppGpp. MglA and SspA form a complex that associates with RNAP in *F. tularensis*. The MglA-SspA complex also associates with a putative DNA-binding protein, PigR, and this interaction between PigR and the MglA-SspA complex is promoted by ppGpp. The ability of (p)ppGpp to modulate the interaction between the MglA-SspA complex and PigR integrates nutritional cues into the regulatory network of virulence gene expression in *F. tularensis*.

In this work, we investigate the physiological relevance of the interaction between the MglA-SspA complex and PigR and whether ppGpp promotes the interaction between the MglA-SspA complex and PigR indirectly through an effect on polyphosphate levels. We identify several mutants of both MglA and SspA that are specifically defective for interaction with PigR. Analysis of these mutants in *F. tularensis* indicates that the interaction between the MglA-SspA complex and PigR is necessary for virulence gene expression. We also found that ppGpp is also required to detect an interaction between the MglA-SspA complex and PigR in a modified version of an *E. coli* two-hybrid assay that permits the detection of an interaction between a protein and a protein complex (a so-called bridge-hybrid assay). These results indicate that ppGpp either directly modulates the interaction between the MglA-SspA complex and PigR or

that ppGpp indirectly modulates this interaction through a mechanism that is conserved between *E. coli* and *F. tularensis*. We investigated whether ppGpp may be indirectly regulating gene expression through the regulation of polyphosphate levels. However, polyphosphate is not required to detect an interaction between the MglA-SspA complex and PigR in the bridge-hybrid assay. Furthermore, polyphosphate appears to be a negative regulator of MglA-, SspA-, and PigR-regulated genes in *F. tularensis*. Overall, the work presented here increases our understanding of how MglA, SspA, PigR, and ppGpp coordinately control virulence gene expression in *F. tularensis*.

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Chapter 2

Coordinate control of virulence gene expression in *Francisella*

***tularensis* involves direct interaction between key regulators**

Author contributions: All of the work in this chapter was performed by Amy Rohlfing, with the following exception. James Charity generated a strain of *F. tularensis* LVS with an in frame deletion of *mglA* which expresses a tagged version of a RNA polymerase subunit

Coordinate control of virulence gene expression in *Francisella tularensis* involves

direct interaction between key regulators

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Running Title: Interaction of key virulence regulators in *Francisella*

Abstract

In *Francisella tularensis* the putative DNA-binding protein PigR works in concert with the SspA protein family members MglA and SspA to control the expression of genes that are essential for the intramacrophage growth and survival of the organism. MglA and SspA form a complex that interacts with RNA polymerase (RNAP) and this interaction between the MglA-SspA complex and RNAP is thought to be critical to its regulatory function. How PigR works in concert with the MglA-SspA complex is not known; previously published findings differ over whether PigR interacts with the MglA-SspA complex leading to disparate models for how PigR and the MglA-SspA complex exert their regulatory effects. Here, using a combination of genetic assays, we identify mutants of MglA and SspA that are specifically defective for interaction with PigR. Analysis of the MglA and SspA mutants in *F. tularensis* reveals that interaction between PigR and the MglA-SspA complex is essential in order for PigR to work coordinately with MglA and SspA to positively regulate the expression of virulence genes. Our findings uncover a surface of the MglA-SspA complex that is important for interaction with PigR and support the idea that PigR exerts its regulatory effects through an interaction with the RNAP-associated MglA-SspA complex.

Introduction

Francisella tularensis, the causative agent of tularemia, is a Gram-negative, intracellular pathogen. Although outbreaks of *F. tularensis* are relatively rare, it is one of the most highly infectious bacterial pathogens known, with as few as ten organisms constituting an infectious dose (Tärnvik and Chu, 2007) Due to its highly infectious nature, as well as its ability to be easily aerosolized, several countries have developed it as a bioweapon and the CDC have categorized *F. tularensis* as a category A select agent. This has led to a renewed research interest in *F. tularensis* over the past decade and an effort to better understand *F. tularensis* pathogenesis (Oyston et al., 2004).

During infection, *F. tularensis* primarily infects and replicates within macrophages. This ability to replicate within macrophages is thought to be essential for virulence (Barel and Charbit, 2013; Chong and Celli, 2010; Gray et al., 2002; Sjöstedt, 2007). One of the first genes that was found to be necessary for this process is *mglA* (macrophage growth locus A) (Baron and Nano, 1998). MglA regulates the expression of many virulence genes, as well as many genes not known to play a role in virulence. Among the genes that are positively regulated by MglA are the genes encoded on the *Francisella* pathogenicity island (FPI) (Brotcke et al., 2006; Charity et al., 2007; Lauriano et al., 2004). The genes on the FPI are necessary for growth in macrophages and appear to encode a secretion system related to the type VI secretion system (Barker et al., 2009; de Bruin et al., 2007; Nano et al., 2004).

MglA is an ortholog of stringent starvation protein A (SspA), a RNA polymerase (RNAP) associated protein from *Escherichia coli* that is thought to play a role in gene regulation during starvation conditions, although the mechanism by which SspA

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influences gene expression is unclear (Baron and Nano, 1998; Ishihama and Saitoh, 1979; Williams et al., 1994). SspA orthologs in several other pathogens have also been shown to be important for virulence (Badger and Miller, 1998; De Reuse and Taha, 1997; Hansen and Jin, 2012; Merrell et al., 2002; Yin et al., 2013). *F. tularensis* encodes two orthologs of SspA, one called MglA and another that is called SspA. MglA and SspA form a heteromeric complex that associates with RNAP in *F. tularensis* (Charity et al., 2007). It is thought that interaction with RNAP is necessary for the function of both MglA and SspA (Charity et al., 2007).

Another key regulator of virulence gene expression in *F. tularensis*, PigR (pathogenicity island gene regulator), appears to function coordinately with the MglA-SspA complex. PigR is a putative DNA-binding protein that was identified in the live vaccine strain (LVS) of *F. tularensis* through a genetic screen for positive regulators of the MglA- and SspA-controlled *iglA* gene present on the FPI (Charity et al., 2009). PigR is identical to FevR from *Francisella novicida*, which was isolated in a genetic screen for positive regulators of the MglA and SspA-controlled *pepO* promoter (Brotcke and Monack, 2008). PigR (FevR) regulates expression of the same set of genes as the MglA-SspA complex, suggesting that PigR, MglA, and SspA function together to regulate gene expression (Brotcke and Monack, 2008; Charity et al., 2009). A direct interaction between the MglA-SspA complex and PigR was detected using a modified version of a bacterial two-hybrid assay, however the physiological relevance of this interaction was not tested (Charity et al., 2009). Furthermore, an independent study in *F. novicida* did not find evidence for an interaction between the MglA-SspA complex and FevR (Brotcke and Monack, 2008). It was therefore unclear whether PigR (FevR)

functions in concert with the MglA-SspA complex through direct protein-protein interaction.

Here we identify amino acid residues within MglA and SspA that are critical for interaction of the MglA-SspA complex with PigR. These residues are within a putative pocket, formed close to the predicted interface between MglA and SspA, which might constitute a binding site for PigR. Furthermore, using mutants of MglA and SspA that are specifically defective for interaction with PigR, we present evidence that the interaction between PigR and the MglA-SspA complex is required for PigR, MglA, and SspA to coordinately control the expression of virulence genes in *F. tularensis*. Our findings support a model for the control of virulence gene expression in *F. tularensis* in which PigR exerts its regulatory effects through a direct interaction with the RNAP-associated MglA-SspA complex.

Results

Genetic screen for MglA mutants specifically defective for interaction with PigR

Using a modified version of a bacterial two-hybrid assay we have shown previously that PigR interacts with the MglA-SspA complex (Charity et al., 2009). In this bridge-hybrid assay PigR is fused to the zinc-finger DNA-binding protein called Zif and MglA is fused to the ω subunit of *E. coli* RNA polymerase (RNAP) (Charity et al., 2009) (Figure 2.1A). The PigR and MglA fusion proteins are synthesized alongside *F. tularensis* SspA in cells of an *E. coli* reporter strain that contain a Zif binding site positioned immediately upstream of a test promoter that drives expression of a linked *lacZ* reporter gene. The MglA-ω fusion protein interacts with *F. tularensis* SspA to form a complex that becomes tethered to the *E. coli* RNAP through the ω moiety of the MglA-ω fusion protein. Interaction between the DNA-bound PigR-Zif fusion protein and the RNAP-tethered MglA-SspA complex stabilizes the binding of RNAP to the test promoter and results in an increase in expression of the *lacZ* reporter (Charity et al., 2009) (Figure 2.1A). In order to determine whether the interaction between the MglA-SspA complex and PigR is necessary for the expression of virulence genes in *F. tularensis*, we first wanted to isolate mutants of MglA that are specifically defective for interaction with PigR and then test whether these mutants are functional in *F. tularensis*.

Our strategy for isolating MglA mutants that are specifically defective for interaction with PigR involved the use of sequential genetic screening steps. In the first screening step we used the bridge-hybrid assay to identify MglA mutants that fail to

form a tripartite complex with PigR and SspA (Figure 2.1A). In the second screening

step we used a bacterial two-hybrid assay to identify those MglA mutants from the first
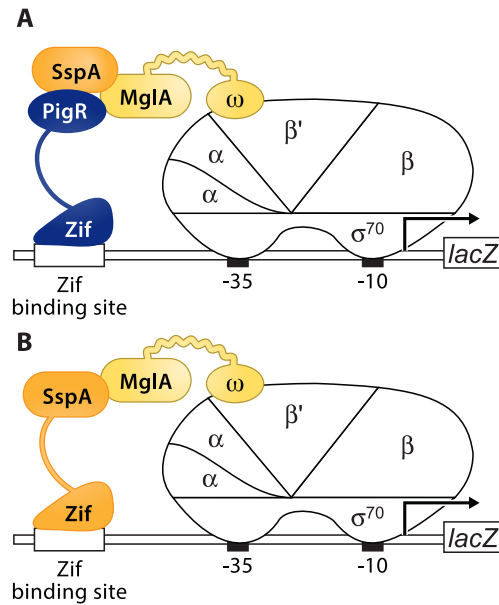
Figure 2.1 Genetic assays for detecting formation of the PigR-MglA-SspA complex and for detecting formation of the MglA-SspA complex (A) The *E. coli* bridge-hybrid assay used to detect formation of the PigR-MglA-SspA complex. In this assay, MglA is fused to the ω subunit of *E. coli* RNAP and PigR is fused to a zinc finger DNA-binding protein referred to as Zif. The MglA-ω and PigR-Zif fusion proteins are produced along with LVS SspA in the *E. coli* reporter strain KDZif1ΔZ. In this reporter strain, a Zif binding site is positioned upstream of a suitable test promoter which drives expression of a linked *lacZ* reporter gene on an F′ episome. MglA-ω and SspA form a heteromeric complex which associates with *E. coli* RNAP through the ω moiety of the MglA-ω fusion protein. DNA-bound PigR-Zif interacts with the RNAP-tethered complex formed between SspA and MglA-ω and stabilizes the binding of RNAP to the test promoter, leading to an increase in *lacZ* expression. (B) The *E. coli* two-hybrid assay used to detect the interaction between MglA and SspA. In this assay, MglA-ω and SspA-Zif are produced in the *E. coli* reporter strain KDZif1ΔZ. Interaction between the DNA-bound Zif-SspA fusion protein and the RNAP-tethered MglA-ω fusion protein leads to an increase in *lacZ* expression.

screen that are unaltered with respect to interaction with SspA; in this assay *F. tularensis* SspA is fused to Zif and MglA is fused to ω (Charity et al., 2007) (Figure 2.1B). As well as enabling us to remove from consideration those MglA mutants that are defective for interaction with SspA, this second screening step also allowed us to eliminate any MglA mutant that no longer interacted with PigR simply because it was misfolded. Specifically, a library of plasmids synthesizing mutant MglA-ω fusion proteins was introduced into cells of the *E. coli* reporter strain that synthesized both *F. tularensis* SspA and the PigR-Zif fusion protein from compatible plasmids. Plasmids containing MglA mutants that no longer permitted formation of the PigR-MglA-SspA complex (and thus gave rise to white colonies on media containing X-Gal) were isolated and pooled. The pool of plasmids encoding these defective MglA-ω fusion proteins was then transformed into cells of the *E. coli* reporter strain that synthesized an SspA-Zif fusion protein. Plasmids containing MglA mutants that could still interact with SspA (and thus gave rise to blue colonies on media containing X-Gal) were isolated.

Three MglA mutants with single amino acid substitutions were isolated using this genetic screen. These mutants had a threonine 47 to alanine [MglA(T47A)] substitution, a proline 48 to serine [MglA(P48S)] substitution, or a lysine 101 to glutamic acid [MglA(K101E)] substitution. Results depicted in Figure 2.2A show that all three mutant MglA-ω fusion proteins were able to interact with SspA-Zif as well as wild-type MglA-ω in the two-hybrid assay. However, as shown in Figure 2.2B, all three of the mutant MglA-ω fusion proteins did not support PigR-Zif-dependent reporter gene activation in the bridge-hybrid assay. These findings suggest that substitutions T47A, P48S, and

K101E in MglA interfere with the interaction between PigR and the MglA-SspA complex but do not interfere with the interaction between MglA and SspA.
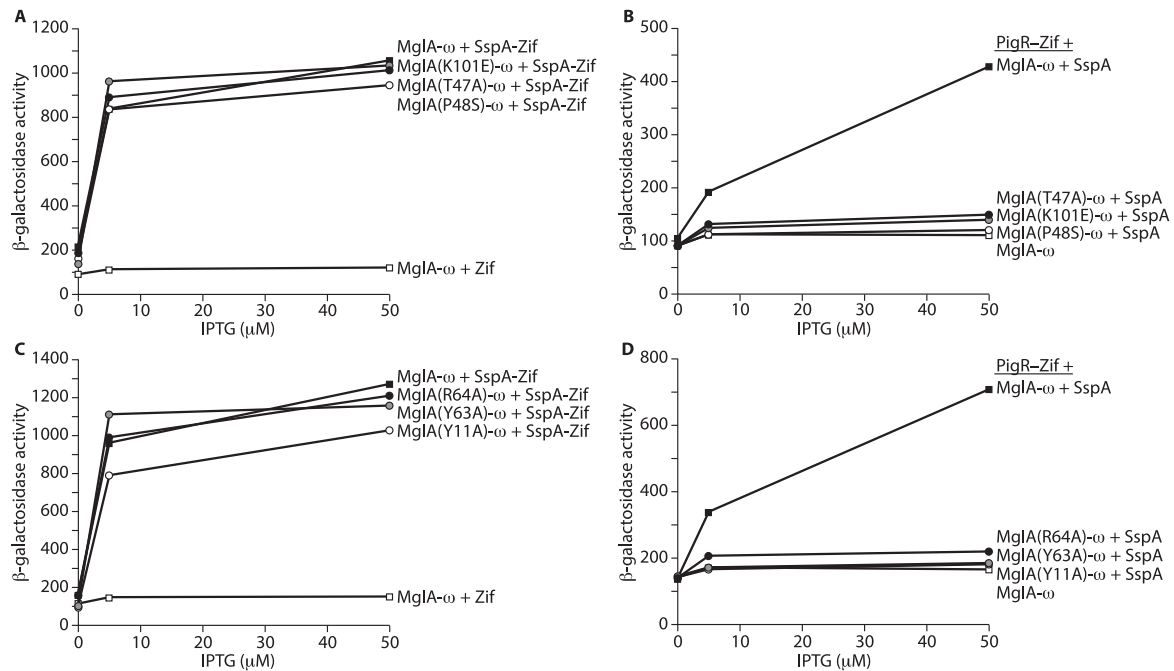
Figure 2.2 Identification of MglA mutants that are specifically defective for interaction with PigR (A) Bacterial two-hybrid assay of the ability of the MglA(T47A)-ω, MglA(P48S)-ω, and MglA(K101E)-ω mutant fusion proteins to interact with SspA-Zif. (B) Bacterial bridge-hybrid assay of the ability of the MglA(T47A)-ω, MglA(P48S)-ω, and MglA(K101E)-ω mutant fusion proteins to form a complex with SspA and PigR-Zif. (C) Bacterial two-hybrid assay of the ability of the MglA(Y11A)-ω, MglA(Y63A)-ω, and MglA(R64A)-ω mutant fusion proteins to interact with SspA-Zif. (D) Bacterial bridge-hybrid assay of the ability of the MglA(Y11A)-ω, MglA(Y63A)-ω, and MglA(R64A)-ω mutant fusion proteins to form a complex with SspA and PigR-Zif.

(A-D) Assays were performed with cells of the *E. coli* reporter strain KDZif1ΔZ containing compatible plasmids directing the IPTG-controlled synthesis of the specified proteins. Cells were grown in the presence of IPTG at the indicated concentration and then assayed for β-galactosidase activity.

MglA residues important for interaction with PigR cluster around a predicted pocket between MglA and SspA

We were interested in knowing whether the location of the MglA residues identified in the screen could provide insight into which surface of the MglA-SspA complex is important for the interaction with PigR. A crystal structure for the MglA-SspA complex from *F. tularensis* has not been solved, but the structure of the SspA homolog from *Yersinia pestis* can be used as a model for the MglA-SspA complex from *F. tularensis* (Hansen et al., 2005). In *Y. pestis*, as in many other bacteria, SspA forms a homodimer. One *Y. pestis* SspA monomer can therefore be used as a surrogate for *F. tularensis* SspA and the other monomer can be used as a surrogate for *F. tularensis* MglA. Phyre prediction software (Kelley and Sternberg, 2009) was also used to predict a structure for a monomer of *F. tularensis* MglA and a monomer of *F. tularensis* SspA. The predicted structures of both the MglA monomer (Figure 2.3A) and SspA monomer (not shown) closely resemble the crystal structure of a *Y. pestis* SspA monomer.

The amino acid sequence of *F. tularensis* MglA was aligned with *Y. pestis* SspA to determine which amino acid residues in the *Y. pestis* structure correspond to the residues in *F. tularensis* MglA identified in the screen. As shown in Figure 2.3B, the residues identified in MglA as being important for the interaction with PigR all lie along one surface of the predicted heterodimer. In the *Y. pestis* SspA homodimer, this surface appears to form a pocket between the two SspA monomers. This pocket lies on the opposite side of the protein from the surface that is predicted to be important for *E. coli* SspA to interact with *E. coli* RNAP (Hansen et al., 2005).
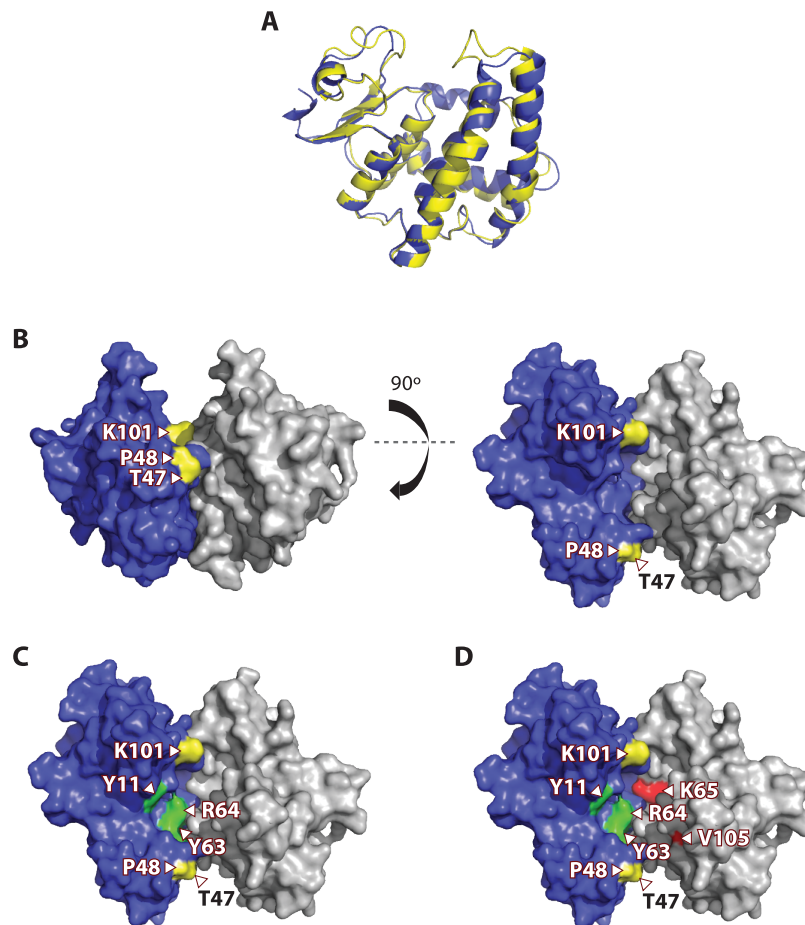
Figure 2.3 Predicted locations of residues in MglA and SspA important for interaction with PigR (A) Alignment of the structure for one *Y. pestis* monomer (shown in blue) with a Phyre predicted structure of a monomer of LVS MglA (shown in yellow). The structures align well, particularly in the dimerization domain for *Y. pestis* SspA shown in the foreground of the figure, indicating that the structure of the SspA homodimer from *Y. pestis* is a suitable model for a SspA-MglA heterodimer. (B) MglA residues T47, P48, and K101 were identified in a genetic screen as being important for interaction between the MglA-SspA complex and PigR. To determine where these residues may be located in the structure of MglA, these residues (colored in yellow) were mapped onto the *Y. pestis* SspA structure. One *Y. pestis* SspA monomer was used as a surrogate for LVS MglA (shown in blue) and the other monomer was used as a surrogate for LVS SspA (shown in gray). In the right panel, the protein structure has been rotated 90° toward the viewer to better visualize the location of the T47, P48, and K101. These residues appear to lie along the edge of a pocket formed between the two proteins. (C) Substitutions were made in MglA to test the importance of specific residues in the predicted pocket for interaction between the MglA-SspA complex and PigR. MglA mutants with substitutions Y11A, Y63A, and R64A (shown in green) were found to be specifically defective for interaction with PigR. Y11, Y63, and R64 are located within the predicted pocket formed between MglA and SspA. (D) SspA Residues K65, V105, and L130 were identified in a genetic screen as being important for interaction between the MglA-SspA complex and PigR. K65 and V105 (shown in red) are also located in the predicted pocket region between the MglA and SspA monomers. L130 is buried beneath the surface of the protein near the pocket in this model and is not shown in this image.

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MglA residues within a predicted pocket are important for interaction with PigR

We next asked whether other residues of MglA that are present within the predicted pocket between MglA and SspA are important for the interaction with PigR. To do this we introduced substitutions tyrosine 11 to alanine [MglA(Y11A)], tyrosine 63 to alanine [MglA(Y63A)], and arginine 64 to alanine [MglA(R64A)] into the MglA-ω fusion protein and tested the abilities of the resulting mutants to interact with SspA or support PigR-Zif-dependent activation using the bacterial two-hybrid and bridge-hybrid assays, respectively. The results presented in Figure 2.2C show that the MglA(Y11A)-ω, the MglA(Y63A)-ω, and the MglA(R64A)-ω fusion proteins were able to interact with the SspA-Zif fusion protein just as well as the wild-type MglA-ω fusion protein in the bacterial two-hybrid assay. The same mutants, however, did not support PigR-Zif-dependent transcription activation in the bridge-hybrid assay (Figure 2.2D). Amino acid substitutions Y11A, Y63A, and R64A in MglA therefore interfere with the interaction between PigR and the MglA-SspA complex but do not interfere with the interaction between MglA and SspA. These findings suggest that MglA residues Y11, Y63, and R64 within the predicted pocket between MglA and SspA (Figure 2.3C) are important for the interaction between the MglA-SspA complex and PigR.

Genetic screen for SspA mutants that are specifically defective for interaction with PigR

To identify residues of SspA that are important for the interaction with PigR, we employed the same genetic screening strategy we had used to identify mutants of MglA that are specifically defective for the interaction with PigR. In particular, we mutagenized the gene specifying the SspA moiety of an SspA-ω fusion protein using error-prone PCR

and then isolated mutants that failed to support PigR-Zif-dependent reporter gene activation in the bridge-hybrid assay (in cells of the *E. coli* reporter strain synthesizing the PigR-Zif fusion protein and *F. tularensis* MglA), but that could still interact with MglA in the two-hybrid assay (in cells of the *E. coli* reporter strain synthesizing an MglA-Zif fusion protein).

Three SspA mutants with single amino acid substitutions were isolated using our genetic screen. These mutants had a lysine 65 to glutamic acid [SspA(K65E)] substitution, a valine 105 to glutamic acid [SspA(V105E)] substitution, or a leucine 130 to serine [SspA(L130S)] substitution. The SspA(K65E)-ω fusion protein was able to interact with MglA-Zif to similar levels as the wild-type SspA-ω fusion protein in the two-hybrid assay (Figure 2.4A). SspA(V105E)-ω and SspA(L130S)-ω were also able to interact with MglA-Zif, although to a lesser extent than the wild-type SspA-ω fusion (Figure 2.4A). All three of the mutant SspA-ω fusion proteins did not support PigR-Zif-dependent transcription activation in the bridge-hybrid assay (Figure 2.4B). Our ability to isolate mutants of both SspA and MglA that were specifically defective for interaction with PigR suggests that both proteins interact with PigR. This is consistent with the hypothesis that MglA and SspA form a heterodimer in LVS that interacts with PigR.
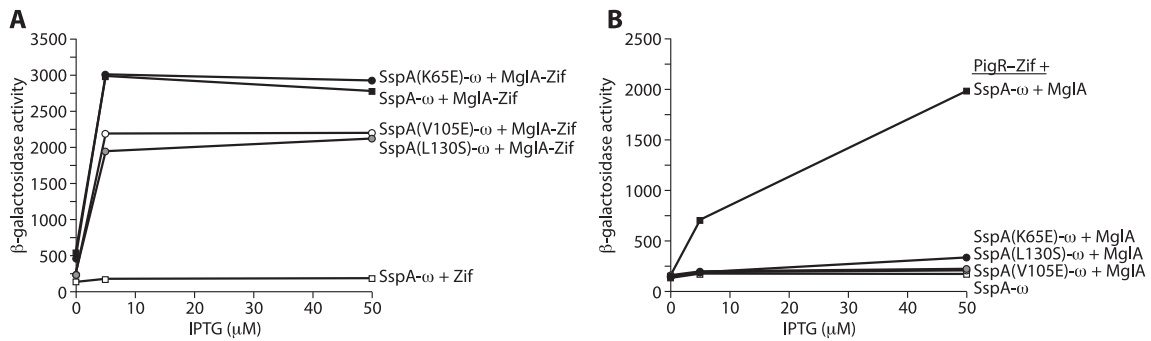
Figure 2.4 Identification of SspA mutants that are specifically defective for interaction with PigR (A) SspA(K65E)-ω, SspA(V105E)-ω, and SspA(L130S)-ω were tested for their ability to interact with MglA-Zif in the *E. coli* two-hybrid assay. SspA(K65E)-ω, SspA(V105E)-ω, and SspA(L130S)-ω were able to interact with MglA-Zif to a similar extent as wild-type SspA-ω. (B) SspA(K65E)-ω, SspA(V105E)-ω, and SspA(L130S)-ω were tested for their ability to interact with MglA and PigR-Zif in the *E. coli* bridge-hybrid assay. SspA(K65E)-ω, SspA(V105E)-ω, and SspA(L130S)-ω did not detectably interact with PigR-Zif.

(A-B) Assays were performed with cells of the *E. coli* reporter strain KDZif1ΔZ containing compatible plasmids directing the IPTG-controlled synthesis of the specified proteins. Cells were grown in the presence of IPTG at the indicated concentration and then assayed for β-galactosidase activity.

To determine the location of the SspA residues that are critical for interaction with PigR within the context of the MglA-SspA complex, the amino acid sequence of *F. tularensis* SspA was aligned with the amino acid sequence of *Y. pestis* SspA to identify the equivalent residues in *Y. pestis* SspA. These residues were then mapped onto the model of the MglA-SspA complex based on the crystal structure of *Y. pestis* SspA. Two of the residues, K65 and V105, are located within the predicted pocket between MglA and SspA (Figure 2.3D). The third residue, L130, is near the predicted pocket but is buried beneath the surface in this model of the MglA-SspA complex. The location of the residues identified in SspA as being important for interaction with PigR further illustrate the importance of this predicted pocket located at the MglA-SspA interface for the interaction with PigR.

MglA and SspA mutants that are specifically defective for interaction with PigR are unable to complement the respective ΔmglA or ΔsspA mutant strain of LVS

If interaction between PigR and the MglA-SspA complex is necessary for PigR, MglA, and SspA to function coordinately, MglA mutants that are specifically defective for interaction with PigR would be expected to be unable to complement the effects of an *mglA* deletion in *F. tularensis*, and SspA mutants that are specifically defective for interaction with PigR would be expected to be unable to complement the effects of an *sspA* deletion. Therefore, in order to determine if the interaction between PigR and the MglA-SspA complex is necessary for virulence gene expression in *F. tularensis*, we tested the ability of the MglA and SspA mutants we had identified to restore the expression of MglA-controlled genes in cells of a *ΔmglA* or *ΔsspA* mutant strain of LVS.

Plasmids directing the synthesis of wild-type MglA, MglA(T47A), MglA(P48S), MglA(K101E), MglA(Y11A), MglA(Y63A), and MglA(R64A), each containing a vesicular stomatitis virus-glycoprotein (VSV-G) epitope-tag fused to its C-terminus, were introduced into cells of the LVS Δ*mglA* mutant strain alongside an empty vector control. RNA was isolated from plasmid-containing cells that were grown to mid-log and the abundance of transcripts from two different PigR/MglA/SspA-controlled virulence genes was determined by qRT-PCR. The results depicted in Figure 2.5A show that wild-type MglA with a C-terminal VSV-G epitope-tag (MglA-V) was able to complement cells of the Δ*mglA* mutant strain and restored expression of both *iglA* and *FTL_1219* to levels near those seen in LVS carrying an empty vector. However, MglA(T47A)-V, MglA(P48S)-V, and MglA(K101E)-V, failed to restore expression of the *FTL_1219* and *iglA* genes in cells of the LVS Δ*mglA* mutant strain. Similarly, Figure 2.5C shows that MglA(Y11A)-V, MglA(Y63A)-V, and MglA(R64A)-V, failed to restore expression of the *FTL_1219* and *iglA* genes in cells of the LVS Δ*mglA* mutant strain, unlike MglA-V.

A similar approach was used to determine if the SspA mutants were able to complement a Δ*sspA* mutant strain of LVS. Plasmids directing the synthesis of VSV-G epitope tagged versions of wild-type SspA, SspA(K65E), SspA(V105E), or SspA(L130S) were introduced into cells of the Δ*sspA* mutant strain of LVS along with an empty vector. Results from qRT-PCR analyses (Figure 2.5E) show that VSV-G epitope tagged wild-type SspA (SspA-V) is able to complement cells of the Δ*sspA* mutant strain and restore expression of *FTL_1219* and *iglA* to levels near those found in cells the wild-type strain of LVS. However, the SspA(K65E)-V, SspA(V105E)-V, and SspA(L130S)-V mutants were unable to restore expression of *FTL_1219* and *iglA* and had similar levels

of expression of these genes as seen in cells of the *ΔsspA* mutant strain containing the

empty vector (Figure 2.5E).

Figure 2.5 MglA or SspA mutants that are specifically defective for interaction with PigR are unable to complement the respective *ΔmglA* or *ΔsspA* mutant strains of LVS (A) The ability of MglA(T47A)-V, MglA(P48S)-V, and MglA(K101E)-V to complement the LVS *ΔmglA* mutant strain was determined by testing the ability of these mutants to restore expression of two MglA-regulated genes, *FTL_1219* (white) and *iglA* (black). Quantitative RT-PCR (qRT-PCR) analysis showed that VSV-G epitope tagged wild-type MglA (MglA-V) was able to restore expression of *FTL_1219* and *iglA* to levels near those in wild-type (WT) LVS. The MglA mutants, MglA(T47A)-V, MglA(P48S)-V, and MglA(K101E)-V, were unable to restore expression of *FTL_1219* and *iglA* in cells of the LVS *ΔmglA* mutant strain (indicated *ΔmglA)* with transcripts being as abundant as those in cells of the LVS *ΔmglA* mutant strain containing the empty vector pF. Figure depicts data from a representative experiment with biological duplicates. Transcripts were normalized to *tul4*, whose expression is not influenced by MglA, SspA, or PigR. Error bars represent +/- 1 SD from the mean ΔΔCt. (B) The abundance of MglA-V, MglA(T47A)-V, MglA(P48S)-V, and MglA(K101E)-V was determined by Western blot analysis with an antibody against the VSV-G epitope tag. MglA(T47A)-V, MglA(P48S)-V, and MglA(K101E)-V were as abundant as wild-type MglA-V. An antibody against *F. tularensis* GroEL was used as a loading control and indicated that similar amounts of protein were loaded from cells of each strain. (C) MglA(Y11A)-V, MglA(Y63A)-V, and MglA(R64A)-V did not restore expression of *FTL_1219* and *iglA* in cells of the LVS *ΔmglA* mutant strain as assessed by qRT-PCR. Figure depicts data from a representative experiment with biological duplicates. (D) MglA(Y11A)-V, MglA(Y63A)-V, and MglA(R64A)-V were as abundant as wild-type MglA-V in cells of the LVS *ΔmglA* mutant strain. (E) VSV-G epitope tagged wild-type SspA (SspA-V) is able to restore expression of *iglA* and *FTL_1219* in cells of the LVS *ΔsspA* mutant strain (indicated *ΔsspA*) to levels similar as in wild-type (WT) LVS as determined by qRT-PCR. SspA(K65E)-V, SspA(V105E)-V, and SspA(L130S)-V do not restore expression of *iglA* and *FTL_1219* in cells of the LVS *ΔsspA* mutant strain with transcripts being as abundant as those in cells of the LVS *ΔsspA* mutant strain containing the empty vector pF. Figure depicts data from a representative experiment with biological duplicates. (F) SspA(K65E)-V and SspA(V105E)-V were as abundant as wild-type SspA-V in cells of the *ΔsspA* mutant strain of LVS. SspA(L130S)-V was also expressed in cells of the *ΔsspA* mutant strain of LVS, although the abundance was less than wild-type SspA-V.
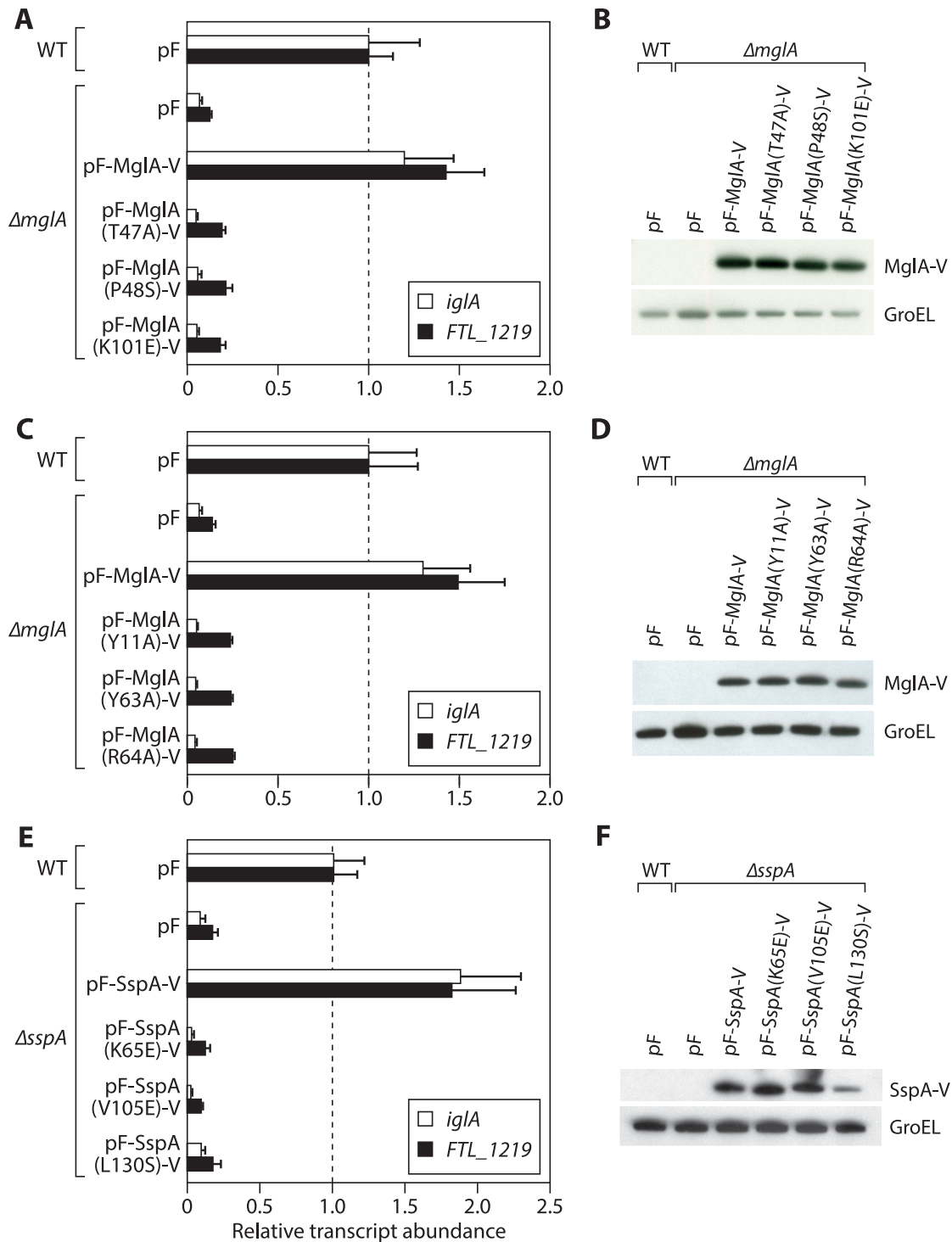
Figure 2.5 (Continued) MgIA or SspA mutants that are specifically defective for interaction with PigR are unable to complement the respective ΔmglA or ΔsspA mutant strains of LVS

Western blot analysis was used to determine whether the abundance of each of the

MglA and SspA mutants in *F. tularensis* was similar to that of the VSV-G epitope tagged

wild-type proteins. Using an antibody against the VSV-G epitope tag on each of the

proteins it was found that each of the MglA mutants was as abundant as wild-type MglA

and SspA(K65E) and SspA(V105E) were as abundant as wild-type SspA (Figure 2.5B,

D, and F). SspA(L130S) was also expressed, although it was less abundant than the

wild-type protein (Figure 2.5F). Thus, all of the MglA mutants that were specifically

defective for interaction with PigR were unable to complement cells of a *ΔmglA* mutant

strain even though they were as abundant as the wild-type proteins. Furthermore, two of

the three SspA mutants that were specifically defective for interaction with PigR were as

abundant as the wild-type protein but were unable to complement cells of a *ΔsspA*

mutant strain. Therefore, the inability of these MglA and SspA mutants to functionally

complement the respective mutant strains of LVS suggests that the interaction between

the MglA-SspA complex and PigR is necessary for virulence gene expression.

MglA mutants that are specifically defective for interaction with PigR still interact with

RNAP in LVS

We have categorized MglA mutants as being specifically defective for interaction

with PigR on the basis that these mutants fail to form a tripartite complex with PigR and

SspA in a bridge-hybrid assay but still interact with SspA in a two-hybrid assay.

However, these assays are unable to report on the ability of the MglA mutants to

interact with *F. tularensis* RNAP. It was therefore possible that the effects of the MglA

mutants on virulence gene expression in *F. tularensis* could be explained by the inability

of these mutants to interact with *F. tularensis* RNAP. To test this possibility, we

determined whether two of the MglA mutants that we had identified as being specifically

defective for interaction with PigR could still interact with RNAP in cells of *F. tularensis*.

To determine the relative amounts of wild-type and mutant MglA that are

associated with RNAP in *F. tularensis*, we synthesized VSV-G-tagged derivatives of

MglA in cells of LVS *ΔmglA* β'-TAP that contain an in-frame deletion of *mglA* and in

which the β' subunit of RNAP contains a tandem affinity purification (TAP)-tag fused to

its C-terminus (Figure 2.6A). RNAP was then isolated from these cells by

immunoprecipitation of β' and the relative amount of MglA-V that co-purified was

determined by Western blotting. Specifically, plasmids directing the synthesis of MglA,

MglA-V, MglA(T47A)-V, and MglA(Y63A)-V were introduced into cells of the LVS *ΔmglA*

β'-TAP strain and RNAP together with any associated proteins was isolated by

immunoprecipitation.

To determine the amount of each MglA mutant that purified with RNAP, relative

to wild-type MglA, samples were analyzed by Western blotting (Figure 2.6B). The

Western blots were quantified to determine the amount of MglA-V relative to the amount

of β'-TAP for each sample. The amounts of each mutant MglA-V relative to β'-TAP were

then normalized to that of wild-type MglA-V. As shown in Figure 6C, both MglA(T47A)-V

and MglA(Y63A)-V co-purified with RNAP. The amount of MglA(T47A)-V that

associated with RNAP was similar to that of wild-type MglA-V, whereas the amount of

MglA(Y63A)-V that associated with RNAP was less than wild-type (~78%). These

findings suggest that the inability of the MglA(T47A)-V mutant (and likely also that of the

MglA(Y63A)-V mutant) to complement a *ΔmglA* strain cannot be explained by the

inabilities of these mutants to interact with *F. tularensis* RNAP. The ability of the MglA-

SspA complex to interact with PigR is therefore critical for PigR, MglA, and SspA to

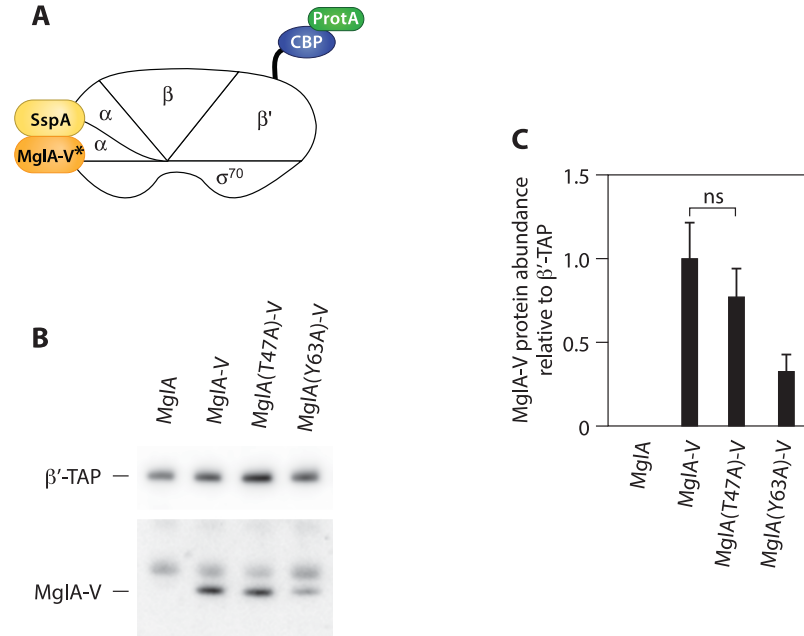control the expression of a common set of genes.

Figure 2.6 MglA mutants containing substitutions T47A or Y63A interact with RNAP in LVS
(A) Schematic of the experimental set-up to determine if MglA mutants are able to interact with RNAP in LVS. Wild-type MglA, MglA-V, MglA(T47A)-V, and MglA(Y63A)-V were synthesized in cells of LVS *ΔmglA* β′-TAP. RNAP, together with any associated proteins, was then purified by immunoprecipitation of the β′ subunit of RNAP. (B) Representative Western blot showing the relative amount of β′-TAP and MglA-V isolated from strains expressing MglA(T47A)-V, MglA(Y63A)-V, MglA(R64A)-V, or wild-type MglA. The VSV-G tagged MglA species were detected with an antibody against the VSV-G epitope tag and β'-TAP was detected with a peroxidase anti-peroxidase (PAP) antibody. (C) Quantification of the amount of MglA-V relative to β′-TAP purified from cells synthesizing either wild-type MglA-V, MglA(T47A)-V, or MglA(Y63A)-V. Western blots were quantified to determine the relative amount of MglA-V species that immunoprecipitated with β'-TAP from each sample. The relative amount of the different MglA-V species purified was then normalized to wild-type MglA-V. Similar amounts of MglA-V and MglA(T47A)-V were purified with β′-TAP. MglA(Y63A)-V also co-purified with β′-TAP. Error bars represent the standard deviation in the relative amount of MglA-V purified compared to β′-TAP between four biological replicates.

Discussion

The SspA family members MglA and SspA as well as a putative DNA-binding protein, PigR, coordinately control virulence gene expression in *F. tularensis* (Brotcke and Monack, 2008; Charity et al., 2009). Conflicting reports in the literature over whether the MglA-SspA complex and PigR interact have led to differing models of how MglA, SspA, and PigR regulate the expression of a common set of genes (Bell et al., 2010; Brotcke and Monack, 2008; Charity et al., 2009; Dai et al., 2010; Meibom et al., 2009). Here we identify mutants of MglA and SspA that are specifically defective for interaction with PigR using a combination of genetic approaches. These mutants identify a set of residues critical for the interaction between the MglA-SspA complex and PigR, all of which cluster around a predicted pocket between MglA and SspA. Thus, our findings have uncovered a surface of the MglA-SspA complex that is important for its interaction with PigR. We were also able to test if the interaction between the MglA-SspA complex and PigR is necessary for virulence gene expression using the MglA and SspA mutants specifically defective for interaction with PigR. The MglA and SspA mutants were unable to functionally complement cells of either a *ΔmglA* mutant strain of LVS or a *ΔsspA* mutant strain of LVS, respectively, indicating that the interaction between the MglA-SspA complex and PigR is necessary for PigR, MglA, and SspA to function coordinately (Figure 2.7).
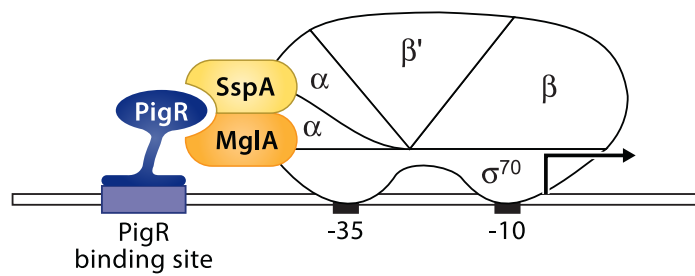
Figure 2.7 Model for how MglA, SspA, and PigR positively control virulence gene expression in a coordinate manner in *F. tularensis* In this model DNA-bound PigR interacts with the RNAP-associated MglA-SspA complex in the pocket formed between MglA and SspA.

Residues in a predicted pocket formed between MglA and SspA are important for interaction with PigR

In order to gain insight into which surface, or surfaces, of the MglA-SspA complex might be important for the interaction with PigR, the structure of the SspA homodimer from *Y. pestis* was used as a model for the MglA-SspA complex. According to this model, the residues that were identified in MglA (Y11, T47, P48, Y63, R64 and K101) and SspA (K65, V105, and L130) in our genetic assays as being critical for the interaction between the MglA-SspA complex and PigR all clustered in and around a putative pocket formed close to the interface between these proteins. PigR might bind directly to the MglA-SspA complex within this putative pocket (Figure 2.7). All of the residues identified in MglA or SspA as being important for the interaction with PigR, except for L130 in SspA, are surface exposed in the model (Figure 2.3D). It is possible that some or all of these residues make direct contact with PigR, or make critical contributions to the charge or structural features of the pocket that are important for interaction with PigR. (Note that if residue L130 of SspA were at the surface of the MglA-SspA complex this would suggest that our current structural model of the MglA-SspA complex does not accurately predict the location of all residues.)

The surface of the MglA-SspA complex that we have identified as being important for interaction with PigR need not interact with PigR directly. This putative pocket region could be important for the binding of another transcription factor, either a protein or small molecule, that is necessary for the interaction between the MglA-SspA complex and PigR. MglA(Y11A), MglA(T47A), MglA(P48S), MglA(Y63A), MglA(R64A), MglA(K101E), SspA(K65E), SspA(V105E), and SspA(L130S) were defective for the

interaction with PigR in the *E. coli* bridge-hybrid assay; therefore, if these substitutions do disrupt binding of another transcription factor, this factor must be conserved in *E. coli*. The molecules guanosine tetraphosphate (ppGpp) and polyphosphate have been proposed to play a role in virulence gene regulation along with MglA, SspA, and PigR (Charity et al., 2009; Faron et al., 2013; Wrench et al., 2013). Previous work suggested that ppGpp regulates the same set of genes as MglA, SspA, and PigR and promotes the interaction between the MglA-SspA complex and PigR in *F. tularensis* (Charity et al., 2009). Another recent study found that polyphosphate binds to the MglA-SspA complex *in vitro* (Wrench et al., 2013). It could be that ppGpp or polyphosphate interacts directly with the MglA-SspA complex to promote the interaction with PigR and one or more of the mutants is defective for binding one of these molecules.

Previous work indicates that MglA and SspA function as a heteromer to regulate the expression of genes in *F. tularensis*. In LVS, SspA is necessary for MglA to interact with RNAP and both MglA and SspA must be present to detect an interaction with PigR in the *E. coli* bridge-hybrid assay. These data suggest that MglA and SspA exist as a heteromer in LVS, although it is possible that homomeric species are also present (Charity et al., 2007). The ability to isolate mutants of both MglA and SspA that are specifically defective for interaction with PigR, further strengthens the model that these proteins function as a heteromer. It also suggests that each protein interacts with PigR or influences the ability of the other protein to interact with PigR.

Previous studies in *F. tularensis* and *F. novicida* have shown that MglA, SspA, and PigR regulate similar sets of genes. By showing that the interaction between the MglA-SspA complex and PigR is necessary for expression of virulence genes, we have

helped elucidate how these proteins coordinately regulate this shared set of genes. However, it is still unknown how these proteins target RNAP to certain promoters. A region of PigR has homology to the helix-turn-helix DNA-binding domain of the MerR family of transcription regulators, so it is possible that PigR is a DNA-binding protein (Brotcke and Monack, 2008; Charity et al., 2009). The MglA-SspA complex would then function as a bridge between RNAP and DNA-bound PigR to stabilize the binding of RNAP to certain promoters (Figure 2.7). However, it has not been demonstrated that PigR functions in *F. tularensis* by binding to DNA. Regardless of whether PigR is a DNA-binding protein, our findings have demonstrated that the ability of PigR to interact with the MglA-SspA complex is crucial for PigR to exert its effects on gene expression in *F. tularensis*.

SspA orthologs from several other bacteria, including *Neisseria gonorrhoeae* (De Reuse and Taha, 1997), *Yersinia enterocolitica* (Badger and Miller, 1998), *Vibrio cholerae* (Merrell et al., 2002), enterohemorrhagic *E. coli* (Hansen and Jin, 2012), and *Pseudomonas aeruginosa* (Yin et al., 2013), have also been implicated in regulating virulence gene expression. The mechanism by which SspA regulates the expression of virulence genes in these other organisms, however, is currently not well understood. It is conceivable that SspA family members in other bacteria also interact with a transcription activator to recruit RNAP to target promoters. There is another example in the literature of SspA coordinately regulating gene expression with a transcription activator. In *E. coli*, the phage P1 late gene activator protein (Lpa) was shown to work with *E. coli* SspA to direct RNAP to the promoters of P1 phage lytic stage late genes (Hansen et al., 2003). Our work provides additional evidence that the interaction

between SspA homologs and a transcription activator may be a common method by which SspA family members control gene expression.

Materials and Methods

Plasmids, strains, and growth conditions

 Francisella tularensis subspecies *holarctica* strain LVS and the strains LVS

ΔmglA and LVS *ΔsspA* have been previously described (Charity et al., 2007). All *F.*

tularensis strains were grown at 37 °C with aeration 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 indicated 5 μg/mL of kanamycin or 5

μg/mL of nourseothricin were used for selection. The *E. coli* strains DH5α F'IQ

(Invitrogen) and XL1-blue (Stratagene) were used for plasmid construction. The *E. coli*

strain KDZif1ΔZ has been previously described (Vallet-Gely et al., 2005) and was used

as the reporter strain for the bacterial two-hybrid and bridge-hybrid assays. When

indicated 100 μg/mL carbenicillin, 10 μg/mL tetracycline, or 100 μg/mL spectinomycin

was used for selection.

Francisella strain for TAP immunoprecipitation

 The strain LVS *ΔmglA* β'-TAP contains an in frame deletion of the *mglA* locus

and the DNA sequence specifying the tandem affinity purification (TAP) tag at the 3' end

of the native locus of *rpoC*, which encodes the β' subunit of RNAP. This strain was

generated from the LVS *ΔmglA* mutant strain which has been previously described

(Charity et al., 2007). The plasmid pEX-RpoC-TAP, which confers resistance to

kanamycin and contains ~400 bp of the 3' end of the *rpoC* gene followed by the TAP-

tag sequence, (Charity et al., 2007) was used to intergrate the TAP tag sequence

downstream of *rpoC*. pEX-RpoC-TAP was electroporated into electrocompetent LVS

ΔmglA cells. Cells were plated on CHA supplemented with hemoglobin and kanamycin

to select for cells that had integrated the pEX-RpoC-TAP plasmid, which is unable to

replicate within cells of *F. tularensis*. PCR was used to confirm that integration of the

TAP vector occurred at the proper chromosomal location.

<u>Plasmids for bacterial two-hybrid and bridge-hybrid assays</u>

The plasmids pBR-MglA-ω, pBR-SspA-ω, pACTR-SspA-Zif, pACTR-MglA-Zif,

pACTR-AP-Zif, pCL-SspA, and pCL have been previously described (Charity et al.,

2007; 2009). The plasmid pCL-MglA, which directs the synthesis of LVS MglA, encodes

mglA under the control of the *lacUV5* promoter and confers resistance to spectinomycin,

was generated by replacing the *sspA* gene from LVS in pCL-SspA with the full-length

mglA gene from LVS.

The plasmids pBR-MglA(T47A)-ω, pBR-MglA(P48S)-ω, pBR-MglA(K101E)-ω,

pBR-SspA(K65E)-ω, pBR-SspA(V105E)-ω, and pBR-SspA(L130S)-ω confer resistance

to carbenicillin and direct the synthesis of the indicated MglA or SspA mutant fused to ω

under the control of the *lacUV5* promoter. These plasmids were isolated in genetic

screens for mutants of MglA-ω or SspA-ω specifically defective for interaction with

PigR-Zif as described below.

Mutations were introduced into the LVS *mglA* gene to generate MglA(Y11A)-ω,

MglA(Y63A)-ω, and MglA(R64A)-ω using splicing by overlap extension (Ho et al.,

1989). The PCR products were digested with NdeI and NotI to insert them into the pBR-

MglA-ω plasmid and generate the plasmids pBR-MglA(Y11A)-ω, pBR-MglA(Y63A)-ω,

and pBR-(R64A)-ω. These plasmids direct the synthesis of MglA(Y11A)-ω,

MglA(Y63A)-ω, and MglA(R64A)-ω, respectively.

<u>Plasmids for complementation analyses</u>

Plasmid pF-MglA confers resistance to kanamycin, directs the synthesis of LVS

MglA under the control of the *groEL* promoter, and has been described previously

(Charity et al., 2007). Plasmid pF is the corresponding empty vector control and has

been described previously (Charity et al., 2007). Plasmids pF-MglA-V and pF-SspA-V

direct the synthesis of LVS MglA or LVS SspA, respectively, with a vesicular stomatitis-

glycoprotein (VSV-G) epitope-tag fused to its C-terminus (MglA-V or SspA-V) under the

control of the *groEL* promoter. Plasmids pF-MglA(T47A)-V, pF-MglA(P48S)-V, pF-

MglA(K101E)-V, pF-MglA(Y11A)-V, pF-MglA(Y63A)-V, and pF-MglA(R64A)-V, direct the

synthesis of mutant MglA-V proteins containing the indicated amino acid substitutions in

MglA. Plasmids pF-MglA-V, pF-MglA(T47A)-V, pF-MglA(P48S)-V, pF-MglA(K101E)-V,

pF-MglA(Y11A)-V, pF-MglA(Y63A)-V, and pF-MglA(R64A)-V were made by cloning the

appropriate EcoRI and BamHI-digested PCR products into EcoRI-BamHI digested

plasmid pF. The PCR products used to construct these plasmids were amplified from

the appropriate wild-type or mutant *mglA* gene from a suitable template using a forward

primer that adds an EcoRI cleavage site and the SD sequence to the 5' end of the *mglA*

gene (5'- ATG AAT TCT TAC TAG GAG GAT ACA ATC TTG CTT TTA TAC ACA AAA

AAA GAT G -3) and using a reverse primer that added DNA specifying the VSV-G

epitope-tag and a BamHI cleavage site to the 3′ end of the *mglA* gene (5'- TAT GGA

TCC TTA TTT ACC TAA TCT ATT CAT TTC AAT ATC AGT ATA TGC GGC CGC AGC

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TCC TTT TGC -3'). The sequences of the PCR-amplified regions of the resulting plasmids were confirmed by DNA sequencing. Plasmids pF-SspA-V, pF-SspA(K65E)-V, pF-SspA(V105E)-V, and pF-SspA(L130S)-V were generated with a similar strategy. PCR products were amplified from a suitable template containing either the appropriate wild-type or mutant *sspA* gene using a forward primer that added an EcoRI cleavage site and SD sequence to the 5' end of the *sspA* gene (5'- ATG AAT TCT TAC TAG GAG GAT ACA ATC TTG ATG AAA GTT ACA TTA TAT ACA ACG -3') and a reverse primer complementary to a region of the template downstream of the *sspA* gene. The resulting PCR products were digested with EcoRI and NotI. The vector pF-MglA(T47A)-V was also digested with EcoRI and NotI to remove the *mglA* gene (a NotI cleavage site is present between the *mglA* gene and the DNA specifying the VSV-G epitope tag) and the EcoRI-NotI digested wild-type or mutant *sspA* genes were ligated into the digested vector.

Plasmids used in TAP-immunoprecipitation

The plasmids pF3-MglA-V, pF3-MglA, pF3-MglA(T47A)-V, and pF3-MglA(Y63A)-V are identical to plasmids pF-MglA-V, pF-MglA, pF-MglA(T47A)-V, and pF-MglA(Y63A)-V except that they confer resistance to nourseothricin. The plasmid pF-MglA has been previously described (Charity et al., 2007). The pF3 plasmids listed above were generated by replacing the kanamycin resistance gene in pF-MglA-V, pF-MglA, pF-MglA(T47A)-V, and pF-MglA(Y63A)-V with the nourseothricin resistance gene from the previously described pF3 plasmid (Charity et al., 2009).

Genetic screens for mutants of MglA or SspA specifically defective for interaction with PigR

The *mglA* portion of the ω fusion in the vector pBR-MglA-ω was mutagenized using error-prone PCR with *Taq* polymerase and primers flanking the *mglA* gene. The PCR product was then digested with the restriction enzymes NdeI and NotI and inserted in the pBR-MglA-ω vector to generate a library of plasmids that direct the synthesis of MglA-ω fusion proteins with random mutations in the *mglA* moiety of the *mglA-ω* fusion gene. This library was transformed into KDZif1ΔZ cells along with plasmids pCL-SspA and pACTR-PigR. Cells were plated on LB containing carbenicillin, spectinomycin, tetracycline, X-Gal (50 µg/mL), IPTG (50 µg/mL), and the X-Gal inhibitor tPEG (125 µg/mL). Approximately 120 colonies were selected in which cells had low levels of *lacZ* expression compared to cells expressing wild-type MglA-ω along with PigR-Zif and LVS SspA. These colonies were struck out on LB plates containing carbenicillin to select only for those cells containing the pBR-MglA-ω vector. The pBR-MglA-ω plasmids expressing various mutant MglA-ω fusion proteins were then isolated, pooled, and subsequently transformed into KDZif1ΔZ cells along with pACTR-SspA-Zif. These cells were then plated on LB containing carbenicillin, tetracycline, X-Gal (50 µg/mL), and IPTG (50 µg/mL). Approximately 40 colonies were selected in which cells expressed similar levels of *lacZ* as cells expressing the wild-type MglA-ω fusion protein and SspA-Zif. pBR-MglA-ω plasmids were isolated from these colonies and transformed back into KDZif1ΔZ cells with either pCL-SspA and pACTR-PigR-Zif or pACTR-SspA-Zif and assayed for β-galactosidase activity. Plasmids directing the synthesis of MglA-ω mutant

proteins that were specifically defective for interaction with PigR were then sequenced to determine the corresponding mutation.

The genetic screen for SspA mutants that were specifically defective for interaction with PigR was essentially the same as that described above for MglA mutants except that a library of SspA-ω mutants was generated using error-prone PCR with *Taq* polymerase and vector pBR-SspA-ω. This library was analyzed first in KDZif1ΔZ cells along with the vectors pCL-MglA and pACTR-Zif-PigR. Then approximately 100 candidate mutants were screened in KDZif1ΔZ cells containing plasmid pACTR-MglA-Zif. Finally, 40 colonies were selected in which cells expressed similar *lacZ* levels as cells expressing the wild-type SspA-ω fusion protein in the presence of MglA-Zif. Plasmids were isolated from cells from these colonies and tested in the bridge-hybrid assay and two-hybrid assays to confirm that the isolated mutants were specifically defective for interaction with PigR. Plasmids directing the synthesis of SspA-ω mutant fusion proteins that were specifically defective for interaction with PigR were sequenced to identify the corresponding mutation.

Bacterial two-hybrid and bridge-hybrid assays

The bacterial two-hybrid and bridge-hybrid assays were performed as previously described (Charity et al., 2007; 2009). Cells were grown with aeration at 37°C in LB supplemented with carbenicillin, tetracycline, and IPTG at the indicated concentration for the two-hybrid assay and with carbenicillin, spectinomycin, 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 (Dove and

Hochschild, 2004). Assays were performed at least three times in duplicate. Duplicate measurements differed by less than 10%. Results shown are averages from a single representative experiment.

Protein structure analysis

All protein structures were analyzed using the PyMOL Molecular Graphics System, Version 1.6.0 Schrödinger, LLC. Phyre2 prediction software (Kelley and Sternberg, 2009) was used to generate a predicted secondary structure for LVS MglA and LVS SspA. The predicted structure for a monomer of LVS MglA was aligned with the structure of *Y. pestis* SspA using PyMOL Molecular Graphics system. ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the protein sequence of *Y. pestis* SspA with LVS MglA and LVS SspA. This alignment was used to determine which residues of *Y. pestis* correspond to the residues identified in MglA and LVS SspA as being important for interaction with PigR.

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 OD_{600} ~0.4. 10 mL of cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4 °C. RNA was isolated using Tri-Reagent (Ambion) as previously described for *Pseudomonas aeruginosa* (Goldman et al., 2011). RNA quality was determined by gel electrophoresis. 3 μg of RNA from each sample were glyoxylated using NorthernMax®-Gly Glyoxal Load Dye (Ambion) and run on a 1% agarose gel using NorthernMax®-Gly Gel Pre/Running Buffer (Ambion).

cDNA synthesis using Superscript IIIIII Reverse transcriptase (Invitrogen) and qRT-PCR using iTaq Universal SYBR Green supermix (Bio-Rad) and Applied Biosystems StepOnePlus detection system were performed essentially as described in (Charity et al., 2007). The abundance of the *iglA* and *FTL_1219* transcripts were measured relative to that of the *tul4* transcript (Charity et al., 2007). qRT-PCR was performed at least twice on sets of biological duplicates. Data shown are from representative experiments.

Immunoprecipitation of β'-TAP

Immunoprecipitation of β'-TAP from LVS was performed using a modified version of the TAP protocol described previously (Rietsch et al., 2005). Cells were grown in liquid culture (100 mL) in the presence of nourseothricin with aeration at 37°C until cultures reached an OD_{600} ~0.4. Cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4 °C. Cells were resuspended in 5 mL buffer 1 (20 mM KHepes pH 7.9, 50 mM KCl, 0.5 mM DTT, 10% glycerol) and then harvested by centrifugation. Cells were resuspended in 500 µL buffer 1 containing a protease inhibitor cocktail (cOmplete Mini, EDTA-free protease inhibitor cocktail, Roche) and then lysed using sonication. Samples were centrifuged at 13000 rpm at 4 °C for 20 minutes. The lysate was transferred to a fresh tube and centrifuged at 13000 rpm at 4 °C for 5 minutes. The lysates were removed and the salt concentration of the lysates was adjusted to 10 mM Tris-HCl pH8 (USB), 150 mM NaCl (Sigma), and 0.1% NP40 (NP40 alternative, Calbiochem) for subsequent steps. Lysates were added to 75 µL IgG sepharose 6 Fast Flow beads (GE healthcare) which had been washed twice and then resuspended in

IPP150 buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP40). Samples were incubated with rocking for 2 hours at 4 °C. Beads were pelleted by centrifugation at 10,000 rpm for 30 seconds and then washed five times with 1 mL IPP150 buffer. Beads were resuspended in 200 µL sample loading buffer (1x NuPAGE® Novex LDS Sample Buffer, 50 mM DTT) and boiled for 10 minutes to elute proteins from beads. Samples were centrifuged to pellet the beads before loading on an SDS-PAGE gel.

Immunoblots

Cell lysates were separated by SDS-PAGE on NuPAGE® 4-12% Bis-Tris protein gels (Novex) with NuPAGE® MES running buffer (Novex). For complementation experiments, proteins were transferred to a PVDF membrane using the iBlot dry blotting system (Invitrogen). Membranes were blocked with 25 mL SuperBlock blocking buffer in TBS (ThermoScientific) with 250 µL Surfact-Amps 20 (ThermoScientific) and washed with TBS with Surfact-Amps. Membranes were probed with either a polyclonal antibody against the VSV-G tag (Sigma) or a primary antibody against GroEL (Karsten Hazlett, Albany Medical College/Daniel L. Clemens, University of California Los Angeles). Goat polyclonal anti-rabbit IgG conjugated with horseradish peroxidase (ThermoScientific) was used to detect proteins. Proteins were then visualized using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific).

To quantify the immunoblots of the immunoprecipitated (IP) material, initial immunoblots were performed using a serial dilution of the IP material to determine the dynamic range of the signal for quantification. For the immunoblots used for quantification, 10 µL of a 1:8 dilution of the IP material was run on a SDS-PAGE

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NuPAGE® 4-12% Bis-Tris protein gels (Novex) with NuPAGE® MES running buffer (Novex). Each sample was run in technical triplicate. Proteins were transferred to a PVDF membrane using the Criterion blotter system (Bio-Rad). Membranes were blocked as above and probed with either a polyclonal antibody against the VSV-G tag (Sigma) or a peroxidase anti-peroxidase (PAP) antibody (Sigma) to visualize the TAP tag. The PAP signal was visualized using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) and the VSV-G signal was visualized using SuperSignal West Femto Chemiluminescent Substrate (ThermoScientific). Blots were imaged using the ChemiDoc XRS+ system (Bio-Rad) and the intensity of the PAP and VSV-G signals were quantified using ImageQuant TL v2005 software (Amersham Biosciences).

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Chapter 3

ppGpp does not promote the interaction between the MglA-SspA complex and PigR through an indirect effect on polyphosphate

Abstract

Virulence gene expression in the pathogen *Francisella tularensis* is regulated by the proteins MglA, SspA, and PigR, as well as the small molecule guanosine tetraphosphate (ppGpp). In *F. tularensis*, MglA and SspA form a complex that associates with RNA polymerase (RNAP) and PigR, a putative DNA-binding protein. A direct interaction between the MglA-SspA complex and PigR is critical for the ability of these proteins to positively regulate gene expression. Previous work in *F. tularensis* has shown that the interaction between the MglA-SspA complex and PigR is promoted by ppGpp. Here, we investigated the importance of ppGpp on the ability of the MglA-SspA complex and PigR to interact in a modified version of an *E. coli* two-hybrid system, referred to as the bridge-hybrid assay, which allows detection of a protein of interest with a protein complex. We found that ppGpp is required to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay, indicating that ppGpp either directly promotes this interaction or indirectly promotes the interaction through a mechanism conserved between *F. tularensis* and *E. coli*. One potential mechanism conserved between *F. tularensis* and *E. coli* by which ppGpp could be functioning is through an effect on the abundance of polyphosphate. We found that polyphosphate was not required for PigR to detectably interact with the MglA-SspA complex in the *E. coli* bridge-hybrid assay. Furthermore, analysis of the effect of polyphosphate on gene expression in *F. tularensis* indicted that polyphosphate negatively regulates expression of PigR-, MglA- and SspA-regulated virulence genes. Our findings suggest that ppGpp is unlikely to exert its regulatory effects in *F. tularensis* by promoting the production of polyphosphate.

Introduction

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 ingestion or inhalation of the bacteria (Sjöstedt, 2007). The most severe form of the disease occurrs after inhalation of aerosolized bacteria. *F. tularensis* is a highly infectious pathogen with the most virulent strains having an infectious dose of as few as 10 bacteria (Tärnvik and Chu, 2007). Due to its highly infectious nature, ability to cause severe disease, and ability to be easily aerosolized, *F. tularensis* has been developed by several countries as a bioweapon, leading the CDC to list *F. tularensis* as a Category A select agent (Oyston et al., 2004).

Previous work from our lab and others has identified several key regulators of virulence gene expression in *F. tularensis*. MglA, SspA, and PigR all control expression of essentially the same set of genes which includes the genes present on the *Francisella* pathogenicity island (FPI), other virulence genes present elsewhere in the genome, and genes not known to play a role in virulence (Brotcke and Monack, 2008; Brotcke et al., 2006; Charity et al., 2009; Lauriano et al., 2004). The proteins MglA and SspA are both members of the stringent starvation protein A (SspA) protein family. In *F. tularensis*, MglA and SspA form a heteromeric complex that binds to RNA polymerase (RNAP) (Charity et al., 2007). PigR, a putative DNA-binding protein, interacts with the RNAP-associated MglA-SspA complex (Charity et al., 2009). PigR appears to confer

sensitivity to a specific sequence found at regulated promoters (Ramsey et al., 2015). The MglA-SspA complex and PigR have been shown to associate with one another in *F. tularensis* and to interact directly in a modified bacterial two-hybrid assay, referred to as a bridge-hybrid assay, that allows detection of an interaction between a protein and a protein complex (Charity et al., 2009). Interaction between the MglA-SspA complex and PigR is critical for the expression of genes regulated by MglA, SspA, and PigR (Rohlfing and Dove, 2014).

The small molecule guanosine tetraphosphate, or ppGpp, also appears to be critical for virulence gene expression in *F. tularensis* (Charity et al., 2009). ppGpp, which is referred to as an alarmone, is produced in response to a variety of stress signals . In *E. coli*, ppGpp is produced by the proteins RelA and SpoT. RelA is a monofunctional enzyme, which synthesizes ppGpp in response to amino acid starvation. RelA mediates the stringent response, where an increase in ppGpp leads to an inhibition of rRNA transcription, increased expression of amino acid biosynthesis genes, and reduction in protein synthesis (Haugen et al., 2008; Potrykus and Cashel, 2008; Srivatsan and Wang, 2008). SpoT is a bifunctional enzyme, capable degrading and synthesizing ppGpp, which responds to conditions of carbon, phosphate, and fatty acid limitation (Potrykus and Cashel, 2008). The effects of ppGpp on transcription in *E. coli* are potentiated by the small RNAP-associated protein DksA (Paul et al., 2004). Recently, direct binding of ppGpp to *E. coli* RNAP has been shown to be critical for ppGpp to inhibit transcription of rRNA genes (Ross et al., 2013). ppGpp has also been shown to regulate virulence gene expression in several other pathogens, including *Legionella pneumophila* (Hammer and Swanson, 1999), *Salmonella* (Thompson et al., 2006),

Vibrio cholerae (Haralalka et al., 2003)*, Mycobacterium tuberculosis* (Primm et al., 2000), *Pseudomonas aeruginosa* (Erickson et al., 2004), and enterohaemorrhagic *E. coli* (Nakanishi et al., 2006). In some cases, DksA has also been shown to be involved in the regulation of virulence gene expression in these organisms (Nakanishi et al., 2006; Pal et al., 2012; Stallings et al., 2009).

Previous work showed that in *F. tularensis*, ppGpp regulates expression of the same set of genes as MglA, SspA, and PigR. A *ΔrelAspoT* mutant strain of the live vaccine strain (LVS) of *F. tularensis*, which produces no ppGpp, has a similar decrease in the expression of virulence genes as seen in a *ΔmglA* mutant strain of LVS (Charity et al., 2009). ppGpp does not regulate expression of MglA controlled genes by regulating expression of either *mglA* or *sspA*, but instead mediates the interaction between the MglA-SspA complex and PigR. In a wild-type strain of LVS, PigR can co-purify with the MglA-SspA complex from LVS after proteins have been crosslinked. However, PigR is no longer able to co-purify with the MglA-SspA complex in a *ΔrelA spoT* mutant strain of LVS (Charity et al., 2009), indicating that ppGpp promotes the interaction between the MglA-SspA complex and PigR. Thus ppGpp appears to control expression of MglA, SspA, and PigR regulated genes by mediating the interaction between the MglA-SspA complex and PigR. However, the mechanism by which ppGpp promotes this interaction is still unknown (Charity et al., 2009).

In addition to its effects on transcription, ppGpp is also known to directly bind to target enzymes to affect their activity, allowing ppGpp to directly affect many cellular processes (reviewed in (Kanjee et al., 2012)). One enzyme that is directly inhibited by ppGpp is polyphosphatase (PPX), an enzyme which breaks down the molecule

polyphosphate, allowing ppGpp to regulate polyphosphate levels within a cell.

Polyphosphate is a chain of tens to hundreds of inorganic phosphate molecules that is

produced in response to stress conditions, including starvation (Brown and Kornberg,

2004). It is generated by the enzyme polyphosphate kinase (PPK) (Kuroda et al., 1997).

Polyphosphate has been shown to be important for expression of many virulence

factors, particularly motility and biofilm formation in *Pseudomonas aeruginosa* (Rashid

and Kornberg, 2000; Rashid et al., 2000a; 2000b). *ppk* (the gene encoding PPK) has

also been found to be important for the virulence of several other pathogens, including

Mycobacterium tuberculosis (Singh et al., 2013; Sureka et al., 2007), *Salmonella* (Kim

et al., 2002), *Helicobator pylori* (Yang et al., 2010), *Vibrio cholerae* (Ogawa et al., 2000),

Shigella flexneri (Kim et al., 2002)*,* and *F. tularensis* (Richards et al., 2008). Although

the effect of polyphosphate on virulence and specific virulence factors have been

determined in several pathogens, the effect of polyphosphate on gene expression has

not been well studied (Rao et al., 2009).

In this study we further investigated the role of ppGpp in promoting the

interaction between the MglA-SspA complex and PigR by determining if ppGpp is

required to detect an interaction between the MglA-SspA complex and PigR in a

heterologous system in *E. coli*. We also investigated whether ppGpp may be working

indirectly to promote the interaction between PigR and the MglA-SspA complex through

its effects on polyphosphate levels. We found that ppGpp, but not polyphosphate, is

required to detect an interaction between the MglA-SspA complex and PigR in a the *E.*

coli bridge-hybrid assay. These results further highlight the importance of ppGpp in

promoting the interaction between PigR and the MglA-SspA complex. Although

polyphosphate is not required for PigR to interact with the MglA-SspA complex, the

effects on polyphosphate on gene expression in *F. tularensis* have not been

investigated. We therefore determined the effects of polyphosphate on expression of

two MglA, SspA, and PigR regulated virulence genes in *F. tularensis*. A mutant of *F.*

tularensis that is predicted to have no polyphosphate had increased expression of two

MglA, SspA, and PigR regulated virulence genes, identifying polyphosphate as a

negative regulator of virulence genes in *F. tularensis*.

Results

ppGpp is required to detect the interaction between the MglA-SspA complex and PigR

in the E. coli bridge-hybrid assay

In the live vaccine strain (LVS) of *Francisella tularensis*, the interaction between

the MglA-SspA complex and PigR is modulated by the small molecule guanosine

tetraphosphate (ppGpp) (Figure 3.1). The ability of PigR to directly interact with the

MglA-SspA complex had previously been shown using a version of the *E. coli* two-

hybrid assay, referred to as the bridge-hybrid assay, that allows for the detection of

interactions between three or more proteins (Charity et al., 2009). We were interested in

further studying the involvement of ppGpp in modulating the interaction between the

MglA-SspA complex and PigR by determining if ppGpp is also required for PigR to

interact with the MglA-SspA complex in the bridge-hybrid assay in *E. coli*.

To determine if ppGpp is necessary to detect an interaction between the MglA-

SspA complex and PigR in the *E. coli* bridge-hybrid assay, we generated a version of

the *E. coli* reporter strain that cannot produce ppGpp. This strain, referred to as ppGpp°,

has deletions of the genes *relA* and *spoT*, which encode the two proteins responsible

for synthesizing ppGpp in *E. coli*. The ability of the MglA-SspA complex and PigR to

interact in the absence of ppGpp in the *E. coli* bridge-hybrid assay was then tested

using the ppGpp° reporter strain. The *E. coli* bridge-hybrid assay, which is a modified

version of the two-hybrid assay, is used to detect an interaction between a protein and a

protein complex (Figure 3.2A). In the bridge-hybrid assay, *F. tularensis* SspA is fused to

the ω subunit of *E. coli* RNAP, allowing SspA to become tethered to *E. coli* RNAP

through the ω moiety of the fusion protein. PigR is fused to a Zinc-finger DNA binding
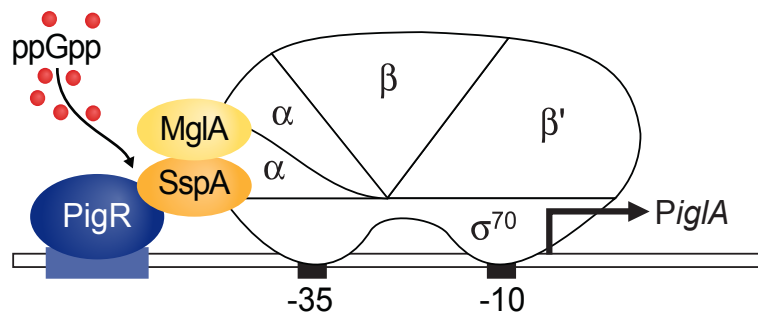
Figure 3.1 Model of virulence gene regulation by MglA, SspA, PigR, and ppGpp In this model, the MglA-SspA complex interacts directly with DNA-bound PigR to positively regulate virulence gene expression in *F. tularensis*. ppGpp, either directly or indirectly, promotes the interaction between the MglA-SspA complex and PigR.
Figure adapted from Charity et al., 2009.

protein called Zif. SspA-ω and PigR-Zif are then expressed alongside *F. tularensis* MglA in an *E. coli* reporter strain containing a Zif binding site positioned upstream of a promoter driving expression of a *lacZ* reporter gene. The interaction between SspA-ω and MglA tethers the MglA-SspA complex to RNAP. DNA bound PigR interacts with the RNAP associated MglA-SspA complex and stabilizes the binding of RNAP at the test promoter to increase expression of *lacZ* (Fig. 3.2A) (Charity et al., 2009).

To determine if ppGpp is necessary to detect an interaction between the MglA-SspA complex and PigR in the bridge-hybrid assay, SspA-ω, MglA, and PigR-Zif were expressed in the original *E. coli* reporter strain that can synthesize ppGpp (referred to here as WT) and expressed in the ppGpp° *E. coli* reporter strain that cannot synthesize ppGpp. As shown in Figure 3.2B, the WT reporter strain supported PigR-Zif dependent activation of the *lacZ* reporter gene while the ppGpp° reporter strain did not support PigR-Zif dependent activation of *lacZ*. Therefore, ppGpp is necessary to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay. This is consistent with previous results indicating that ppGpp promotes the interaction between the MglA-SspA complex and PigR in *F. tularensis* (Charity et al., 2009). ppGpp is either directly promoting the interaction between the MglA-SspA complex and PigR or ppGpp is working indirectly through a mechanism that is conserved between *F. tularensis* and *E. coli*.

It is possible that the inability to detect an interaction between the MglA-SspA complex and PigR in the ppGpp° reporter strain is due to an inability of MglA and SspA to interact in this strain. To test this possibility, the ability of MglA and SspA to interact in the ppGpp° *E. coli* reporter strain was tested using the two-hybrid assay. In this assay,

which has been used to show that MglA and SspA directly interact with one another (Charity et al., 2007), *F. tularensis* SspA is fused to the ω subunit of *E. coli* RNAP and MglA is fused to Zif. An interaction between DNA-bound MglA-Zif and RNAP-tethered SspA in the *E. coli* reporter strain, stabilizes the binding of RNAP to the promoter and leads to an increase in *lacZ* expression (Figure 3.2C). As shown in Figure 3.2D, SspA-ω and MglA-Zif are able to interact in both the WT and ppGpp° reporter strains. Although the absolute level of β-galactosidase activity measured for the interaction between SspA-ω and MglA-Zif differs between the two strains, the fold activation by the interaction between SspA-ω and MglA-Zif is similar between the two strains. For the WT reporter strain, the fold activation was about 18-fold at the two highest concentrations of inducer (IPTG) tested. The fold activation for the ppGpp° reporter strain was about 20-fold at the two highest concentrations of IPTG tested. Thus, the ppGpp° reporter strain is not generally defective for detecting protein-protein interactions and the inability of the MglA-SspA complex to detectably interact with PigR in the ppGpp° reporter strain is not due to an inability of MglA and SspA to interact in this strain.
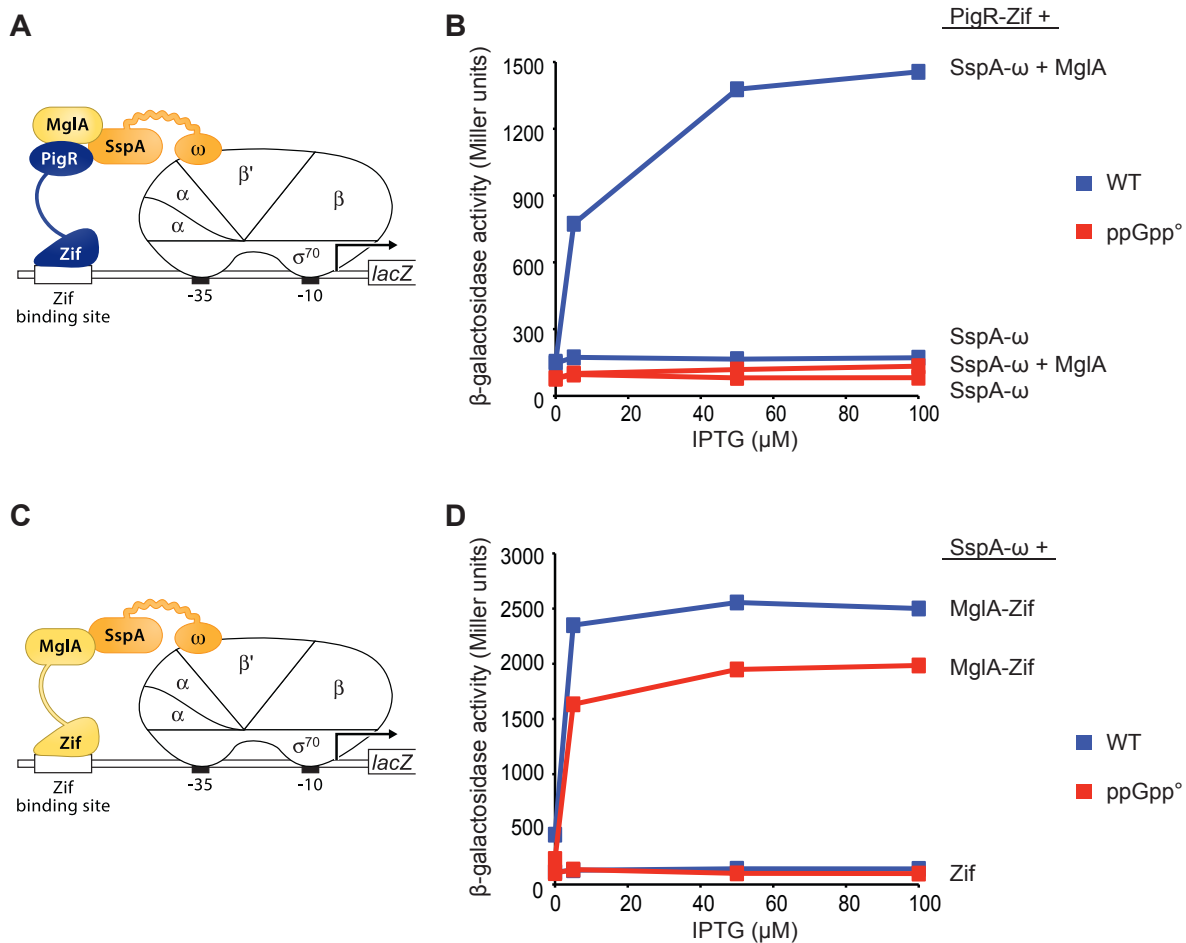
Figure 3.2 ppGpp is required to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay (A) Schematic of the *E. coli* bridge-hybrid assay to detect an interaction between PigR and the MglA-SspA complex. In this assay, SspA is fused to the ω subunit of *E. coli* RNAP and PigR is fused to a zinc-finger containing DNA-binding protein called Zif. The fusion proteins are expressed alongside *F. tularensis* MglA in the strain KDZif1ΔZ which contains a Zif-binding site upstream of a promoter driving expression of *lacZ*. Interaction between SspA-ω and MglA tethers the MglA-SspA complex to RNAP through ω. Interaction between the RNAP-bound MglA-SspA complex and DNA-bound PigR stabilizes binding of RNAP to the promoter and leads to an increase in *lacZ* expression. (B) The ability of SspA-ω and MglA to interact with PigR-Zif in the *E. coli* bridge hybrid assay in a *ΔrelA spoT* version of the *E. coli* reporter strain (referred to as ppGpp°) was tested. The MglA-SspA complex did not detectably interact with PigR in the ppGpp° reporter strain. The MglA-SspA complex was able to interact with PigR in the KDZif1ΔZ *E. coli* reporter strain (referred to as WT). (C) Schematic of the *E. coli* two-hybrid assay to detect an interaction between MglA and SspA. In this assay, SspA-ω and MglA-Zif are expressed in the KDZif1ΔZ reporter strain. Interaction between DNA-bound MglA and RNAP-bound SspA stabilizes binding of RNAP to the promoter and leads to an increase in *lacZ* expression. (D) The ability of SspA-ω and MglA-Zif to interact in the ppGpp° reporter strain compared to the WT reporter strain in the *E. coli* two-hybrid assay was tested. SspA-ω and MglA-Zif were able to interact to a similar extent in the ppGpp° reporter strain and the WT reporter strain (B and D) Assays were performed in the indicated reporter strains. The proteins were expressed in an IPTG-controlled manner from compatible plasmids. Cells were grown in the indicated IPTG concentrations and then assayed for β-galactosidase activity. Results from the KDZif1ΔZ reporter strains (WT) are shown in blue and results from the *ΔrelA spoT* version of the strain (ppGpp°) are shown in red.
(A and C) Adapted from Rohlfing and Dove, 2014.

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Polyphosphate is not required to detect an interaction between the MglA-SspA complex and PigR

A potential pathway conserved between *E. coli* and *F. tularensis* by which ppGpp may be indirectly influencing the interaction between the MglA-SspA complex and PigR is through regulation of polyphosphate levels. The enzyme polyphosphatase (PPX), which removes phosphate molecules from a polyphosphate chain, is inhibited by ppGpp (Figure 3.3A) (Kuroda et al., 1997). In a ppGpp null strain, inhibition of PPX is relieved, allowing the enzyme to degrade polyphosphate. Thus, cells of an *E. coli ΔrelAspoT* mutant strain cannot synthesize ppGpp and effectively do not synthesize polyphosphate either (Kuroda et al., 1997).

We were interested in determining if ppGpp may be indirectly influencing the interaction between the MglA-SspA complex and PigR in our bridge-hybrid assay through an effect on polyphosphate production. To test this, we determined whether PigR could interact with the MglA-SspA complex in cells of an *E. coli* reporter strain that could no longer synthesize polyphosphate. This version of the *E. coli* reporter strain was generated by deleting the gene *ppk*, which encodes polyphosphate kinase (PPK), the enzyme responsible for synthesis of polyphophate in *E. coli*. We also generated a *Δppx* mutant version of the *E. coli* reporter strain in which polyphosphate is made but cannot be degraded.

We then tested the ability of MglA-ω and SspA to interact with PigR-Zif in cells that cannot produce polyphosphate using the bridge-hybrid assay. As shown in Figure 3.3B, the *Δppk* and *Δppx E. coli* reporter strains were able to support PigR-Zif-dependent reporter gene activation in the bridge-hybrid assay to a similar extent as in

the WT reporter strain (Figure 3.3B). MglA-ω and SspA-Zif were also able to interact to the same extent in the two-hybrid assay in the Δppk and Δppx E. coli reporter strains as they were in the WT reporter strain (Figure 3.3C). Therefore, polyphosphate is not required in order for the MglA-SspA complex to interact with PigR in the E. coli bridge-hybrid assay and it is unlikely that ppGpp is acting indirectly through polyphosphate to modulate the interaction between PigR and the MglA-SspA complex.

As an additional test to show that the loss of polyphosphate in the ppGpp° strain does not effect the ability of the MglA-SspA complex to interact with PigR in the bridge-hybrid assay, we generated a Δppx ppGpp° version of the E. coli reporter strain. Since the loss of inhibition of PPX in the ppGpp° strain leads to increased degradation of polyphosphate by PPX, the deletion of ppx should restore polyphosphate levels in the E. coli ppGpp° strain. If polyphosphate is involved in promoting the interaction between the MglA-SspA complex and PigR, we would expect to see the interaction between the MglA-SspA complex and PigR in the Δppx ppGpp° reporter strain restored to levels near that seen in the WT reporter strain. However, as shown in Figure 3.3D, the Δppx ppGpp° reporter strain did not support PigR-Zif dependent reporter gene activation and had similar β-galactosidase activity as was seen in the ppGpp° reporter strain.

We then looked at the ability of MglA and SspA to interact in the Δppx ppGpp° reporter strain to ensure that the inability of the MglA-SspA complex to detectably interact with PigR in this strain was not due to a defect in the ability of MglA and SspA to interact. As seen previously with the ppGpp° reporter strain, MglA-ω and SspA-Zif were able to interact to a similar extent in the Δppx ppGpp° reporter strain as seen in the WT reporter strains (Figure 3.3E). Therefore, the deletion of ppx in the ppGpp° reporter
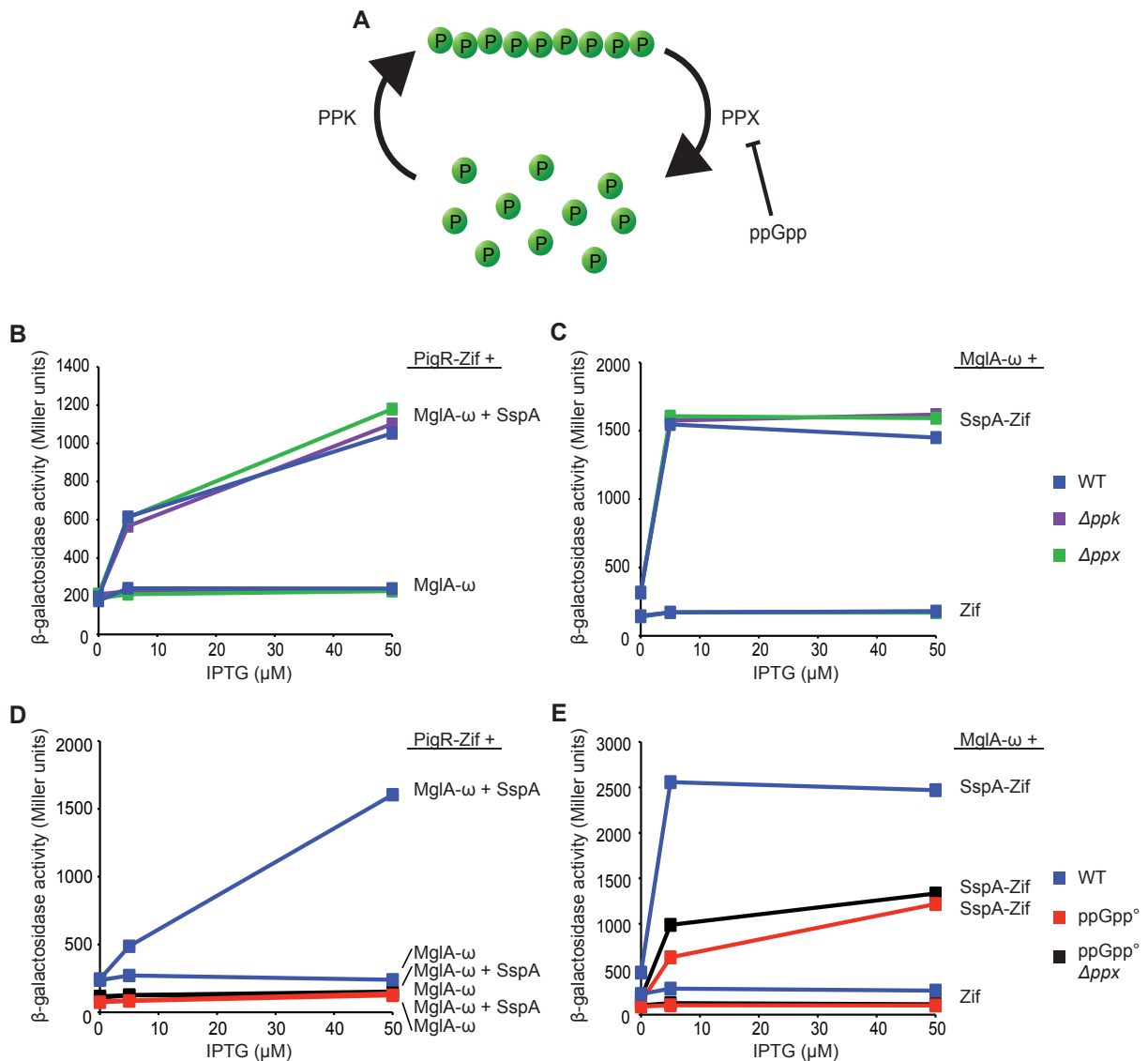
Figure 3.3 ppGpp does not work indirectly through polyphosphate to influence the interaction between the MglA-SspA complex and PigR in the bridge-hybrid assay (A) Polyphosphate is synthesized by the protein polyphosphate kinase (PPK) and degraded by the protein polyphosphate phosphatase (PPX). PPX is inhibited ppGpp. (B) The ability of MglA-ω and SspA to interact with PigR-Zif in the bridge-hybrid assay in *Δppk* and *Δppx* versions of the *E. coli* reporter strain compared to the WT reporter strain was tested. The MglA-SspA complex was able to interact with PigR to a similar extent in the WT (shown in blue), *Δppk* (shown in purple), and *Δppx* (shown in green) reporter strains. (C) The ability of MglA-ω and SspA-Zif to interact in the two-hybrid assay in the *Δppk* and *Δppx* reporter strains compared the WT reporter strain was tested. MglA-ω and SspA-Zif were able to interact to a similar extent in the *Δppk* (purple) and *Δppx* (green) reporter strains as in the WT strain (blue). (D) The ability of MglA-ω and SspA to interact with PigR in the bridge-hybrid assay in a *Δppx relA spoT* version of the *E. coli* reporter strain (referred to as ppGpp° *Δppx*) compared to the WT and ppGpp° reporter strains was tested. The MglA-SspA complex and PigR did not detectably interact in either the ppGpp° (red) or ppGpp° *Δppx* (black) reporter strains. (E) The ability of MglA-ω and SspA-Zif to interact in the WT (blue), ppGpp° (red), and ppGpp° *Δppx* (black) reporter strains was tested. MglA-ω and SspA-Zif were able to interact in all three strains. (B-E) Assays were performed in the indicated reporter strains. Proteins were expressed in an IPTG-controlled manner from compatible plasmids. Cells were grown in the indicated IPTG concentrations and assayed for β-galactosidase activity.

strain does not restore the interaction between the MglA-SspA complex and PigR. These findings support the idea that it is the absence of ppGpp, and not the loss of polyphoshphate, in the ppGpp° reporter strain that leads to the inability to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay.

Polyphosphate is a negative regulator of MglA, SspA, and PigR-regulated virulence genes

We have shown that polyphosphate is not required for the interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay, however the effect of polyphosphate on gene expression in *F. tularensis* has not been investigated. Based on our findings, we would not expect polyphosphate to be required for expression of those genes that are positively regulated by PigR, MglA, SspA, and ppGpp. To test whether polyphosphate influences the expression of MglA-, SspA-, PigR-, and ppGpp-regulated genes, we determined expression of two MglA-, SspA-, PigR-, and ppGpp-regulated genes in a *Δppk* mutant strain of LVS.

To generate a polyphosphate null strain of LVS, we deleted the predicted *ppk* gene (*FTL_0554*). Computational analysis of *F. tularensis* genomes has predicted this gene to encode the only PPK in *F. tularensis* (Rao et al., 2009). The homologous gene in a related strain of *F. tularensis* (*FTN_1472*) has previously been shown to encode a functional polyphosphate kinase (Richards et al., 2008). To determine if polyphosphate has an effect on expression of MglA, SspA, and PigR controlled virulence genes, we measured the expression of two MglA/SspA/PigR controlled virulence genes in WT LVS cells, cells of a *ΔmglA* mutant strain, and cells of a *Δppk* mutant strain. RNA was

isolated from WT LVS, LVS *ΔmglA*, and LVS *Δppk* cells grown to mid-log phase. The relative transcript numbers of two MglA/SspA/PigR controlled genes was determined using qRT-PCR. As previously shown (Charity et al., 2007) (Charity et al., 2009), expression of *iglA* and *FTL_1219* is reduced in cells of the *ΔmglA* mutant strain compared to cells of WT LVS. We predicted that polyphosphate would have little to no effect on expression of these genes positively regulated by MglA, SspA, and PigR and that the expression of *iglA* and *FTL_1219* would be similar between WT LVS and the *Δppk* mutant strain. However, expression of *iglA* and *FTL_1219* increased by about 17-fold and 9-fold, respectively, in the *Δppk* mutant strain compared to WT LVS. These findings indicate that polyphosphate is functioning as a negative regulator of MglA/SspA/PigR controlled virulence genes in *F. tularensis*. The ability of polyphosphate to negatively regulate expression of MglA/SspA/PigR regulated genes was not expected based on data from the *E. coli* bridge- and two-hybrid assays, which indicated that polyphosphate has no detectable effect on the interaction between the MglA-SspA complex and PigR.
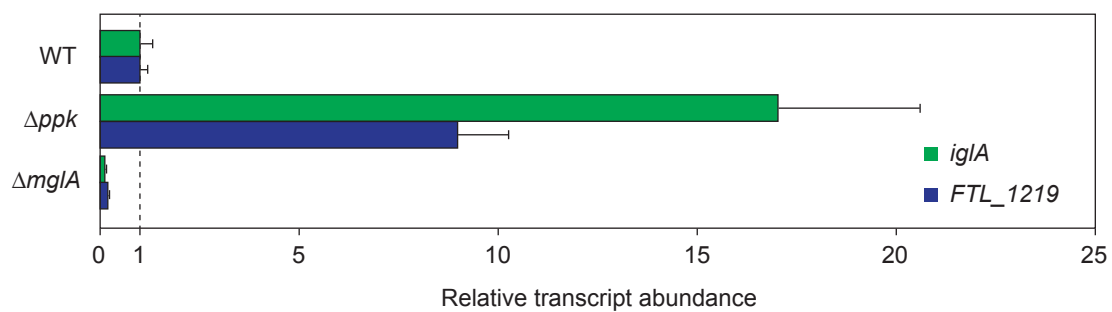
Figure 3.4 Polyphosphate is a negative regulator of virulence gene expression in LVS The effect of polyphosphate on gene expression in LVS was determined by comparing expression of two MglA-regulated virulence genes, *iglA* and *FTL_1219*, in WT LVS, a *Δppk* mutant strain of LVS, and a *ΔmglA* mutant strain of LVS. Quantitative RT-PCR (qRT-PCR) was used to determine the relative transcript abundance of *iglA* and *FTL_1219* in the WT, *Δppk*, and *Δppx* strains of LVS. Expression of both *iglA* (shown in green) and *FTL_1219* (shown in blue) was decreased in the *ΔmglA* mutant strain of LVS compared to WT. Expression of *iglA* and *FTL_1219* was increased in the *Δppk* strain compared to WT. Error bars represent ±1 standard deviation from the mean (calculated using the mean threshold). Figure includes representative data from an experiment performed on biological triplicates.

Discussion

The proteins MglA, SspA, and PigR, along with the small molecule ppGpp, coordinately regulate expression of a set of genes in *F. tularensis*. Previous work has shown that ppGpp promotes the interaction between the MglA-SspA complex and PigR. To further investigate the mechanism by which ppGpp modulates this interaction, we investigated whether ppGpp is required to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay. We found that ppGpp also promotes the interaction between PigR and the MglA-SspA complex in this *E. coli* assay, indicating that either ppGpp is directly involved in promoting this interaction or that it is working indirectly through a mechanism conserved between *F. tularensis* and *E. coli*. We investigated one potential mechanism which may be conserved between *F. tularensis* and *E. coli* through which ppGpp may be working indirectly, by looking at the ability of polyphosphate to modulate the interaction between the MglA-SspA complex and PigR. Polyphosphate, however, was not required to detect an interaction between the MglA-SspA complex and PigR. Furthermore, we found that polyphosphate appears to be a negative regulator of MglA/SspA/PigR regulated virulence genes in LVS.

The results presented in this work further highlight the importance of ppGpp in modulating the interaction between the MglA-SspA complex and PigR to regulate expression of a common set of genes in *F. tularensis*. Although our data do not explicitly address whether ppGpp directly modulates the interaction between the MglA-SspA complex and PigR, the data are consistent with the idea that ppGpp may be directly influencing the interaction between PigR and the MglA-SspA complex. ppGpp is required to detect an interaction between the MglA-SspA complex and PigR in both *F.*

tularensis and in the bridge-hybrid assay in *E. coli*. We have also shown that ppGpp is not working indirectly through polyphosphate to promote the interaction between the MglA-SspA complex and PigR. One mechanism by which ppGpp could influence the interaction between the MglA-SspA complex and PigR is by binding directly to one or more of these proteins. In the previous chapter, we indentified residues in both MglA and SspA which are critical in order for the MglA-SspA complex to interact with PigR. It is possible these residues may be important for binding of ppGpp to the MglA-SspA complex to promote interaction with PigR. One or more of the MglA and SspA mutants that are specifically defective for interaction with PigR may therefore be unable to interact with PigR because they are unable to bind ppGpp.

Our results directly contradict the results from a recent paper from Wrench and colleagues, who found that polyphosphate is required to detect an interaction between MglA and SspA in the *E. coli* two-hybrid assay. They also reported that ppGpp was not required to detect an interaction between the MglA-SspA complex and PigR in the bridge-hybrid (Wrench et al., 2013). Although it is difficult to completely reconcile these contradictory results, our findings that polyphosphate functions as a negative regulator of MglA/SspA/PigR regulated genes in LVS indicate that polyphosphate can not be required for MglA and SspA to interact.

If polyphosphate is indeed a negative regulator of all MglA/SspA/PigR controlled genes, it is possible that polyphosphate may regulate this set of genes by affecting intracellular levels of ppGpp. The PPK encoded in *F. tularensis* is a member of the PPK2 family, indicating that it is able to add phosphate molecules to polyphosphate using either ATP or GTP as a substrate (Brown and Kornberg, 2008; Rao et al., 2009).

It is possible that the loss of polyphosphate in the *Δppk* strain frees up GTP in the cell which may now be used to produce ppGpp. An increase in intracellular ppGpp has previously been shown to correlate with an increase in expression of *iglA*, a gene that is positively regulated by MglA/SspA/PigR (Faron et al., 2013). It is also possible that polyphosphate itself inhibits the interaction between the MglA-SspA complex and PigR, but polyphosphate is not produced in the conditions of the *E. coli* two- and bridge-hybrid assays. It is possible that a combination of an increase in ppGpp pools and the loss of an inhibitory effect of polyphosphate on the interaction between the MglA-SspA complex and PigR in the *Δppk* mutant strain is responsible for the large increase in *iglA* and *FTL_1219* gene expression seen in this strain.

The effects on virulence of *Δppk* mutants of related strains of *F. tularensis* have previously been investigated. It was found that a *Δppk* mutant strain of *F. tularensis* subsp. *novicida* had a similar ability to replicate within macrophages as a wild-type strain, but had decreased virulence in mice (Richards et al., 2008). A *Δppk* mutant of the virulent *F. tularensis* subsp. *tularensis* was also avirulent in mice, but had a slight decrease in the ability to replicate within macrophages (Richards et al., 2008). Although the effect of polyphosphate on gene expression in these *F. tularensis* subspecies was not investigated, we predict that polyphosphate would have a similar inhibitory effect on virulence gene expression in both *F. tularensis* subsp. *novicida* and in *F. tularensis* subsp. *tularensis*. Thus the attenuation of the *Δppk* mutant strains of these related strains may be due to an overproduction of virulence genes. This seemingly contradictory idea that an inhibitor of virulence gene negatively affects virulence

suggests that virulence gene expression must be tightly controlled for *F. tularensis* to cause disease.

As previously mentioned, ppGpp has been found to be important for virulence gene expression in several other pathogens, including *Legionella pneumophila* (Hammer and Swanson, 1999), *Salmonella* (Thompson et al., 2006), *Vibrio cholerae* (Haralalka et al., 2003)*, Mycobacterium tuberculosis* (Primm et al., 2000), *Pseudomonas aeruginosa* (Erickson et al., 2004), and enterohaemorrhagic *E. coli* (EHEC) (Nakanishi et al., 2006). In EHEC, DksA has been shown to be required for expression of ppGpp regulated genes located on a pathogenicity island (Nakanishi et al., 2006). In *Salmonella*, however, DksA is not required for expression of ppGpp regulated virulence genes located on a *Salmonella* Pathogenicity Island (Song et al., 2010). Thus ppGpp has been shown to regulate virulence gene expression in both a DksA dependent and independent manner and little is known about how ppGpp functions to regulate gene expression independently of DksA. At least in the case of *F. tularensis*, ppGpp exerts effects on gene expression by mediating the interaction between other transcription factors. It is possible that ppGpp may regulate interactions between other SspA family members and transcription activators in other bacteria. These findings from *F. tularensis* may be relevant for understanding virulence gene expression in other pathogens.

Materials and Methods

Plasmids, strains, and growth conditions

 Francisella tularensis subspecies *holarctica* strain LVS and the strain LVS *ΔmglA* have been previously described (Charity et al., 2007). All *F. tularensis* strains were grown at 37 °C with aeration 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 indicated 10 µg/mL of kanamycin was used for selection. The *E. coli* strain XL1-blue (Stratagene) was used for plasmid construction. The *E. coli* strain KDZif1ΔZ has been previously described (Vallet-Gely et al., 2005) and was used as the reporter strain for the bacterial two-hybrid and bridge-hybrid assays. When indicated 100 µg/mL carbenicillin, 10 µg/mL tetracycline, or 100 µg/mL spectinomycin was used for selection.

LVS deletion constructs and strains

 The allelic replacement vector to used to generate an inframe deletion of *ppk* (pEX-*ppk*) was generated as previously described (Charity et al., 2007). (Note that the pEX-*ppk* is derivative of the plasmid pEX, not pEX2, and contains a single copy of the *sacB* gene (Charity et al., 2007)). The vector pEX-*ppk* was used to generate the strain LVS *Δppk* using allelic exchange (Golovliov et al., 2003). The strain LVS *Δppk* was confirmed by PCR and southern blotting.

Plasmids for bacterial two-hybrid and bridge-hybrid assays

The plasmids pBR-MglA-ω, pBR-SspA-ω, pACTR-SspA-Zif, pACTR-MglA-Zif, pACTR-AP-Zif, pCL-SspA, pCL-MglA, and pCL have been previously described (Charity et al., 2007; 2009; Rohlfing and Dove, 2014).

E. coli strains for two-hybrid and bridge-hybrid assays

The mutant versions of the *E. coli* two-hybrid and bridge-hybrid reporter strains referred to in the text as *ΔrelA spoT*, *Δppk*, *Δppx*, *Δppx relA spoT* were all derived from the strain FW102 (Whipple, 1998). The strain *relA::kan* was generated by P1-mediated transduction of the allele *relA::kan* from the *relA* mutant strain of the Keio collection (Baba et al., 2006) 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 (Datsenko and Wanner, 2000) in the strain FW102 *relA::kan* to excise the kanamycin resistance gene and generate the strain *ΔrelA*, which contains an inframe deletion of the *relA* gene. The λ red recombinase system (Datsenko and Wanner, 2000) was used to generate the strain *ΔrelA rpoZspoT::cat*. Specifically, polymerase chain reaction (PCR) was used to amplify the *cat* gene from the pKD3 plasmid (Datsenko and Wanner, 2000) with a primer pair that included about 40 nucleotides homology to the 5' flanking region of *rpoZ* and about 40 nucleotides homology to the 3' flanking region of *spoT*. 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 the strain *ΔrelA* containing λ red helper plasmids as described in (Datsenko and Wanner, 2000). The desired *ΔrelA rpoZspoT::cat* mutants were selected for as previously described (Datsenko and

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Wanner, 2000). The previously described F' episome containing the *lacUV5* promoter derivative p*lac*Zif1-61 driving expression of a linked *lacZ* reporter gene (Vallet-Gely et al., 2005) was mated into the strain *ΔrelA rpoZspoT::cat* to generate the strain ARZif1ΔAZT (referred to as the *ΔrelAspoT* mutant version of the *E. coli* two-hybrid reporter strain in the text).

The strain *rpoZ::cat* was generated by P1-mediated transduction of the D*spoS3::cat* allele from the strain KDZif1ΔZ into the recipient strain FW102. P1-mediated transduction of the *ppk::kan* allele from the *ppk* mutant strain of the Keio collection (Baba et al., 2006) into the recipient strain *rpoZ::cat* generated the strain *ppk::kan rpoZ::cat*. A similar strategy was used to generate the strain *ppx::kan rpoZ::cat*. FLP recombinase was expressed in the strains *ppk::kan rpoZ::cat* and *ppx:kan rpoZ::cat* to excise the kanamycin resistant genes and generate the strains *Δppk rpoZ::cat* and *Δppx rpoZ::cat*, respectively. The F' reporter construct (Vallet-Gely et al., 2005) was mated into *Δppk rpoZ::cat* and *Δppx rpoZ::cat* to generate the strains ARZif1ΔKZ and ARZif1ΔXZ, respectively. ARZif1ΔKZ is referred to as the *Δppk* mutant version of the *E. coli* reporter strain in the text of this chapter. ARZif1ΔXZ is referred to as the *Δppx* mutant version of the *E. coli* reporter strain in the text of this chapter.

P1-mediated transduction of the *ppx::kan* allele from the *ppx* mutant strain of the Keio collection (Baba et al., 2006) into the recipient strain *ΔrelA* generated the strain *ppx::kan ΔrelA*. FLP recombinase was expressed in the strain *ppx::kan ΔrelA* to excise the kanamycin resistance gene to generate the strain *Δppx relA*. The λ red recombinase system was used with the PCR product described above to generate the strain *Δppx relA rpoZspoT::cat*. The F' reporter construct (Vallet-Gely et al., 2005) was mated into

the strain *Δppx relA rpoZspoT::cat* to generate the strain ARZif1ΔXAZT (referred to as the *Δppx relA spoT* mutant version of the reporter strain in the text of this chapter). The strains ARZif1ΔKZ, ARZif1ΔXZ, ARZif1ΔAZT, and ARZif1ΔXAZT were all confirmed by southern blotting.

Bacterial two-hybrid and bridge-hybrid assays

The bacterial two-hybrid and bridge-hybrid assays were performed as previously described (Charity et al., 2007; 2009). Cells were grown with aeration at 37°C in LB supplemented with carbenicillin, tetracycline, and IPTG at the indicated concentration for the two-hybrid assay and with carbenicillin, spectinomycin, 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 (Dove and Hochschild, 2004). 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 OD_{600} ~0.25. 10 mL of cells were harvested by centrifugation at 4000 rpm for 20 minutes at 4 °C. RNA was isolated using Tri-Reagent (Ambion) as previously described for *Pseudomonas aeruginosa* (Goldman et al., 2011). RNA quality was determined by gel electrophoresis.

cDNA synthesis using Superscript III Reverse transcriptase (Invitrogen) and qRT-PCR using iTaq Universal SYBR Green supermix (Bio-Rad) and Applied Biosystems StepOnePlus detection system were performed essentially as described in (Charity et al., 2007). The abundance of the *iglA* and *FTL_1219* transcripts were measured relative to that of the *tul4* transcript (Charity et al., 2007). qRT-PCR was performed at least twice on sets of biological triplicates. Data shown are from representative experiments.

Acknowledgements

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Chapter 4

Summary and Future Directions

Summary

In the Gram-negative, intracellular pathogen *F. tularensis*, virulence gene expression is regulated by the MglA-SspA complex, PigR, and ppGpp. These factors function in concert with one another to regulate expression of a common set of genes, including the genes present on the *Francisella* pathogenicity island (FPI) (Baron and Nano, 1998; Brotcke and Monack, 2008; Brotcke et al., 2006; Charity et al., 2009; 2007). The genes present on the FPI, as well as other genes in the MglA-, SspA-, and PigR-regulon, have previously been shown to be necessary for intracellular growth of *F. tularensis* and for virulence in animal models of infection (Lauriano et al., 2004){Charity:2009eh}. Thus, the ability of these factors to regulate expression of genes necessary for intramacrophage growth of the organism, makes them critical regulators of virulence in *F. tularensis*.

Previous work had led to a model of how MglA, SspA, PigR, and ppGpp regulate gene expression in *F. tularensis* (Charity et al., 2009). In this model, the RNAP-associated MglA-SspA complex interacts with the putative DNA-binding protein PigR. The interaction between DNA-bound PigR and the RNAP-associated MglA-SspA complex would stabilize the binding of RNAP to promoters of regulated genes, leading to an increase in transcription initiating from these promoters. Furthermore, according to this model, ppGpp regulates the expression of virulence genes by promoting the interaction between the MglA-SspA complex and PigR, although the model does not specify how this might occur (Charity et al., 2009). The work presented in this dissertation has further refined this model of gene regulation and has increased our

understanding of how MglA, SspA, PigR, and ppGpp coordinately control expression of virulence gene expression in *F. tularensis*.

A crucial point of the model described above is that the MglA-SspA complex directly interacts with PigR. Evidence for this interaction came from a modified version of a bacterial two-hybrid assay, referred to as a bridge-hybrid assay, which allows detection of an interaction between a protein of interest and a protein complex. However, an independent study in *F. novicida* did not find evidence for an interaction between the MglA-SspA complex and FevR (the PigR homolog from *F. novicida*) (Brotcke and Monack, 2008). It was therefore uncertain if PigR functions through a direct interaction with the MglA-SspA complex. To address the physiological role of the interaction between PigR and the MglA-SspA complex observed in the bridge hybrid assay, I identified mutants of MglA and SspA that were specifically defective for interaction with PigR and then tested whether these mutants were functional in *F. tularensis*.

To identify mutants of MglA or SspA that were specifically defective for interaction with PigR, we developed a genetic screen that took advantage of the *E. coli* two-hybrid and bridge-hybrid assays. I used error-prone PCR to generate a library of MglA or SspA mutants and used two rounds of screening to identify the desired mutants. In the first round of the screen, I isolated mutants of MglA or SspA that were no longer able to form a complex with PigR in the bridge-hybrid assay. Then in the second round of the screen, I identified MglA mutants that were still able to interact with SspA, and SspA mutants that could still interact with MglA in the two-hybrid assay. Using this approach, I identified three mutants of MglA [MglA(T47A), MglA(P48S), and

MglA(K101E)] and three mutants of SspA [SspA(K65E), SspA(V105E), and SspA(L130S)] that were specifically defective for interaction with PigR.

To determine where the residues of MglA and SspA that were identified as being important for interaction with PigR may be located on the structure of the MglA-SspA complex, I used the structure of the SspA homolog from *Yersinia pestis* (Hansen et al., 2005) as a model for the MglA-SspA complex. SspA from *Y. pestis* forms a homodimer (Hansen et al., 2005), so I was able to use one monomer of *Y. pestis* SspA as a surrogate for *F. tularensis* MglA and the other monomer of *Y. pestis* SspA as a surrogate for *F. tularensis* SspA. The residues identified in the screens for mutants of MglA and SspA specifically defective for interaction with PigR clustered around a putative pocket formed near the predicted interface between MglA and SspA, indicating that this pocket may be involved in binding PigR.

To determine if residues within the putative pocket were important for the interaction with PigR, I made alanine substitutions at several positions within the putative pocket (specifically Y11, Y63, and R64) and tested the ability of the resulting mutants to interact with SspA and PigR. The MglA mutants with substitutions within the putative pocket were able to interact with SspA to a similar extent as wild-type MglA in the two-hybrid assay. However, these MglA mutants were unable to detectably form a complex with SspA and PigR in the bridge-hybrid assay and were therefore also specifically defective for interaction with PigR. Thus, residues within the putative pocket were also important for the interaction between the MglA-SspA complex and PigR, further highlighting the importance of this pocket for the interaction with PigR. This surface may constitute a binding surface for PigR on the MglA-SspA complex.

I next tested the ability of the MglA and SspA mutants specifically defective for interaction with PigR to function in *F. tularensis*. The ability of the MglA or SspA mutants to complement a *ΔmglA* or *ΔsspA* mutant strain of LVS, respectively, and restore expression of two MglA- and SspA-regulated virulence genes was determined. All of the MglA and SspA mutants were unable to complement the corresponding mutant strains and did not restore expression of two PigR/MglA/SspA-controlled virulence genes, one present on the FPI and another present elsewhere in the genome. Therefore the interaction between the MglA-SspA complex and PigR is required for these proteins to positively regulate virulence gene expression.

I had categorized the MglA mutants specifically defective for interaction with PigR based on their inability to form a complex with PigR and SspA in the bridge-hybrid assay and their ability to still form a complex with SspA in the two-hybrid assay. However, neither of these assays is able to report on the ability of these MglA mutants to interact with RNAP. It is therefore formally possible that the MglA mutants are unable to complement the *ΔmglA* mutant strain of LVS due to an inability to interact with RNAP. In order to test whether the MglA mutants were able to interact with RNAP in *F. tularensis*, I expressed epitope tagged versions of wild-type MglA and two of the MglA mutants in a *ΔmglA* β'-TAP strain of LVS. I used immunoprecipitation to purify β'-TAP and associated proteins. Western blot analysis was used to determine the amount of each of the epitope tagged MglA species that co-purified with β'-TAP. Wild-type MglA and one of the MglA mutants [MglA(T47A)] co-purified to a similar extent with β'-TAP. The other mutant tested [MglA(Y63A)] also co-purified with β'-TAP, although to a lesser extent. Thus, the inability of at least MglA(T47A) [and also likely MglA(Y63A)] to support

MglA-regulated gene expression in *F. tularensis* is not due to a defect in the ability to interact with RNAP.

In this work, I also further examined the ability of ppGpp to promote the interaction between the MglA-SspA complex and PigR. Previous work showed that ppGpp was necessary to detect an interaction between the MglA-SspA complex and PigR in *F. tularensis*. I was interested in determining if ppGpp was also required to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay. To address this, I generated a *ΔrelA ΔspoT* mutant version (referred to as ppGpp°) of the *E. coli* reporter strain for the two-hybrid and bridge-hybrid assays that cannot produce ppGpp. The MglA-SspA complex and PigR did not detectably interact in the bridge-hybrid assay in the ppGpp° reporter strain. Thus, ppGpp is required to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay. MglA and SspA were able to interact to a similar extent in the ppGpp° reporter strain as in the wild-type reporter strain, indicating that the inability of the MglA-SspA complex to interact with PigR in the bridge-hybrid assay is not due to a defect in the interaction between MglA and SspA in this strain background. Therefore, ppGpp is either working directly to promote the interaction between the MglA-SspA complex and PigR or ppGpp works indirectly via a mechanism that is conserved between *F. tularensis* and *E. coli*.

One potential conserved pathway between *F. tularensis* and *E. coli* through which ppGpp may be influencing the interaction between the MglA-SspA complex and PigR is through the regulation of polyphosphate levels. ppGpp inhibits the activity of polyphosphatase (PPX), the enzyme responsible for degradation of polyphosphate. In a

ppGpp° mutant in *E. coli*, PPX is activated and polyphosphate is degraded. A ppGpp°

mutant is therefore also essentially a polyphosphate null mutant. I was interested in

testing whether ppGpp may indirectly regulate the interaction between the MglA-SspA

complex and PigR by regulating accumulation of polyphosphate.

To determine the effects of polyphosphate on the interaction between the MglA-

SspA complex and PigR, I generated a polyphosphate null strain of the *E. coli* reporter

strain by deleting *ppk*, the gene encoding polyphosphate kinase (PPK). I also generated

a *Δppx* mutant version of the reporter strain in which polyphosphate could not be

degraded. I tested the ability of MglA, SspA, and PigR to form a tripartite complex in the

Δppk and *Δppx* mutant versions of the reporter strains compared to the wild-type

reporter strain and found that the MglA-SspA complex and PigR were able to interact in

the bridge-hybrid assay to a similar extent in each strain. MglA and SspA were also able

to interact in the two-hybrid assay in the wild-type, *Δppk*, and *Δppx* versions of the *E.*

coli reporter strain. Therefore, polyphosphate is not required to detect an interaction

between the MglA-SspA complex and PigR in the bridge-hybrid assay.

As an additional test to show that the loss of polyphosphate in the ppGpp°

version of the *E. coli* reporter strain does not explain the inability of the MglA-SspA

complex to interact with PigR in the ppGpp° version of the *E. coli* reporter strain, I

generated a *ΔrelA ΔspoT Δppx* version of the reporter strain. The deletion of *ppx* in the

ΔrelA ΔspoT mutant version of the reporter strain should restore levels of

polyphosphate. However, the MglA-SspA complex was still unable to detectably interact

in the *ΔrelA ΔspoT Δppx* version of the reporter strain and the results from this strain

were similar to those seen in the ppGpp° version of the reporter strain. Therefore, it is

not the loss of polyphosphate in the ppGpp° reporter strain that results in the inability of the MglA-SspA complex and PigR to interact in the absence of ppGpp.

It has previously been shown that a ppGpp° mutant of *F. tularensis* has decreased expression of MglA-, SspA-, and PigR-regulated genes, presumably because PigR does not interact as well with the MglA-SspA complex in the absence of ppGpp (Charity et al., 2009). The effects of polyphosphate on gene expression in *F. tularensis* have not been determined. Since polyphosphate is not necessary to detect an interaction between the MglA-SspA complex and PigR in the bacterial bridge-hybrid assay in *E. coli*, I hypothesized that polyphosphate would not be required for the expression of MglA-, SspA-, and PigR-regulated virulence genes. To test the effects of polyphosphate on gene expression in *F. tularensis*, I generated a *Δppk* mutant strain of LVS and compared the expression of two MglA-regulated virulence genes between a *ΔmglA* mutant strain, a *Δppk* mutant strain, and wild-type LVS. As previously reported (Charity et al., 2007; 2009), expression of the two tested virulence genes was decreased in the *ΔmglA* mutant strain. However, expression of these two virulence genes was greatly increased in the *Δppk* mutant compared to wild-type LVS, indicating that polyphosphate functions as a negative regulator of MglA-, SspA-, and PigR-regulated virulence genes.

My work presented here has furthered our understanding of how the MglA-SspA complex, PigR, and ppGpp coordinately control virulence gene expression in *F. tularensis*. My findings show that a direct interaction between PigR and the MglA-SspA complex is critical to the ability of PigR to exert its regulatory effects. My results also support the idea that the ability of ppGpp to promote the interaction between the MglA-

SspA complex and PigR, either directly or indirectly, may be the main mechanism through which ppGpp regulates virulence gene expression in *F. tularensis*.

Future Directions

The data presented in Chapters 2 and 3 of this dissertation have increased our knowledge of how the MglA-SspA complex, PigR, and ppGpp coordinately control virulence gene expression in *F. tularensis*. There are, however, many remaining questions about the mechanism by which MglA, SspA, PigR, and ppGpp regulate gene expression. Particularly, more work needs to be done to understand how ppGpp promotes the interaction between the MglA-SspA complex and PigR and how polyphosphate functions as a negative regulator of virulence gene expression in *F. tularensis*. Discussed below are potential future studies which could help answer some of these open questions.

Does ppGpp bind directly to MglA, SspA, or PigR?

The ability of ppGpp to modulate the interaction between the MglA-SspA complex and PigR in the bacterial bridge-hybrid assay suggests that ppGpp may be working directly to promote this interaction. A potential mechanism by which ppGpp could be directly influencing the interaction between the MglA-SspA complex and PigR is by binding to MglA, SspA, and/or PigR. A photo-crosslinkable, radiolabeled version of ppGpp, 6-thio-P^{32}-ppGpp, has previously been used to localize binding of ppGpp to *E. coli* RNAP (Ross et al., 2013; Toulokhonov et al., 2001). Similar techniques using 6-thio-P^{32}-ppGpp could be used to determine if ppGpp is able to bind to purified MglA,

SspA, and/or PigR *in vitro*. Previous attempts in our lab to purify PigR from *E. coli* have indicated that PigR is insoluble when expressed in *E. coli*. Expressing the MglA-SspA complex alongside PigR in *E. coli* may increase the solubility of PigR and it may be necessary to attempt to purify PigR along with the MglA-SspA complex. By comparing the ability of the MglA-SspA complex to bind 6-thio-P^{32}-ppGpp and the ability of the MglA-SspA complex along with PigR to bind 6-thio-P^{32}-ppGpp, it could be determined whether PigR contributes to the ability of the tripartite complex to bind ppGpp.

If the MglA-SspA complex does bind to ppGpp, it would be interesting to test if any of the MglA or SspA mutants specifically defective for interaction with PigR are also defective for ppGpp binding. In Chapter 3, I found that ppGpp is required to detect an interaction between the MglA-SspA complex and PigR in the *E. coli* bridge-hybrid assay. It is therefore possible that one or more of the MglA or SspA mutants identified in Chapter 2 may be unable to bind PigR because they are unable to bind ppGpp. Tyrosine and arginine residues have previously been shown to be important for ppGpp binding to other proteins (Artsimovitch et al., 2004; Kanjee et al., 2011; Ross et al., 2013; Vrentas et al., 2008). The three MglA mutants specifically defective for interaction with PigR with substitutions within the predicted pocket have substitutions at tyrosine and arginine residues. It is possible that one of more of these mutants could be defective for binding ppGpp.

Can the effects of ppGpp on expression of MglA-, SspA-, and PigR-regulated genes be recapitulated in vitro?

The ability of ppGpp to directly control the transcription of rRNA genes from *E. coli* has been studied using *in vitro* transcription assays for many decades (Bartlett et al., 1998; Oostra et al., 1977; Ross et al., 2013). The ability of ppGpp to directly influence the transcription of MglA-, SspA-, and PigR-regulated genes could also be tested in an *in vitro* transcription assay using purified *F. tularensis* RNAP, purified MglA-SspA complex, and purified PigR (if soluble). If ppGpp is able to directly promote the interaction between the MglA-SspA complex and PigR, I would expect that *in vitro* transcription reactions containing RNAP, purified MglA-SspA complex, purified PigR, and ppGpp would have higher levels of transcription from promoters of MglA-, SspA-, and PigR- regulated genes (such as the promoter for *iglA*) than from promoters of non-regulated genes. I would also expect that *in vitro* transcription reactions containing RNAP, purified MglA-SspA complex, purified PigR, and ppGpp would have higher levels of transcription than reactions containing only RNAP, reactions containing RNAP and ppGpp, reactions containing RNAP and the MglA-SspA complex, or reactions containing RNAP, the MglA-SspA complex, and ppGpp. Determining if ppGpp can activate transcription in an *in vitro* transcription assay could provide strong evidence that ppGpp is acting directly to regulate expression of MglA-, SspA-, and PigR-regulated genes.

What is the polyphosphate regulon in F. tularensis?

In Chapter 3, I found that polyphosphate can function as a negative regulator of two MglA-, SspA-, and PigR-regulated genes. It is unknown whether polyphosphate inhibits all MglA-, SspA-, and PigR-regulated genes and if polyphosphate regulates

genes outside the MglA, SspA, and PigR regulon. In order to identify all genes whose expression is influenced by *ppk* (and thus polyphosphate), RNA-Seq could be used to obtain a genome wide analysis of the differences in transcription between wild-type LVS, *Δppk, ΔmglA*, and *ΔrelA ΔspoT* mutant strains of LVS. Identifying the full set of genes whose expression is influenced by *ppk* may help elucidate a potential mechanism through which polyphosphate is functioning. Although MglA, SspA, and PigR regulate essentially the same set of genes, there is a slight difference between the set of genes regulated by PigR and the set of genes regulated by the MglA-SspA complex. PigR appears to function only as a positive regulator, while MglA and SspA can exert, in a PigR-independent manner, negative effects on the expression of a subset of genes (K. Ramsey and S.L.D. unpublished results). If polyphosphate also regulates expression of this set of genes negatively regulated by the MglA-SspA complex, it would suggest that polyphosphate might affect the MglA-SspA complex. However, if polyphosphate regulates expression of the genes positively regulated by MglA, SspA, and PigR (and not the genes negatively regulated by the MglA-SspA complex), it could suggest that polyphosphate influences the ability of the MglA-SspA complex and PigR to interact. Two potential ways that polyphosphate could inhibit gene expression though modulation of the interaction between the MglA-SspA complex and PigR are by inhibiting the interaction between the MglA-SspA complex and PigR or by decreasing ppGpp pools in the cell.

It is also possible that polyphosphate regulates a set of genes outside of those genes regulated by MglA, SspA, and PigR and that polyphosphate may have both positive and negative effects on gene expression in *F. tularensis*. If that is the case,

comparing RNA-Seq data identifying the set of genes influenced by *ppk* with ChIP-Seq data from the lab identifying promoters associated with over 30 transcription factors from *F. tularensis* (K. Ramsey and S.L.D.) unpublished data), may provide a hint as to how polyphosphate is regulating gene expression in *F. tularensis.*

Does polyphosphate negatively regulate gene expression in F. tularensis through an effect on ppGpp levels?

It is possible that polyphosphate may negatively regulate expression of MglA-, SspA-, and PigR-regulated genes by decreasing the intracellular ppGpp pool. *F. tularensis* encodes a PPK2 homolog; PPK2 family members are able to generate polyphosphate using either ATP or GTP (Rao et al., 2009). The use of GTP by PPK2 to generate polyphosphate may decrease the amount of GTP available in the cell to synthesize ppGpp. To determine if PPK influences the amount of ppGpp produced in *F. tularensis*, the amount of ppGpp in the cell can be measured by growing wild-type LVS, *ΔrelA ΔspoT*, and *Δppk* mutant strains in the presence of radiolabeled phosphate. Nucleotides extracted from these strains would be analyzed by thin layer chromatography (TLC) to determine the relative amount of ppGpp and GTP present in the wild-type, *ΔrelA ΔspoT*, and *Δppk* mutant strains of LVS. If PPK does influence the amount of ppGpp present in the cell, I would expect the *Δppk* mutant strain to produce more ppGpp than wild-type LVS grown in the same conditions. *F. tularensis* PPK2 could also be ectopically expressed in wild-type LVS to attempt to see whether it might result in an increase in the amount of polyphosphate produced in the cell. It is possible that a

strain that overproduces polyphosphate would have lower levels of ppGpp than wild-type LVS.

Another prediction of the hypothesis that PPK2 affects ppGpp pools is that a PPK1 enzyme, which only produces polyphosphate from ATP, would not have an effect on ppGpp levels in *F. tularensis*. To test this, PPK1 from *E. coli* could be ectopically expressed in a *Δppk* mutant strain of LVS to restore polyphosphate levels in these cells. If PPK2 does decrease ppGpp pools through a decrease in cellular GTP, I would expect that a *Δppk* mutant strain expressing *E. coli* PPK1 would have similar ppGpp levels as the *Δppk* mutant strain. The ability of *E. coli* PPK1 to function and produce polyphosphate in *F. tularensis* would need to be determined by comparing polyphosphate levels between a wild-type strain of LVS and a *Δppk* mutant strain of LVS expressing *E. coli* PPK1.

The ability of PPK2 to influence ppGpp levels can also be tested using the *E. coli* two-hybrid and bridge-hybrid assays. Since the interaction between the MglA-SspA complex and PigR is dependent upon ppGpp in the *E. coli* bridge-hybrid assay, a decrease in ppGpp in the *E. coli* reporter strain would be predicted to result in a decrease in the ability of MglA, SspA, and PigR to form a complex in the bridge-hybrid assay. If PPK2 does influence the level of intracellular GTP pools (and consequently ppGpp pools), ectopic expression of *F. tularensis* PPK2 in the *Δppk* mutant version of the *E. coli* reporter strain would be predicted to lead to a decrease in ppGpp and a decrease in the ability of MglA, SspA, and PigR to form a complex in this strain.

It is also a possibility that polyphosphate may negatively regulate expression of MglA-, SspA-, and PigR-regulated genes by positively regulating a negative regulator of

MglA, SspA, and/or PigR. However, a negative regulator of MglA, SspA, and PigR has not yet been identified.

Concluding remarks

In *F. tularensis*, MglA and SspA, two stringent starvation protein A family members, and PigR, a putative DNA binding protein, work in concert with the small molecule ppGpp to control a set of genes required for intramacrophage growth and survival of the organism. In this work, I have further refined the model for how these factors coordinately control virulence gene expression in *F. tularensis*. A direct interaction between the RNAP-associated MglA-SspA complex and PigR is necessary for PigR to exert its regulatory effects and promote expression of MglA-, SspA-, and PigR-regulated genes. The main function of the RNAP-associated MglA-SspA complex may be to provide a point of contact on RNAP for PigR, a transcription activator. An important role for ppGpp in regulated virulence gene expression is to promote the interaction between the MglA-SspA complex and PigR. The ability of ppGpp to modulate the interaction between the MglA-SspA complex and PigR integrates nutritional cues into the regulatory network of virulence gene expression in *F. tularensis*. I have also identified the molecule polyphosphate as a negative regulator of MglA-, SspA-, and PigR-regulated virulence genes in *F. tularensis*. The data presented in this dissertation provide a framework to better understand the mechanism by which MglA, SspA, PigR, and ppGpp regulate gene expression in *F. tularensis* and may provide insight into how SspA family members and ppGpp function to regulate gene expression in other organisms.

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