Coordinate Control of Virulence Gene Expression in *Francisella tularensis* Involves Direct Interaction between Key Regulators

Amy E. Rohlfing, Simon L. Dove

Division of Infectious Diseases, Boston Children's Hospital, Harvard Medical School, Boston, Massachusetts, USA

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.

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 10 organisms constituting an infectious dose (1). 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 has 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 (2).

During infection, *F. tularensis* primarily infects and replicates within macrophages. This ability to replicate within macrophages is thought to be essential for virulence (3–6). One of the first genes that was found to be necessary for this process is *mglA* (macrophage growth locus A) (7). 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 contained on the *Francisella* pathogenicity island (FPI) (8–10). 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 (11–13).

MglA is an ortholog of stringent starvation protein A (SspA), an RNA polymerase (RNAP)-associated protein from *Escherichia coli* that is thought to play a role in gene regulation under starvation conditions, although the mechanism by which SspA influences gene expression is unclear (7, 14, 15). SspA orthologs in several other pathogens have also been shown to be important for virulence (16–20). *F. tularensis* genes encode 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* (10). It is thought that interaction with RNAP is necessary for the function of both MglA and SspA (10).

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 regula-

tors of the MglA- and SspA-controlled *iglA* gene present on the FPI (21). 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 (22). PigR (FevR) regulates expression of the same set of genes as that regulated by the MglA-SspA complex, suggesting that PigR, MglA, and SspA function together to regulate gene expression (21, 22). 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 (21). Furthermore, an independent study in *F. novicida* did not find evidence for an interaction between the MglA-SspA complex and FevR (22). 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.

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Address correspondence to Simon L. Dove, simon.dove@childrens.harvard.edu.

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MATERIALS AND METHODS

Plasmids, strains, and growth conditions. *Francisella tularensis* subsp. *holarctica* strain LVS and the strains LVS Δ *mglA* and LVS Δ *sspA* have been previously described (10). 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% IsoVitalX (BD Biosciences) or on cysteine heart agar (CHA; Difco) supplemented with 1% hemoglobin solution (BD Biosciences). When indicated, 5 μ g/ml of kanamycin or 5 μ g/ml of nourseothricin was used for selection. The *E. coli* strains DH5 α F'IQ (Invitrogen) and XL1-Blue (Stratagene) were used for plasmid construction. The *E. coli* strain KDZif1 Δ Z has been previously described (23) and was used as the reporter strain for the bacterial two-hybrid and bridge-hybrid assays. When indicated, 100 μ g/ml carbenicillin, 10 μ g/ml tetracycline, or 100 μ g/ml spectinomycin was used for selection.

Francisella strain for TAP immunoprecipitation. The strain LVS Δ *mglA* 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 Δ *mglA* mutant strain which has been previously described (10). 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 (10), was used to integrate the TAP tag sequence downstream of *rpoC*. pEX-RpoC-TAP was electroporated into electrocompetent LVS Δ *mglA* 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.

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, and pCL have been previously described (10, 21). The plasmid pCL-MglA, which directs the synthesis of LVS MglA, carries *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 (24). 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-MglA(R64A)- ω . These plasmids direct the synthesis of MglA(Y11A)- ω , MglA(Y63A)- ω , and MglA(R64A)- ω , respectively.

Plasmids for complementation analyses. 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 (10). Plasmid pF is the corresponding empty vector control and has been described previously (10). Plasmids pF-MglA-V and pF-SspA-V direct the synthesis of LVS MglA or LVS SspA, respectively, with a vesicular stomatitis virus 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 Shine-Dalgarno (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 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, respectively, except that they confer resistance to nourseothricin. The plasmid pF-MglA has been previously described (10). 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 (21).

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 KDZif1 Δ Z cells along with plasmids pCL-SspA and pACTR-PigR. Cells were plated on LB containing carbenicillin, spectinomycin, tetracycline, X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 50 μ g/ml), IPTG (isopropyl- β -D-thiogalactopyranoside; 50 μ g/ml), and the X-Gal inhibitor tPEG (2-phenylethyl β -D-thiogalactoside; 125 μ g/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 for only 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 KDZif1 Δ Z cells along with pACTR-SspA-Zif. These cells were then plated on LB containing carbenicillin, tetracycline, X-Gal (50 μ g/ml), and IPTG (50 μ g/ml). Approximately 40 colonies were selected in which cells expressed levels of *lacZ* similar to those in 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 KDZif1 Δ Z 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 KDZif1 Δ Z cells along with the vectors pCL-MglA and pACTR-PigR-Zif. Then, approximately 100 candidate mutants were screened in KDZif1 Δ Z cells containing plasmid pACTR-MglA-Zif. Finally, 40 colonies were selected in which cells expressed *lacZ* levels similar to those in 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 (10, 21). 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 CHCl₃ and assayed for β -galactosidase activity as previously described (25). 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 (26) 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 *Yersinia pestis* SspA using the 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* SspA correspond to the residues identified in MglA and LVS SspA as being important for interaction with PigR.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR). LVS cells were grown in liquid culture (50 ml) in the presence of kanamycin with aeration at 37°C until cultures reached an optical density at 600 nm (OD₆₀₀) of ~0.4. Ten milliliters of cells was harvested by centrifugation at 4,000 rpm for 20 min at 4°C. RNA was isolated using Tri reagent (Ambion) as previously described for *Pseudomonas aeruginosa* (27). RNA quality was determined by gel electrophoresis. Three micrograms of RNA from each sample was 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 III reverse transcriptase (Invitrogen) and qRT-PCR using iTaq Universal SYBR green supermix (Bio-Rad) and the Applied Biosystems StepOnePlus detection system were performed essentially as described in reference 10. The abundances of the *iglA* and *FTL_1219* transcripts were measured relative to that of the *tul4* transcript (10). 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 (28). Cells were grown in liquid culture (100 ml) in the presence of nourseothricin with aeration at 37°C until cultures reached an OD₆₀₀ of ~0.4. Cells were harvested by centrifugation at 4,000 rpm for 20 min at 4°C. Cells were resuspended in 5 ml buffer 1 (20 mM K-HEPES, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol [DTT], 10% glycerol) and then harvested by centrifugation. Cells were resuspended in 500 μ l buffer 1 containing a protease inhibitor cocktail (cOmplete Mini, EDTA-free protease inhibitor cocktail; Roche) and then lysed using sonication. Samples were centrifuged at 13,000 rpm at 4°C for 20 min. The lysate was transferred to a fresh tube and centrifuged at 13,000 rpm at 4°C for 5 min. The lysates were removed, and the salt concentration of the lysates was adjusted to 10 mM Tris-HCl, pH 8 (USB), 150 mM NaCl

(Sigma), and 0.1% NP-40 (NP-40 alternative; Calbiochem) for subsequent steps. Lysates were added to 75 μ l 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% NP-40). Samples were incubated with rocking for 2 h at 4°C. Beads were pelleted by centrifugation at 10,000 rpm for 30 s and then washed five times with 1 ml IPP150 buffer. Beads were resuspended in 200 μ l sample loading buffer (1 $\times$ NuPAGE Novex LDS sample buffer, 50 mM DTT) and boiled for 10 min 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 to 12% Bis-Tris protein gels (Novex) with NuPAGE morpholinoethanesulfonic acid (MES) running buffer (Novex). For complementation experiments, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot dry blotting system (Invitrogen). Membranes were blocked with 25 ml SuperBlock blocking buffer in Tris-buffered saline (TBS) (Thermo Scientific) with 250 μ l Surfact-Amps 20 (Thermo Scientific) 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, and Daniel L. Clemens, University of California Los Angeles). Goat polyclonal anti-rabbit IgG conjugated with horseradish peroxidase (Thermo Scientific) was used to detect proteins. Proteins were then visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

To quantify the immunoblot data of the immunoprecipitated (IP) material, initial immunoblot assays were performed using a serial dilution of the IP material to determine the dynamic range of the signal for quantification. For the immunoblot assays used for quantification, 10 μ l of a 1:8 dilution of the IP material was run on an SDS-PAGE NuPAGE 4 to 12% Bis-Tris protein gel (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 described 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 (Thermo Scientific), and the VSV-G signal was visualized using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). Blots were imaged using the ChemiDoc XRS+ system (Bio-Rad), and the intensities of the PAP and VSV-G signals were quantified using ImageQuant TL v2005 software (Amersham Biosciences).

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 (21). 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) (21) (Fig. 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 (21) (Fig. 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

FIG 1 Genetic assays for detecting formation of the PigR-MglA-SspA complex and for detecting formation of the MglA-SspA complex. (A) *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 KDZif1 Δ Z. 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 KDZif1 Δ Z. Interaction between the DNA-bound Zif-SspA fusion protein and the RNAP-tethered MglA- ω fusion protein leads to an increase in *lacZ* expression.

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 (Fig. 1A). In the second screening step, we used a bacterial two-hybrid assay to identify those MglA mutants from the first 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 (10) (Fig. 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 medium 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 medium containing X-Gal) were isolated.

Three MglA mutants with single amino acid substitutions were isolated using this genetic screen. These mutants had a threonine 17-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 Fig. 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 Fig. 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.

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* (29). 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 (26) 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 (Fig. 3A) and the SspA monomer (data 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 Fig. 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 (29).

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 Fig. 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 (Fig. 2D). Amino acid substitutions Y11A, Y63A, and R64A in MglA therefore interfere with the

FIG 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 to 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.

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 (Fig. 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 that 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 levels similar to those of the

wild-type SspA-ω fusion protein in the two-hybrid assay (Fig. 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 (Fig. 4A). All three of the mutant SspA-ω fusion proteins did not support PigR-Zif-dependent transcription activation in the bridge-hybrid assay (Fig. 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.

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 (Fig. 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 illustrates the importance of this predicted pocket located at the MglA-SspA interface for the interaction with PigR.

FIG 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 an 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 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.

MglA and SspA mutants that are specifically defective for interaction with PigR are unable to complement the respective $\Delta mglA$ or $\Delta sspA$ 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 that we had identified to restore the expression of MglA-controlled genes in cells of a $\Delta mglA$ or $\Delta sspA$ 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 $\Delta mglA$ mutant strain alongside an empty vector control. RNA was isolated from plasmid-containing cells that were grown to mid-log phase, and the abundance of transcripts from two different PigR/MglA/SspA-controlled virulence genes was determined by qRT-PCR. The results depicted in Fig. 5A show that wild-type MglA with a C-terminal VSV-G epitope tag (MglA-V) was able to complement cells of the $\Delta mglA$ 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

FIG 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 and 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.

LVS $\Delta mglA$ mutant strain. Similarly, Fig. 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 $\Delta mglA$ mutant strain, unlike MglA-V.

A similar approach was used to determine if the SspA mutants were able to complement a $\Delta sspA$ 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 $\Delta sspA$ mutant strain of LVS along with an empty vector. Results from qRT-PCR analyses (Fig. 5E) show that VSV-G epitope-tagged wild-type SspA (SspA-V) is able to complement cells of the $\Delta sspA$ mutant strain and restore expression of *FTL_1219* and *iglA* to levels near those found in cells of 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 $\Delta sspA$ mutant strain containing the empty vector (Fig. 5E).

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 that SspA(K65E) and SspA(V105E) were as abundant as wild-type SspA (Fig. 5B, D, and F). SspA(L130S) was also expressed, although it was less abundant than the wild-type protein (Fig. 5F). Thus, all of the MglA mutants that were specifically defective for interaction with PigR were unable to complement cells of a $\Delta mglA$ 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 $\Delta sspA$ 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 $\Delta mglA$ 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 (Fig. 6A). RNAP was then isolated from these cells by immunoprecipitation of β', and the relative amount of MglA-V that copurified 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 $\Delta mglA$ 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 (Fig. 6B). The Western blot data 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 Fig. 6C, both MglA(T47A)-V and MglA(Y63A)-V copurified 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 that of the 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 $\Delta mglA$ strain cannot be explained by the inabilities of these

FIG 5 MglA or SspA mutants that are specifically defective for interaction with PigR are unable to complement the respective $\Delta mglA$ or $\Delta sspA$ mutant strains of LVS. (A) The ability of MglA(T47A)-V, MglA(P48S)-V, and MglA(K101E)-V to complement the LVS $\Delta mglA$ mutant strain was determined by testing the ability of these mutants to restore expression of two MglA-regulated genes, *FTL_1219* and *iglA*. 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 $\Delta mglA$ mutant strain (indicated as $\Delta mglA$), with transcripts being as abundant as those in cells of the LVS $\Delta mglA$ mutant strain containing the empty vector pF. The 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 standard deviation from the value (calculated using the mean threshold cycle). (B) The abundance of MglA-V, MglA(T47A)-V, MglA(P48S)-V, and MglA(K101E)-V was determined by Western blotting 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 $\Delta mglA$ mutant strain as assessed by qRT-PCR. The 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 $\Delta mglA$ 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 $\Delta sspA$ mutant strain (indicated as $\Delta sspA$) to levels similar to those 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 $\Delta sspA$ mutant strain, with transcripts being as abundant as those in cells of the LVS $\Delta sspA$ mutant strain containing the empty vector pF. The 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 $\Delta sspA$ mutant strain of LVS. SspA(L130S)-V was also expressed in cells of the $\Delta sspA$ mutant strain of LVS, although the abundance was less than that of wild-type SspA-V.

FIG 6 MglA mutants containing substitution T47A or Y63A interact with RNAP in LVS. (A) Schematic of the experimental setup 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 $\Delta mglA$ 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 amounts of β' -TAP and MglA-V isolated from strains expressing MglA(T47A)-V, MglA(Y63A)-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 antiperoxidase 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 blotting data 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 copurified with β' -TAP. Error bars represent the standard deviations in the relative amount of MglA-V purified compared to β' -TAP between four biological replicates.

FIG 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.

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.

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* (21, 22). 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 (21, 22, 30–32). 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 $\Delta mglA$ mutant strain of LVS or a $\Delta sspA$ 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.

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 (Fig. 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 (Fig. 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 (21, 33, 34). Previous work suggested that ppGpp regulates the same set of genes as that regulated by MglA, SspA, and PigR and promotes the interaction between the MglA-SspA complex and PigR in *F. tularensis* (21). Another recent study found that polyphosphate binds to the MglA-SspA complex *in vitro* (33). It could be that ppGpp or polyphosphate interacts directly with the MglA-SspA complex to promote the interaction with PigR and that 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 (10). 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 (21, 22). 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 (Fig. 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* (16), *Yersinia enterocolitica* (17), *Vibrio cholerae* (18), enterohemorrhagic *E. coli* (20), and *Pseudomonas aeruginosa* (19), 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 (35). 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.

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