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Identification of *fevR*, a Novel Regulator of Virulence Gene Expression in *Francisella novicida*▽†

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Francisella tularensis **infects wild animals and humans to cause tularemia. This pathogen targets the cytosol of macrophages, where it replicates using the genes in the *Francisella* pathogenicity island (FPI). Virulence gene regulation in *Francisella* is complex, but transcriptional regulators MglA and SspA have been shown to regulate the expression of approximately 100 genes, including the entire FPI. We utilized a *Francisella novicida* transposon mutant library to identify additional regulatory factors and identified five additional genes that are essential for virulence gene expression. One regulatory gene, FTN_0480 (*fevR*, *Francisella e*ffector of *v*irulence *r*egulation), present in all *Francisella* species, is required for expression of the FPI genes and other genes in the MglA/SspA regulon. The expression of *fevR* is positively regulated by MglA. However, constitutive expression of *fevR* in an *mglA* mutant strain did not restore expression of the MglA/SspA regulon, demonstrating that *mglA* and *fevR* act in parallel to positively regulate virulence gene expression. Virulence studies revealed that *fevR* is essential for bacterial replication in macrophages and in mice, where we additionally show that *fevR* is required for the expression of genes in the MglA/SspA regulon in vivo. Thus, *fevR* is a crucial virulence gene in *Francisella*, required for the expression of virulence factors known to be essential for this pathogen's subversion of host defenses and pathogenesis in vivo.**

Francisella tularensis is a gram-negative facultative intracellular pathogen that causes the zoonotic disease tularemia. *F. tularensis* can be contracted from ticks, contaminated water, or infected mammals, particularly rabbits. Since *F. tularensis* can survive in a wide range of environments, the bacterium must be able to sense and respond appropriately to each niche by altering expression of survival genes. Indeed, it is known that stresses, such as nutrient and iron limitation, influence the expression of *Francisella* virulence genes (7, 12, 20). During infections, *F. tularensis* replicates in host cells, and the virulence of the pathogen is linked to its ability to replicate intracellularly (1). Intracellular bacteria initially reside within a vacuole that stains with cellular markers typical of a late endosome/early lysosome, but the vacuole does not acidify appreciably and does not contain cathepsin D, suggesting that the vacuole is modified (10, 15, 26, 27). *F. tularensis* escapes this vacuole within 5 h and replicates to high numbers in the cytosol (11, 15). After replication in the cytosol, *F. tularensis* is found in an autophagic vacuole that fuses with lysosomes, though without harm to the bacteria (9). Thus, it seems likely that *F. tularensis* senses specific phases of the intracellular life cycle and alters its gene expression accordingly.

Francisella tularensis subsp. *tularensis* is the most virulent subspecies of *Francisella* and is found throughout North America and in parts of Europe. *Francisella novicida*, a close relative of *F. tularensis*, causes severe disease in immunocompromised humans but rarely does so in immunocompetent individuals (24). However, *F. novicida* has the same families of virulence genes as *F. tularensis*, causes a similar disease in mice, and thereby serves as a good experimental model with which to study *Francisella* pathogenesis (18, 23).

The transcriptional regulator macrophage growth locus A (*mglA*) was discovered in *F. novicida* (4). *mglA* is upregulated inside macrophages 1.5 h postinfection (3), suggesting that the MglA regulon represents a subset of *Francisella* genes involved in interactions with macrophages. Indeed, MglA regulates the expression of 102 genes, several of which are necessary for intracellular replication, including a large cluster of 17 genes called the *Francisella* pathogenicity island (FPI) (6, 19). MglA interacts with stringent starvation protein A (SspA) to form a heterodimer that binds to RNA polymerase, consistent with a direct role in transcriptional regulation (8). In addition, it was recently reported that the two-component regulator *pmrA* regulates the expression of the FPI and other virulence genes (22). However, MglA does not regulate *pmrA*, nor does PmrA regulate MglA. During growth in broth, *mglA* and *sspA* mRNAs are expressed during the lag phase and levels decrease over time (3, 6, 8). These kinetics are opposite to those of MgA/SspA-regulated transcripts, whose levels start low and increase over time. Taken together, these results suggest that additional factors or signals are necessary for regulon expression.

We devised a genetic screen using an *F. novicida* transposon library (33) to identify genes involved in the expression of the MglA/SspA regulon. We report here that one novel gene, FTN_0480 (*fevR*, for *Francisella e*ffector of *v*irulence *r*egulation), which is positively regulated by MglA and SspA (6, 8), is required for replication in macrophages and for virulence in mice. We also demonstrate that *fevR* functions in mice to regulate the known virulence determinants in the MglA/SspA regulon. Thus, we have identified *fevR*, a novel regulator of virulence genes which is essential for *Francisella* pathogenesis.

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MATERIALS AND METHODS

Bacterial strains and growth. Wild-type *F. novicida* strain U112 and an isogenic *mglA* point mutant (GB2) have been described previously (4). Bacteria were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) supplemented with 0.2% cysteine or plated on tryptic soy agar (Becton Dickinson, Sparks, MD) supplemented with 0.1% cysteine (Sigma, St. Louis, MO) or modified Mueller-Hinton (MH) agar (Difco) supplemented with 0.025% ferric pyrophosphate (Sigma), 0.02% IsoVitaleX (Becton Dickinson), 0.1% glucose, and 0.025% calf serum (Gibco, Carlsbad, CA). Kanamycin (15 μg/ml), chloramphenicol (3 μg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 50 μg/ml) (Sigma) were added to the growth media when appropriate. *Escherichia coli* strain DH12S was used for cloning and grown in Luria broth (Difco Laboratories, Detroit, MI).

Screen to identify transcriptional regulators. An MglA-regulated β-galactosidase reporter plasmid was constructed by fusing the promoter of *pepO* to *lacZY* (*pepO*::*lacZY*). The region 5′ to the *pepO* open reading frame (FTN_1186) was amplified using pepO F and pepO R (see Table S1 in the supplemental material), introducing BglI and XhoI sites, respectively. *lacZY* was amplified from pCE37 using lacZ F and lacY R (see Table S1 in the supplemental material), introducing XhoI and PstI sites, respectively. PCR products were digested and ligated to pDSC (6) digested with BlgI and PstI. The ligations were transformed into *E. coli* DH12S. Chloramphenicol-resistant colonies were sequenced, and plasmid DNA was isolated using a DNA midiprep kit (Qiagen) and transformed into U112 by chemical transformation. Among the 15 chloramphenicol-resistant U112 colonies, only 2 turned blue on MH agar supplemented with X-Gal (termed U112-B1 and U112-B2). To test whether the MglA pathway was still functional in U112-B1, we deleted *mglA*. The *mglA* mutant in the U112-B1 strain background did not express β-galactosidase and the colonies were white on indicator plates, consistent with the role of MglA in regulating *pepO*::*lacZY* expression. Thus, we performed our genetic screen with the U112-B1(pDSC3) strain. To create a library of mutants in the U112-B1 background, genomic DNA was isolated from the previously described U112 transposon library (33). Genomic DNA (gDNA; 0.65 μg) from the total library was chemically transformed into U112-B1, and transformants were plated on MH agar (3 μg/ml chloramphenicol, 15 μg/ml kanamycin, and X-Gal). White colonies were picked 2 days after plating and verified to have only one transposon insertion by Southern blotting (data not shown). Candidate transposon mutants were additionally screened by quantitative reverse transcription-PCR (RT-PCR). The wild type and transposon mutants were subcultured to an optical density (OD) of 0.03. RNA was isolated from transposon mutants grown for 7 h to an OD of 2.0, on average.

Mutagenesis, epitope-tagged clonings, and complementation. *F. novicida* deletion and complemented mutants were made as described previously using the primers listed in Table S1 in the supplemental material (6). The *groES*::*fevR* constructs, C-terminal epitope-tagged constructs FevR-glutathione *S*-transferase (GST), MglA-hemagglutinin (HA), and SspA-His, were made by overlapping PCR using the primers listed in Table S1 in the supplemental material. Chemically competent *F. novicida* was used in transformations (2). Mutants were assessed by PCR to verify homologous recombination and sequenced.

Identification of transposon insertion. The sites of transposon insertion were identified by either inverse PCR or direct sequencing of gDNA. gDNA was isolated from the mutants using DNeasy (Qiagen). Next the gDNA was either sent for sequencing using the transposon primer TnF or cut with XbaI and ligated in a large volume to promote self-ligation of DNA fragments. The ligations were purified using Zymo DNA cleaner and concentrator, used as the template in a PCR with TnF and TnR (primers that extended out from the transposon), and then sequenced using primer TnF.

Time courses and RNA isolation. Overnight cultures of *F. novicida* strains were grown in TSB–0.2% cysteine at 37°C with shaking and subcultured to an OD at 600 nm (OD_{600}) of 0.01 in 200 ml. Samples were taken at various time points throughout the 8-h time course for RNA isolation and determination of the OD_{600}. Five samples were taken during the time course at 1, 3, 5, and 7.5, and 10 h for characterization of single mutants or at 2, 4, 6, and 8 h in *gro*::*fevR* bypass experiments. RNA was harvested as previously described (6).

Preparation of cDNA probes, hybridization, and analysis. The *Francisella* microarray and hybridization conditions were described previously (6). Normalized data were collected using the Stanford Microarray Database (30). Spots were excluded from analysis due to obvious abnormalities, a regression correlation of <0.6, or a Cy3 net mean intensity of <100. Only spots with at least 70% good data across the experiment were included for analysis. The normalized $\log_2$ (Cy5/Cy3) ratio was used for hierarchical clustering using the CLUSTER program (14). Results were visualized using the TREEVIEW program (14). Significant differences in gene transcript between the *mglA* and *fevR* mutant growth curves were determined using Significance Analysis for Microarrays program, version 1.21 (32), with the two-class statistical analysis tool and a calculated false-discovery rate of 0. Microarray results are available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

Quantitative RT-PCR. Quantitative RT-PCR for bacteria grown in broth was performed as previously described (6). To quantify transcripts from infected mice, cDNA was created using the high-capacity cDNA kit and quantified using Power Sybr green PCR master mix, both from Applied Biosystems. The gene-specific message was calculated by plotting a standard curve for each primer set using a dilution series of RNA from a sample known to contain the message of interest. To compare transcript abundances for the wild type and mutants, values were normalized to that for DNA helicase *uvrD* (FTT0121) to obtain relative quantities of message.

Macrophage replication assay. Bone marrow-derived macrophages were prepared as described elsewhere (28). Bone marrow-derived macrophages were seeded in 24-well plates at a density of 2.5×10^5 macrophages per well and incubated at 37°C and 5% CO_2 overnight. The macrophages were then infected at a multiplicity of infection of 25:1 (bacteria-to-macrophage ratio) and centrifuged at $730 \times g$ for 15 min to mediate attachment. The macrophages were incubated (time zero) for 1 h and washed three times with warm media. Macrophages were lysed 0.5, 4, 8, and 12 h postinfection with 1% saponin in water for 5 min and then diluted in phosphate-buffered saline and plated on tryptic soy agar with 0.1% cysteine.

Mouse infections. Six- to 8-week-old, female wild-type C57BL/6 (Jackson Laboratories, Bar Harbor, ME) mice were kept under specific-pathogen-free conditions in filter top cages. Mice were provided with sterile water and food ad libitum. Competitive-index (CI) experiments were performed as previously described (6). CI values were calculated as the ratio of mutant to wild-type output, normalized for the input, and significance was calculated by comparing the log of the CI to 0. Standard errors were calculated, and significance of results was determined by application of the Mann-Whitney statistical test on the $\log_{10}$ value of the CI. For RNA isolation from the skin, hair was removed from the mouse abdomen and mice were infected subcutaneously with 10^7 CFU in two spots. After 24 h, skin was harvested and one side was taken for bacterial enumeration, while the other was placed immediately in 1 ml of Trizol and homogenized. RNA was then extracted as described previously (6). All animal infection experiments were approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of Stanford University.

Coimmunoprecipitations. MglA-HA/SspA-His-, MglA-HA/FevR-GST-, and SspA-His/FevR-GST-tagged strains were grown in 100 ml at 37°C until an OD of 1.0. Bacteria were pelleted and resuspended in 5 ml of Bugbuster (Novagen) and 80 μl of protease inhibitor cocktail (Novagen). Cell extracts were sonicated four times with 15-second bursts at a 70 output setting on a Kontes ultrasonic cell disruptor to decrease viscosity. Cell extracts were spun for 1 h at ~150,000 × *g* in a Beckman ultracentrifuge to separate soluble and insoluble fractions. To purify SspA-His- and SspA-interacting partners, 1.5 ml of the soluble fraction was incubated with 80 μl of His-Bind resin (Novagen) overnight at 4°C and purified by following the manufacturer's instructions. To purify MglA-HA and MglA-HA binding partners, 5 μl of anti-HA antibody (Sigma; H3663) was noncovalently attached to 80 μl protein G plus agarose (Calbiochem) by following Sigma's instructions. The soluble fraction (1.5 ml) was incubated overnight at 4°C with the HA antibody and beads. To purify FevR-GST and copurifying partners, 1.5 ml of the soluble fraction was added to 80 μl BaculoGold glutathione-agarose beads (BD Biosciences) by following the manufacturer's instructions and incubated overnight at 4°C. The HA- and glutathione-containing beads were washed five times with 500 μl of radioimmunoprecipitation assay (RIPA) buffer. Fifty microliters of sodium dodecyl sulfate sample buffer was then added to the beads, and 15 μl of sample was run on a 12% acrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose. Immunoblots were blocked overnight with Odyssey blocking buffer (LI-COR Biosciences) and probed with anti-HA antibody at 1:2,000 dilution (Roche), anti-GST antibody at 1:4,000 dilution (Sigma; G1160), and anti-His antibody at 1:2,000 dilution (R&D Systems; MAB050).

RESULTS

Genetic screen to identify genes that affect *F. novicida* virulence gene expression. Since MglA and SspA were known to regulate the expression of many virulence genes in *F. tularensis*, including the FPI (6, 8, 19), we devised a screen to identify additional regulators involved in the MglA/SspA pathway us-

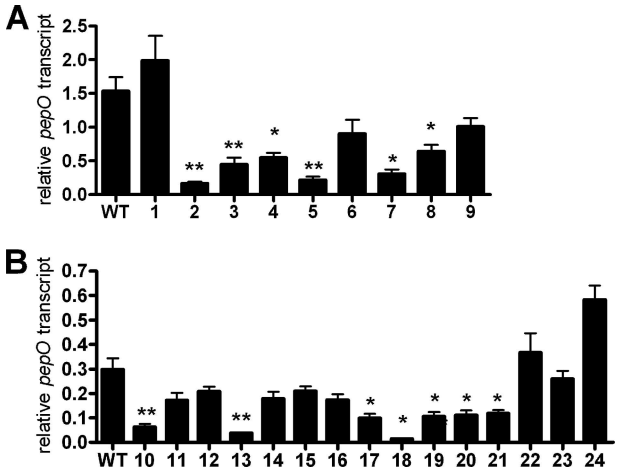

FIG. 1. Screen for *F. novicida* transposon mutants that regulate *pepO*. (A and B) RNA was isolated from transposon mutants grown in broth at 37°C to early stationary phase. *pepO* mRNA levels were determined by quantitative RT-PCR and normalized to that for the *uvrD* transcript. Thirteen of 24 transposon mutants had significantly lower levels of *pepO* transcript than the wild type (WT). Samples were obtained in triplicate. Experiments were performed at least three times. Means and standard deviations from a representative experiment are shown. *, $P < 0.05$; **, $P < 0.01$.

ing an MglA-regulated β-galactosidase reporter vector. The *pepO* promoter, which is expressed roughly 50-fold higher in the wild-type strain than in an *mglA* mutant (6), was fused to *lacZY* (pDSC3) and introduced into our *F. novicida* transposon library, which represents eightfold coverage of the nonessential genome (33). Transposon mutants with wild-type expression of *pepO* formed blue colonies on plates containing the indicator X-Gal, due to *pepO* promoter-dependent expression of *lacZY*. Mutants defective in the induction of the *pepO* promoter formed white colonies. Approximately 40,000 colonies were screened, representing approximately 25-fold coverage of the genome; 24 white colonies were isolated and designated W1 to W24. All candidate mutant clones were additionally screened by quantitative RT-PCR for decreased *pepO* expression to eliminate false positives. Thirteen of the 24 isolates contained significantly lower levels of *pepO* transcript than the wild type (Fig. 1A and B). Thus, this genetic screen led to the identification of genes required for the expression of the MglA-regulated gene *pepO*.

***fevR* positively regulates expression of the MglA/SspA regulon in vitro.** The transposon insertion sites were determined for the 13 mutants identified in the screen and were mapped to five distinct genes: two hypothetical genes (FTN_0480 and FTN_1069), *caiC* (encoding AMP binding protein), *uhpC* (encoding a major facilitator superfamily transport protein), and *cphA* (encoding cyanophycin synthetase) (Table 1). We did not identify *mglA* or *sspA* in our screen; thus, our results may represent an underestimate of genes involved in the regulation of MglA/SspA-dependent gene expression. However, we focused our efforts on hypothetical gene FTN_0480 for several reasons: (i) it encodes a novel protein; (ii) its transcript is positively regulated by MglA and SspA, which are required for virulence (6, 8); and (iii) it is induced by iron depletion, a condition which induces the expression of critical virulence genes in many bacterial pathogens (6, 8, 12).

TABLE 1. Identity of transposon insertion sites in mutants with significantly lower levels of *pepO*

Clone(s)	U112 designation	Gene	Description of product
3, 4, 13	FTN_0480	*fevR*	Hypothetical protein
2, 5, 7, 18, 21	FTN_0604	*caiC*	AMP-binding protein
8	FTN_1069		Protein of unknown function
17, 19, 20	FTN_1112	*cphA*	Cyanophycin synthetase
10	FTN_1611	*uhpC*	Major facilitator superfamily transport protein

To determine the role of FTN_0480 in the regulation of *F. novicida* gene expression, we compared the transcriptomes of the wild type, an FTN_0480 deletion mutant, and an *mglA* point mutant (GB2) (4) by microarray analysis. RNA was isolated from the bacterial strains during a time course in broth at 37°C. Similar to our previous findings (6), the majority of MglA-regulated genes were downregulated in the *mglA* point mutant (92), while 10 genes were expressed at higher levels than in wild-type bacteria (Fig. 2). The ΔFTN_0480 mutant transcriptome was strikingly similar to that of the *mglA* mutant. Indeed, statistical analysis of the microarray data using Significance Analysis for Microarrays revealed that the only gene whose expression varied significantly between the *mglA* and ΔFTN_0480 mutant strains was FTN_0480 itself. Thus, under the conditions used, the *mglA* and Δ*fevR* mutant transcriptomes appear identical. This result demonstrates that expression of the genes within the FPI, all of which are required for *Francisella* virulence, is dependent on FTN_0480. Thus, we annotate the FTN_0480 locus as *fevR*.

***mglA* and *fevR* act in parallel to positively regulate virulence gene expression in vitro.** MglA and SspA positively regulate transcript expression of *fevR* (6, 8). *mglA* and *sspA* transcript levels are themselves unaffected in a Δ*fevR* mutant (Fig. 2), suggesting that *fevR* does not positively regulate the transcrip-

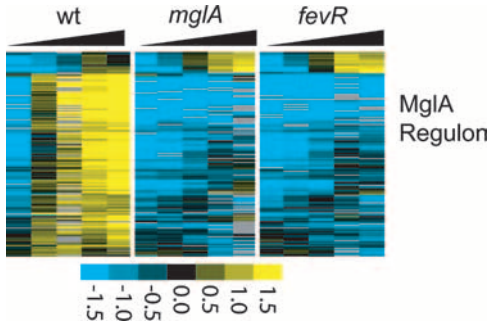

FIG. 2. The MglA/SspA regulon is not expressed in *mglA* and Δ*fevR* mutants grown in broth. RNA was isolated from wild-type, *mglA*, and Δ*fevR* mutants during growth in TSB at 37°C to compare transcriptomes by microarray. The levels of the gene transcripts of the previously published MglA regulon (6) are shown. The levels of these transcripts in *mglA* and *fevR* mutants are identical and demonstrate that these genes contribute to MglA-regulated gene expression in broth. Columns represent individual time points increasing from left to right during the time course at 1, 3, 5, 7, and 9 h. Rows represent individual genes.

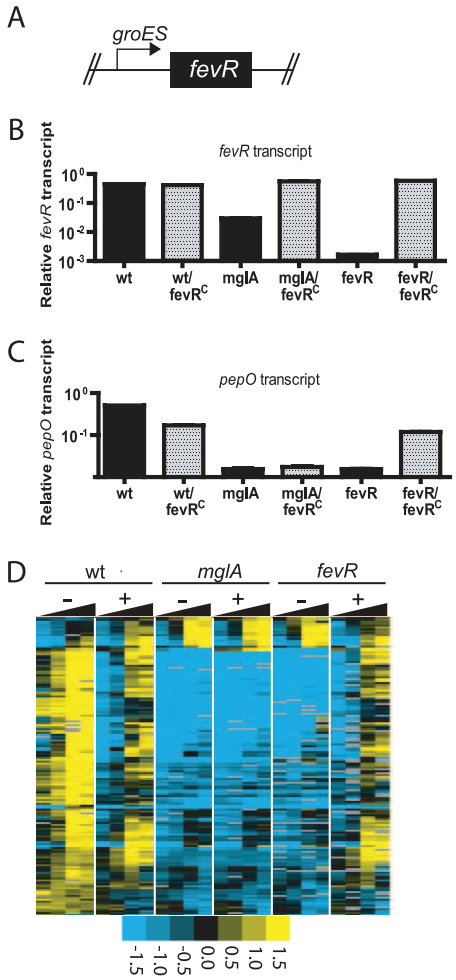

FIG. 3. Constitutive expression of an *fevR* transcript does not rescue expression of the MglA/SspA regulon in an *mglA* mutant. (A) The *fevR* promoter was replaced with the *groES* promoter and the resulting gene was inserted into the chromosome of wild-type (wt) and *mglA* and Δ*fevR* mutant backgrounds to create wild-type *fevR*(Con) (fevR^C), *mglA fevR*(Con), and Δ*fevR fevR*(Con) mutants. Strains were grown in TSB at 37°C, and RNA was isolated at 2, 4, 6, and 8 h. Quantitative RT-PCR of *fevR* (B) and *pepO* (C) mRNA was performed at the 8-h time point, and levels were normalized to that for the *uvrD* control transcript. Although the *mglA* mutant containing the *fevR*(Con) construct expresses the *fevR* transcript, *pepO* expression is not rescued. Sampling was performed in triplicate. Means and standard deviations from a representative experiment are shown. (D) Microarray analysis of MglA regulon expression throughout the growth curves confirms the quantitative RT-PCR findings that both *mglA* and *fevR* are necessary for MglA/SspA regulon expression.

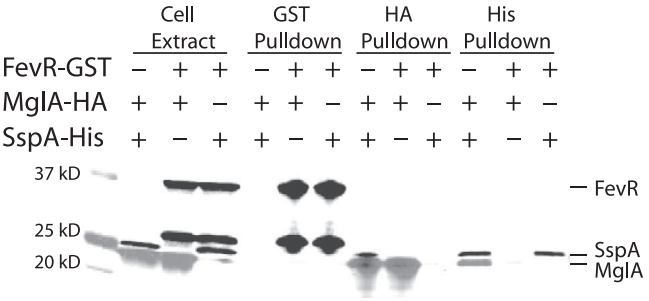

FIG. 4. MglA and SspA do not coprecipitate with FevR. MglA-HA/SspA-His-, MglA-HA/FevR-GST-, and SspA-His/FevR-GST-tagged strains were created by placing the corresponding epitope tag at the C termini of the endogenous copies of products of *mglA*, *sspA*, and *fevR*. Cell extracts were prepared from double-tagged strains grown to late exponential phase. Each cell extract was used in coprecipitations with nickel beads, anti-HA antibody/protein G resin, or glutathione beads and immunoblotted to detect His, HA, and GST tags. The anti-GST antibody stained two bands, a high-molecular-weight band representing FevR-GST and a low-molecular-weight band likely to be a GST cleavage product.

tion of *mglA* and *sspA*. One hypothesis to explain this result is that FevR is a transcriptional activator that is downstream of MglA/SspA in a regulatory cascade and that FevR can act independently of MglA/SspA to induce the expression of the regulon. To test this model, we designed a strain that expresses *fevR* and contains a mutated *mglA*. Rescue of FPI expression in this strain lacking *mglA* would indicate that FevR does indeed act downstream and independently of MglA. We replaced the *fevR* promoter with that of *groES*, which is constitutively active in the wild-type and *mglA* and Δ*fevR* mutant backgrounds, resulting in a *fevR*(Con) strain (Fig. 3A). RNA was then isolated during growth, and the transcriptomes were analyzed by quantitative RT-PCR and microarray analysis. As expected, the *fevR* transcript is expressed in the *mglA fevR*(Con) and Δ*fevR fevR*(Con) mutant backgrounds, establishing that MglA no longer regulates expression of the *fevR* transcript in these strains (Fig. 3B). To test whether uncoupling *fevR* expression from MglA would result in expression of *pepO* and the MglA/SspA regulon, we measured gene expression by quantitative RT-PCR and microarray analysis, respectively. In the wild-type *fevR fevR*(Con) and Δ*fevR fevR*(Con) backgrounds the MglA/SspA regulon was expressed, albeit at lower levels than in the wild-type strain (Fig. 3C and D). The physiological cause of the decreased *pepO* transcript expression is unclear. The *mglA fevR*(Con) mutant, however, did not express *pepO* or the entire MglA regulon. Thus, *fevR* is not sufficient to induce the MglA/SspA regulon in the absence of *mglA*. Instead, these data suggest that MglA positively regulates the expression of *fevR* and that, once expressed, MglA and FevR are both required to initiate transcription of the genes in the MglA/SspA regulon.

FevR does coprecipitate with MglA or SspA. Since MglA and FevR are both required for expression of the MglA/SspA regulon and constitutive expression of *fevR* did not lead to MglA-independent virulence gene expression, we proposed that MglA and FevR may interact. To determine whether MglA and FevR directly interact, we constructed epitope-tagged versions of these proteins. The C terminus of endogenous MglA was tagged with HA_2 and FevR was tagged with GST. As a positive control for coprecipitation, the C terminus of endogenous SspA was also tagged with an eight-histidine epitope (8). Insertion of the epitope tags did not disrupt regulatory functions, as MglA/SspA-regulated transcripts were still expressed in each of the strains (data not shown). Strains were grown to late exponential phase, and cell extracts were prepared under nondenaturing conditions (Fig. 4). Similar to published studies, MglA-HA coimmunoprecipated with SspA-His and vice versa, demonstrating that conditions were sufficient for identifying direct interactions within this complex (Fig. 4) (8). However, neither MglA-HA nor SspA-His copu-

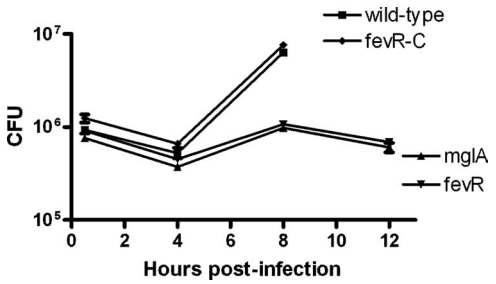

FIG. 5. *mglA* and *fevR* contribute to replication in macrophages. Bone marrow-derived macrophages were infected with either the wild-type, *mglA*, Δ*fevR*, or Δ*fevR* complemented (fevR-C) bacterial strain at a multiplicity of infection of 20 bacteria per macrophage. Macrophages were washed at 30 min postinfection, lysed at 2, 4, 8, and 12 h postinfection, and plated to enumerate the number of CFU. The means from a single experiment are shown and are representative of three independent experiments. The standard deviations are shown and are less than 10%.

rified with FevR-GST. Likewise, FevR-GST did not copurify with MglA-HA or SspA-His. These data suggest that, while MglA and FevR function in parallel, FevR does not bind MglA or SspA to promote expression of the MglA/SspA regulon.

***fevR* is required for intracellular growth in macrophages.** MglA and several MglA-regulated genes, including many FPI genes and a putative transglutaminase gene (FTN_0869), are necessary for replication of *F. novicida* in macrophages (6, 16, 19). Since *fevR* contributes to the expression of these genes in vitro, we predicted that a Δ*fevR* mutant would be unable to grow in macrophages. To test this, we infected bone marrow-derived macrophages with the wild-type, *mglA*, Δ*fevR*, and Δ*fevR* complemented strains (Fig. 5). The numbers of both the wild-type and the complemented Δ*fevR* mutant bacteria increased 10-fold inside macrophages over 8 h (Fig. 5A), resulting in the death of infected macrophages at 9 h postinfection (data not shown). In contrast, the numbers of *mglA* and Δ*fevR* mutant bacteria did not increase inside macrophages, nor did the *mglA* and Δ*fevR* mutant-infected macrophages die throughout the course of the experiment. These data demonstrate that *fevR* is required for *Francisella* replication within macrophages, a critical virulence trait of this pathogen.

***fevR* is required for virulence in a mouse model of infection.** MglA positively regulates the expression of many virulence factors, including the FPI. Indeed, an *mglA* mutant strain is severely attenuated in a mouse model of infection (6, 22, 33). Likewise, if *fevR* is involved in regulating this pathway in vivo, we anticipate that a Δ*fevR* mutant would also be severely attenuated. To measure the contribution of *fevR* to virulence, we performed competition experiments in which mice were infected subcutaneously with a 1:1 mixture of wild-type bacteria and either the *mglA* or Δ*fevR* mutant bacteria. Forty-eight hours postinfection, the skin and spleens were collected and the numbers of wild-type and mutant bacteria in each sample were enumerated. The Δ*fevR* mutant exhibited a severe decrease in fitness compared to the wild type in both the spleen and the skin, with a CI similar to that of an *mglA* or Δ*fpi* mutant (Fig. 6A and B). The attenuation of the Δ*fevR* mutant could be rescued by adding back a wild-type copy of *fevR*. These data show that a Δ*fevR* mutant has one of the most severe attenuations described for *Francisella* and that therefore *fevR* is critical for *F. novicida* virulence.

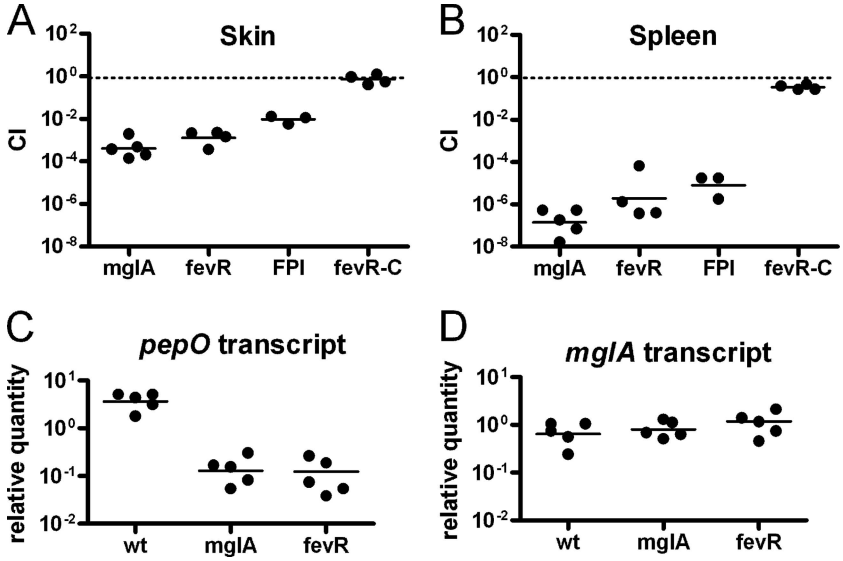

FIG. 6. *mglA* and *fevR* are necessary for virulence gene expression and survival in vivo. (A and B) Mice were infected subcutaneously with 5×10^4 CFU of wild-type bacteria and 5×10^4 CFU of *mglA* (point mutant), Δ*fevR*, Δ*fevR* complemented (fevR-C), and FPI deletion mutant bacteria, for a total of 10^5 bacteria/mouse. After 2 days, the skin (A) and the spleens (B) were taken for counts and the CI was calculated. Each dot represents the CI value for one mouse. Bars represent the geometric means. (C and D) Mice were infected subcutaneously in two different spots on the abdomen with 10^7 CFU of wild-type (wt) bacteria or *mglA* or Δ*fevR* mutants. After 24 h, one injection site was taken for counts, while the other was taken to isolate total RNA. Transcript levels of *pepO* (C) and *mglA* (D) were determined by quantitative RT-PCR and normalized to that for control transcript *uvrD*. Since the *mglA* mutant is a point mutant, the *mglA* transcript is still expressed in this strain. Each dot represents the average of quantitative PCR values from experiments performed triplicate for one mouse. Bars represent the geometric means.

Virulence gene expression in tissue requires *mglA* and *fevR*. To conclusively demonstrate that both *mglA* and *fevR* are necessary for expression of MglA/SspA-regulated genes in vivo, we quantified the transcripts from MglA-regulated gene *pepO* during an infection in mice. Mice were injected subcutaneously in two spots on the abdomen with 10^7 CFU of the wild-type, *mglA*, or Δ*fevR* strain. At 24 h postinfection, one injection site was taken to quantify bacterial load, while the other was taken for total RNA isolation. The wild-type strain replicated to $\sim 10^9$ bacteria per gram in infected skin in 24 h, whereas *mglA* and Δ*fevR* mutant counts were 100-fold lower. These single-infection results are in agreement with our data from CI experiments and conclusively demonstrate that *fevR* is required for *F. novicida* virulence in vivo. Quantitative RT-PCR was performed to determine the levels of *pepO* and *mglA* transcripts, which were normalized to those for control gene *uvrD*, which is expressed at similar levels in these strains. *mglA* was included as a control because microarray results suggested that the gene was not differentially regulated between the wild-type, *mglA* point mutant, and Δ*fevR* mutant bacteria. Indeed, the *mglA* transcript did not vary significantly between the strains (Fig. 6D). However, the expression of the *pepO* transcript was 10 times higher in the wild type than in the *mglA* and Δ*fevR* mutants (Fig. 6C). These results establish a function for *fevR* as a critical regulator of virulence gene expression in vivo.

DISCUSSION

Francisella is a highly infectious pathogen that possesses the ability to survive in various environments including freshwater, arthropods, and mammals, suggesting that this organism has the ability to regulate gene expression in response to various external signals. Nutrient and iron limitation has been shown to influence the expression of *Francisella* virulence genes (7, 12, 20). Several *F. tularensis* regulatory factors have been described. MglA and SspA act together to regulate the expression of at least 100 genes, including FPI genes, which are essential for virulence. In addition, the two-component regulator *pmrA* regulates the expression of the FPI and other virulence genes (22). *mglA* and *pmrA* are required for the ability of *F. tularensis* to survive within mammalian hosts, yet we do not understand how their gene products integrate to control virulence. These regulators likely play roles in *F. tularensis* subversion of the immune system, including growth within macrophages and dendritic cells and modulation of innate immunity signaling pathways within macrophages (1, 5, 25, 31).

To further elucidate the MglA/SspA signaling cascade, we screened for transposon mutants unable to induce transcription from the MglA-regulated promoter *pepO*. The majority of the transposon mutants we identified contained insertions in either FTN_0480 (*fevR*), *caiC*, or *cphA*. We have shown that *fevR* is required for the expression of the MglA/SspA regulon during growth in rich media and in tissue. In addition, we demonstrate that a Δ*fevR* mutant is unable to replicate within macrophages and exhibits one of the most severe attenuations described for *Francisella* during a mouse infection. Previously, all regulation studies of *Francisella* have utilized broth or in vitro tissue culture systems to track virulence regulation. Since we do not know what all of the environmental signals that integrate to control virulence are, it was important to address the regulation of known virulence factors, such as *pepO*, in the host. In this study, we demonstrate that *fevR* regulates known virulence factors in the environmental context of a natural infection.

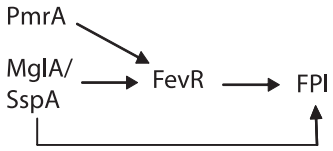

FIG. 7. Model for the MglA/SspA pathway. MglA and SspA positively regulate *fevR* expression, and *fevR* regulates expression of the MglA/SspA regulon. Constitutive expression of the *fevR* transcript in an *mglA* mutant did not rescue MglA/SspA regulon expression, suggesting that MglA/SspA and FevR work in parallel to activate downstream genes. Furthermore, while MglA and SspA copurify with each other, neither copurifies with FevR-GST. These data suggest that the MglA/SspA regulon is under the control of a common network motif called an FFL.

The expression of *fevR* is positively regulated by MglA and SspA, suggesting that FevR may be downstream of these proteins in a pathway. However, the requirement of MglA for expression of genes in the MglA/SspA regulon could not be bypassed by constitutively expressing an *fevR* transcript in an *mglA* mutant (Fig. 3), leading us to test if FevR may directly interact with the MglA/SspA complex. We created epitope-tagged versions of MglA, SspA, and FevR in *F. novicida* to assay for interactions between these proteins. Similar to results from previously published work for another organism (*Francisella tularensis* subspecies *holarctica* strain LVS) (8), MglA and SspA copurified. However, we could not demonstrate MglA or SspA protein interactions with FevR in coprecipitation assays, suggesting that MglA and SspA do not directly interact with FevR. Taken together our data indicate that MglA/SspA and FevR function in parallel to regulate the transcription of the MglA/SspA regulon (Fig. 7). This resembles a network motif called a feed forward loop (FFL), where one inducer activates the other and then the two inducers act together to activate the downstream genes (29). FFLs have been shown to accelerate the time for response to a given stimulus, as well as decrease responses to transient signals so that the bacterium responds only to persistent environmental cues (21). Previous work has also shown that the orphan response regulator PmrA contributes to expression of *fevR* (22), suggesting that expression of the *fevR* transcript requires the integration of multiple input signals and that the upregulation of *fevR* may represent a commitment step for FPI transcript expression. Thus, we have identified an essential novel regulator of *Francisella* virulence.

The identification of *fevR* as a regulator may help to explain an interesting observation. When *Francisella* is grown in TSB with 0.2% cysteine at 37°C, *mglA* and *sspA* are maximally expressed during lag phase and the transcript levels decrease throughout the growth curve (3, 6). Curiously, MglA/SspA-regulated genes, including *fevR*, follow a pattern of gene expression that is opposite of *mglA* and *sspA* transcript expression, with maximal levels occurring during stationary phase. This suggests that MglA/SspA regulatory activity increases as levels of *fevR* transcript and presumably levels of FevR protein increase in the bacterium. Since *mglA-sspA* and *fevR* tran-

scripts display different patterns of gene expression, it also suggests that their upregulation may result from different environmental cues. The future study of how *F. tularensis* incorporates these signals in order to regulate virulence gene expression during infection will be very instructive.

FevR is annotated as a hypothetical protein and shows little similarity to proteins from non-*Francisella* species. However, iterative Psi-BLAST searches indicate that FevR is weakly homologous to the MerR family of transcription factors. The observed similarity was highly driven by a putative DNA binding domain in FevR (see Fig. S1 in the supplemental material). The MerR family of transcription factors typically contain a conserved N terminus with a helix-turn-helix domain followed by a highly divergent C-terminal metal binding domain that regulates MerR function. FevR is 111 amino acids, which is smaller than typical MerRs, and alignments suggest that FevR may lack a metal binding domain (see Fig. S1 in the supplemental material). In fact, the similarity between FevR and MerR transcription factors is mostly contained within a 22-amino-acid region that encompasses the MerR helix-turn-helix domain (13). So, while FevR exhibits some degree of homology to MerRs in the helix-turn-helix region, the remainder of the FevR predicted protein appears divergent, and it is unclear if FevR would have a mechanism of action similar to that of MerRs. However, MerRs act by binding DNA independently of RNA polymerase, which may explain why we were unable to coprecipitate FevR with MglA or SspA. We are currently conducting experiments to test the model that FevR positively regulates gene expression in *F. tularensis* by binding DNA.

SspA homologs in *Francisella* are required for expression of the FPI and other virulence genes. SspA is highly conserved in gram-negative bacteria, including pathogenic *Neisseria* and *Yersinia* spp. and *E. coli*. Although SspA is involved in virulence gene regulation in *Neisseria* and *Yersinia* species, the specific mechanism of SspA-mediated gene regulation is not known. However, in *E. coli* SspA requires a coactivator for binding to phage promoters (17). Given the integration of SspA/MglA and FevR in *Francisella*, the work presented here supports the idea that SspA homologs require a coactivator for gene regulation. It is possible that *Neisseria* and *Yersinia* spp. encode a functional homolog of FevR and that this could be a conserved mechanism of SspA-mediated gene regulation in bacterial pathogens.

Our screen identified four other genes, *uhpC*, *cphA*, FTN_1069, and *caiC*, that are involved in the regulating the expression of the virulence factor *pepO* and possibly the MglA/SspA regulon. The further characterization of FevR and these additional genes will lead to further advances in understanding how *F. tularensis* regulates the expression of genes important for survival and virulence in response to various environmental cues. As we learn more about the complex signaling cascades that regulate gene expression, we may also gain further understanding of virulence regulation in related gram-negative pathogens.

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REFERENCES

1. **Anthony, L. D., R. D. Burke, and F. E. Nano.** 1991. Growth of *Francisella* spp. in rodent macrophages. Infect. Immun. **59:**3291–3296.
2. **Anthony, L. S., M. Z. Gu, S. C. Cowley, W. W. Leung, and F. E. Nano.** 1991. Transformation and allelic replacement in *Francisella* spp. J. Gen. Microbiol. **137:**2697–2703.
3. **Baron, G. S., and F. E. Nano.** 1999. An erythromycin resistance cassette and mini-transposon for constructing transcriptional fusions to cat. Gene **229:** 59–65.
4. **Baron, G. S., and F. E. Nano.** 1998. MglA and MglB are required for the intramacrophage growth of *Francisella novicida*. Mol. Microbiol. **29:**247–259.
5. **Bosio, C. M., and S. W. Dow.** 2005. *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. J. Immunol. **175:**6792–6801.
6. **Brotcke, A., D. S. Weiss, C. C. Kim, P. Chain, S. Malfatti, E. Garcia, and D. M. Monack.** 2006. Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. Infect. Immun. **74:**6642–6655.
7. **Buchan, B. W., M. K. McLendon, and B. D. Jones.** 2008. Identification of differentially regulated *Francisella tularensis* genes using a newly developed Tn*5*-based transposon delivery system. Appl. Environ. Microbiol. **74:**2637–2645.
8. **Charity, J. C., M. M. Costante-Hamm, E. L. Balon, D. H. Boyd, E. J. Rubin, and S. L. Dove.** 2007. Twin RNA polymerase-associated proteins control virulence gene expression in *Francisella tularensis*. PLoS Pathog. **3:**e84.
9. **Checroun, C., T. D. Wehrly, E. R. Fischer, S. F. Hayes, and J. Celli.** 2006. Autophagy-mediated reentry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. Proc. Natl. Acad. Sci. USA **103:** 14578–14583.
10. **Clemens, D. L., and M. A. Horwitz.** 2007. Uptake and intracellular fate of *Francisella tularensis* in human macrophages. Ann. N. Y. Acad. Sci. **1105:** 160–186.
11. **Clemens, D. L., B. Y. Lee, and M. A. Horwitz.** 2004. Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect. Immun. **72:**3204–3217.
12. **Deng, K., R. J. Blick, W. Liu, and E. J. Hansen.** 2006. Identification of *Francisella tularensis* genes affected by iron limitation. Infect. Immun. **74:** 4224–4236.
13. **Dodd, I. B., and J. B. Egan.** 1990. Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. Nucleic Acids Res. **18:**5019–5026.
14. **Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein.** 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA **95:**14863–14868.
15. **Golovliov, I., V. Baranov, Z. Krocova, H. Kovarova, and A. Sjostedt.** 2003. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect. Immun. **71:**5940–5950.
16. **Gray, C. G., S. C. Cowley, K. K. Cheung, and F. E. Nano.** 2002. The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. FEMS Microbiol. Lett. **215:**53–56.
17. **Hansen, A. M., H. Lehnherr, X. Wang, V. Mobley, and D. J. Jin.** 2003. *Escherichia coli* SspA is a transcription activator for bacteriophage P1 late genes. Mol. Microbiol. **48:**1621–1631.
18. **Kieffer, T. L., S. Cowley, F. E. Nano, and K. L. Elkins.** 2003. *Francisella novicida* LPS has greater immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. Microbes Infect. **5:**397–403.
19. **Lauriano, C. M., J. R. Barker, S. S. Yoon, F. E. Nano, B. P. Arulanandam, D. J. Hassett, and K. E. Klose.** 2004. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. Proc. Natl. Acad. Sci. USA **101:**4246–4249.
20. **Lenco, J., M. Hubalek, P. Larsson, A. Fucikova, M. Brychta, A. Macela, and J. Stulik.** 2007. Proteomics analysis of the *Francisella tularensis* LVS response to iron restriction: induction of the *F. tularensis* pathogenicity island proteins IglABC. FEMS Microbiol. Lett. **269:**11–21.
21. **Mangan, S., and U. Alon.** 2003. Structure and function of the feed-forward loop network motif. Proc. Natl. Acad. Sci. USA **100:**11980–11985.
22. **Mohapatra, N. P., S. Soni, B. L. Bell, R. Warren, R. K. Ernst, A. Muszynski, R. W. Carlson, and J. S. Gunn.** 2007. Identification of an orphan response regulator required for the virulence of *Francisella* spp. and transcription of pathogenicity island genes. Infect. Immun. **75:**3305–3314.
23. **Nano, F. E., N. Zhang, S. C. Cowley, K. E. Klose, K. K. Cheung, M. J. Roberts, J. S. Ludu, G. W. Letendre, A. I. Meierovics, G. Stephens, and K. L. Elkins.** 2004. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J. Bacteriol. **186:**6430–6436.
24. **Owen, C. R., E. O. Buker, W. L. Jellison, D. B. Lackman, and J. F. Bell.** 1964. Comparative studies of *Francisella tularensis* and *Francisella novicida*. J. Bacteriol. **87:**676–683.
25. **Reilly, T. J., G. S. Baron, F. E. Nano, and M. S. Kuhlenschmidt.** 1996.

Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. J. Biol. Chem. **271:**10973–10983.
26. **Santic, M., M. Molmeret, and Y. Abu Kwaik.** 2005. Modulation of biogenesis of the *Francisella tularensis* subsp. *novicida*-containing phagosome in quiescent human macrophages and its maturation into a phagolysosome upon activation by IFN-gamma. Cell. Microbiol. **7:**957–967.
27. **Santic, M., M. Molmeret, K. E. Klose, S. Jones, and Y. A. Kwaik.** 2005. The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell. Microbiol. **7:**969–979.
28. **Schaible, U. E., and S. H. E. Kaufmann.** 2002. Studying trafficking of intracellular pathogens in antigen-presenting cells. Methods Microbiol. **31:**343–360.
29. **Shen-Orr, S. S., R. Milo, S. Mangan, and U. Alon.** 2002. Network motifs in the transcriptional regulation network of *Escherichia coli*. Nat. Genet. **31:** 64–68.
30. **Sherlock, G., T. Hernandez-Boussard, A. Kasarskis, G. Binkley, J. C. Matese, S. S. Dwight, M. Kaloper, S. Weng, H. Jin, C. A. Ball, M. B. Eisen, P. T. Spellman, P. O. Brown, D. Botstein, and J. M. Cherry.** 2001. The Stanford Microarray Database. Nucleic Acids Res. **29:**152–155.
31. **Telepnev, M., I. Golovliov, T. Grundstrom, A. Tarnvik, and A. Sjostedt.** 2003. *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages. Cell. Microbiol. **5:**41–51.
32. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98:**5116–5121.
33. **Weiss, D. S., A. Brotcke, T. Henry, J. J. Margolis, K. Chan, and D. M. Monack.** 2007. In vivo negative selection screen identifies genes required for *Francisella* virulence. Proc. Natl. Acad. Sci. USA **104:**6037–6042.

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