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RESEARCH ARTICLE

An AraC/XylS Family Transcriptional Regulator Modulates the Oxidative Stress Response of *Francisella tularensis*

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Dina Marghani and Zhuo Ma contributed equally to this work. Zhuo Ma generated the *osrR* mutant and the transcomplemented strains; Dina Marghani characterized these strains.

ABSTRACT *Francisella tularensis* is a Gram-negative bacterium that causes a fatal human disease known as tularemia. The Centers for Disease Control and Prevention have classified *F. tularensis* as a category A tier 1 select agent. The virulence mechanisms of *Francisella* are not entirely understood. *Francisella* possesses very few transcription regulators, and most of these regulate the expression of genes involved in intracellular survival and virulence. The *F. tularensis* genome sequence analysis reveals an AraC (FTL_0689) transcriptional regulator homologous to the AraC/XylS family of transcriptional regulators. In Gram-negative bacteria, AraC activates genes required for L-arabinose utilization and catabolism. The role of the FTL_0689 regulator in *F. tularensis* is not known. In this study, we characterized the role of FTL_0689 in the gene regulation of *F. tularensis* and investigated its contribution to intracellular survival and virulence. The results demonstrate that FTL_0689 in *Francisella* is not required for L-arabinose utilization. Instead, FTL_0689 specifically regulates the expression of the oxidative and global stress response, virulence, metabolism, and other key pathways genes required by *Francisella* when exposed to oxidative stress. The *FTL_0689* mutant is attenuated for intramacrophage growth and virulence in mice. Based on the deletion mutant phenotype, *FTL_0689* was termed *osrR* (oxidative stress response regulator). Altogether, this study elucidates the role of the *osrR* transcriptional regulator in tularemia pathogenesis.

IMPORTANCE The virulence mechanisms of category A select agent *Francisella tularensis*, the causative agent of a fatal human disease known as tularemia, remain largely undefined. The present study investigated the role of a transcriptional regulator and its overall contribution to the oxidative stress resistance of *F. tularensis*. The results provide an insight into a novel gene regulatory mechanism, especially when *Francisella* is exposed to oxidative stress conditions. Understanding such *Francisella*-specific regulatory mechanisms will help identify potential targets for developing effective therapies and vaccines to prevent tularemia.

KEYWORDS AraC/XylS, *Francisella tularensis*, oxidative stress, pathogenesis, transcriptional regulation, virulence

Francisella tularensis is a Gram-negative bacterium that causes a fatal human disease known as tularemia. Based on its extreme virulence and potential to be used as a bioweapon or a bioterror agent, the Centers for Disease Control and Prevention have classified *F. tularensis* as category A tier 1 select agent (1). The genus *Francisella* is divided into two species, *F. tularensis* and *F. philomiragia* (2, 3). *F. tularensis* has four major subspecies; *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) are virulent for humans, while the other two subsp., *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *mediasiatica*, do not cause disease in immunocompetent individuals (3–5). Type A *F. tularensis* strains are found in North America and Central Europe (6–8), while

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type B strains are less common in North America but are prevalent throughout Europe and Asia. Both type A and type B strains of *F. tularensis* can be transmitted through insect bites, direct contact with infected animals, ingestion of contaminated food or water, or inhaling bioaerosols. The live vaccine strain (LVS) is derived from *F. tularensis* subsp. *holarctica*.

F. tularensis is an intracellular bacterium and has a unique intracellular life cycle. *Francisella* can infect macrophages, neutrophils, dendritic cells, and several other cell types (9–12); however, macrophages are targeted primarily to initiate the infection. Upon phagocytosis, *Francisella* remains in phagosomes for a short duration, prevents the maturation of phagosomes, disintegrates the phagosomal wall, and escapes into the cytosol, where the replication occurs (12). The genes required to escape from the phagosome are encoded on a *Francisella* pathogenicity island (FPI) (13). The regulation of virulence mechanisms of *Francisella* is not entirely understood. *Francisella* possesses very few transcription regulators. The three well-characterized transcriptional regulators, the macrophage growth locus protein A (MglA), the stringent starvation protein A (SspA), and the pathogenicity island gene regulator (PigR), regulate the expression of genes encoded on the FPI (14–16). The other transcriptional regulators, PmrA and QseC, function as response regulators of the two-component system and regulate the expression of virulence-associated genes in *F. tularensis* (17, 18). Additionally, *F. tularensis* encodes specialized transcriptional regulators such as Fur, which regulate the expression of genes involved in iron uptake (19), and OxyR, which plays a central role in regulating the essential genes required for oxidative stress resistance and virulence (20).

The *F. tularensis* genome sequence analysis revealed a transcriptional regulator homologous to the AraC/XylS family of regulators encoded by the *FTL_0689* gene. The AraC/XylS family of transcriptional regulators in several Gram-negative bacteria exhibit high amino acid sequence homology. They contain two unique and highly conserved helix-turn-helix DNA binding motifs. The first well-characterized AraC/XylS transcriptional regulator is the one that controls the L-arabinose operon in *Escherichia coli* (21, 22). It functions as an activator of the *araBAD* and other operons, the *araFGH* and *araE* (21), that are required for L-arabinose utilization and catabolism in *E. coli*. Like *E. coli*, the AraC regulator also functions in arabinose utilization in *Erwinia chrysanthemum*, *Citrobacter freundii*, and *Salmonella* (23, 24).

AraC/XylS regulators also regulate the expression of genes involved in metabolic processes, secretion of siderophores, urease, and virulence factors in several Gram-negative bacteria. Members of the AraC/XylS transcriptional regulators also regulate the bacterial responses to alkylating agents and oxidative and other stresses, including antibiotics (25). The role of the AraC/XylS regulator encoded by the *FTL_0689* gene of *F. tularensis* is not known. In this study, we elucidated the role of FTL_0689 in the regulation of oxidative stress response and its contribution to intracellular survival and virulence of *F. tularensis*. We named *FTL_0689* an oxidative stress response regulator (*osrR*) based on its deletion mutant phenotype. We report a novel role of *osrR* in the oxidative stress response of *F. tularensis*.

RESULTS

Sequence analysis, confirmation of gene deletion, and transcomplementation of the *osrR* gene of *F. tularensis* LVS. The scanning of the PROSITE motif database (expasy.org) with the OsrR (FTL_0689) motif confirmed that *osrR* belongs to the AraC/XylS family of transcriptional regulators. The AraC/XylS family comprises over 260 members with a characteristic C-terminal helix-turn-helix DNA binding domain. Further bioinformatic analysis using the UniProtKB database did not identify additional hits or matches in the *F. tularensis* genome, indicating that *Francisella* possesses only one AraC-like transcriptional regulator. Multiple sequence alignment using ClustalW demonstrated that OsrR of *F. tularensis* has a conserved C-terminal domain with a consensus motif GXXXXXXFXXXXXXXXXXP ($GX_6FX_{11}P$). This consensus sequence starts at amino acid 244 and ends at amino acid 263 of OsrR (see Fig. S1A in the supplemental material). The sequence comparison of *osrR* DNA and amino acid sequences within *Francisella* subspecies revealed high similarity, ranging from

98% to 99.65%. However, OsrR of *F. tularensis* LVS showed less homology when aligned with the transcriptional regulator present in other pathogenic Gram-negative bacteria. OsrR of *F. tularensis* exhibits 33%, 26%, 28%, and 20% amino acid sequence homology with the transcriptional regulators of *E. coli*, *Salmonella enterica* serovar Typhi, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa*, respectively (Fig. S1B). Multiple Em for Motif Elicitation (MEME) software identified the C-terminal and N-terminal conserved sequences of OsrR protein of *F. tularensis* LVS similar to those observed in AraC of *E. coli* and VirF of *Yersinia pestis* (Fig. S1C). Collectively, bioinformatic analysis of OsrR of *F. tularensis* demonstrates conserved features of the AraC/XylS transcriptional regulators from other bacterial pathogens. The *osrR* gene deletion and transcomplementation were confirmed by PCR (Fig. S1D).

OsrR of *F. tularensis* is not required for arabinose utilization. AraC activates *araBAD* and other operons required for L-arabinose utilization and catabolism in *E. coli* (21). To determine if OsrR of *F. tularensis* is required for L-arabinose utilization, we tested the ability of the Δ*osrR* mutant to grow in the presence of L-arabinose-containing media. We prepared Mueller-Hinton (MH)–chocolate agar plates containing 10 g (1%) of either the D-glucose or L-arabinose. The plates were then streaked with *F. tularensis* LVS, the Δ*osrR* mutant, or the transcomplemented strain. Two strains of *E. coli*, DH-5α and S17.1, were used as positive controls, as both these strains can grow in the presence of arabinose. Our results demonstrate that the Δ*osrR* mutant, the wild-type *F. tularensis* LVS, and the transcomplemented strain, as well as *E. coli* DH-5α and S17.1 strains, all grew sufficiently in the presence of D-glucose (Fig. 1A). Scant growth for the wild-type *F. tularensis*, the Δ*osrR* mutant, and the transcomplemented strain was observed when grown on MH-chocolate agar plates containing L-arabinose as the sole sugar source (Fig. 1B). These results indicated that either the *Francisella* strains poorly utilize L-arabinose or components of the MH-agar were supporting this scant growth. To address this notion, we generated bacterial growth curves using Chamberlain's chemically defined medium (CDM) with three different compositions containing D-glucose only, L-arabinose only, and neither D-glucose nor L-arabinose. Both the Δ*osrR* mutant and the wild-type *F. tularensis* LVS grew very well in glucose-containing CDM (Fig. 1C). Both strains grew at a similar rate and reached an approximate optical density at 600 nm (OD_{600}) of 2.4 after 40 h of growth. However, when the CDM was supplemented with L-arabinose only (Fig. 1D) or with no sugar (Fig. 1E), both the wild type and the mutant strains reached an OD_{600} of 0.5 after a short period of growth and then entered a stationary phase, indicating their growth was largely affected by the lack of glucose in the medium. Collectively, these results demonstrate that *F. tularensis* cannot utilize L-arabinose that and D-glucose is essential for its growth. Furthermore, these results also show that OsrR of *F. tularensis* LVS is not involved in L-arabinose metabolism.

The Δ*osrR* mutant of *F. tularensis* is susceptible to oxidative stress. The genomic organization revealed that the *osrR* of *F. tularensis* is divergently transcribed from the genes encoding major facilitator superfamily (MFS) type Emr multidrug efflux pump (MEP) genes—*emrB*, *emrA1*, and *silC* (Fig. 2A). Such a unique organization indicated that *osrR* might be involved in the regulation of these downstream genes. Both the *emrA1* and *silC* mutants characterized previously exhibit extreme sensitivity to oxidants (26, 27). We hypothesized that if OsrR regulates the expression of MEP genes, the Δ*osrR* mutant should mirror the oxidant-sensitive phenotype of the *emrA1* mutant of *F. tularensis* LVS. We investigated the sensitivity of the Δ*osrR* mutant to peroxides and superoxide ($O_2^{-\cdot}$)-generating compounds. The *emrA1* mutant was used for comparison. We performed a spot assay by exposing wild-type *F. tularensis* LVS, the Δ*osrR* mutant, the Δ*osrR*+p*osrR* transcomplemented strain, and the *emrA1* mutant to various concentrations of H_2O_2, incubating them for 1 and 3 h, and plating them on MH-chocolate agar to determine the bacterial viability. The results showed enhanced killing of the Δ*osrR* mutant when exposed to increasing concentrations of H_2O_2 for 1 and 3 h compared to the wild-type *F. tularensis* LVS. The *emrA1* mutant showed enhanced killing compared to the wild-type *F. tularensis* LVS after 1 h of exposure to H_2O_2. However, the Δ*osrR* mutant exhibited a higher sensitivity to H_2O_2 than the *emrA1* mutant. The transcomplementation restored the wild-type phenotype (Fig. 2B). Similar results were

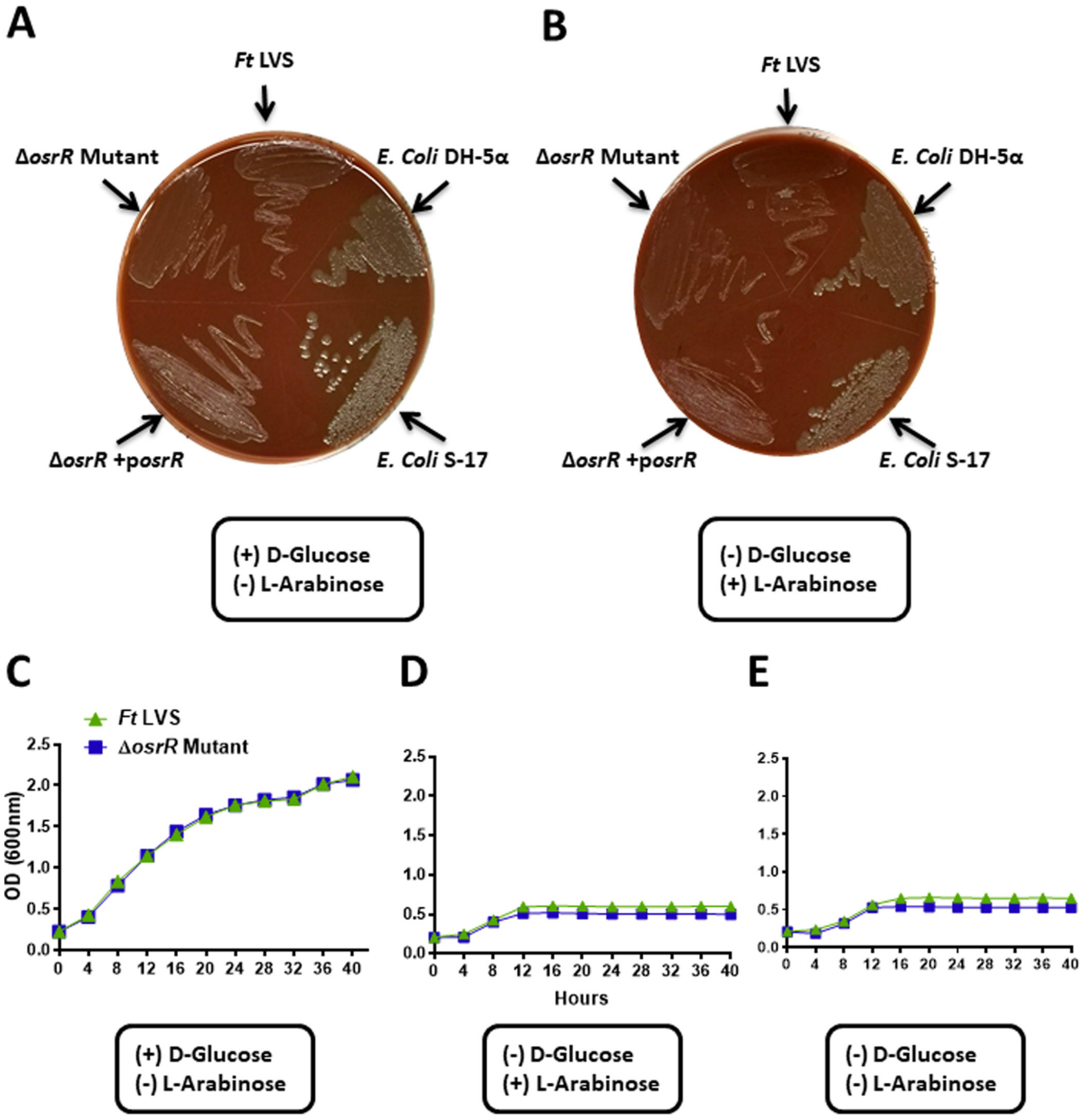

FIG 1 OsrR of *F. tularensis* is not required for arabinose utilization. (A and B) The wild-type *F. tularensis* (*Ft*) LVS, the Δ*osrR* mutant, and the transcomplemented strain (Δ*osrR* +p*osrR*) were grown in MH-chocolate agar containing D-glucose (A) or L-arabinose (B). *E. coli* DH-5α and *E. coli* S17.1 were used as positive controls. (C to E) Growth curves of the wild-type *F. tularensis* LVS and the Δ*osrR* mutant in Chamberlain's defined medium supplemented with D-glucose (C) or L-arabinose (D) or in the absence of both sugars (E). The results shown are representative of three independent experiments conducted with similar results.

observed in a bacterial killing assay. Wild-type *F. tularensis* LVS, the Δ*osrR* mutant, the transcomplemented strain, and the *emrA1* mutant (adjusted to an OD_{600} of 0.2) were exposed to 1 mM H_2O_2. The results showed a significant reduction in the numbers of Δ*osrR* and *emrA1* mutant bacteria compared to the wild-type *F. tularensis* LVS after 1 and 3 h of exposure to H_2O_2. Transcomplementation restored the wild-type phenotype (Fig. 2C). As reported earlier for both the *emrA1* and Δ*silC* mutants of *F. tularensis* (26, 27), the Δ*osrR* mutant also showed enhanced sensitivity to the organic peroxides

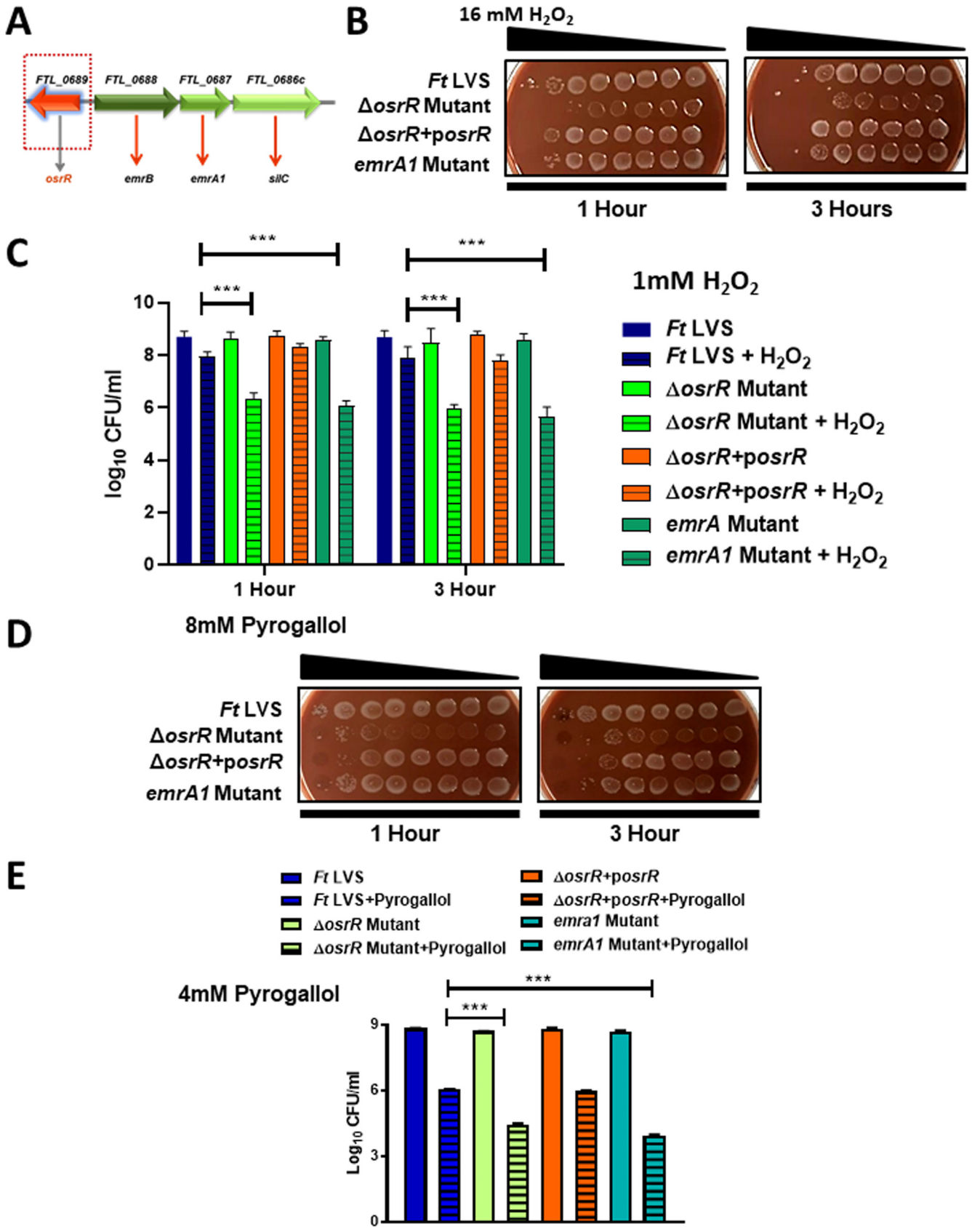

FIG 2 The Δ*osrR* mutant of *F. tularensis* is sensitive to hydrogen peroxide and pyrogallol. (A) Genomic organization of the *osrR* gene of *F. tularensis* LVS. (B) Wild-type *F. tularensis* (*Ft*) LVS, the Δ*osrR* mutant, the Δ*osrR* +*posrR* strain, and the *emrA1* mutant were exposed to H_2O_2 diluted 2-fold from a starting

(Continued on next page)

cumene hydroperoxide (CHP) and tert-butyl hydroquinone (TBH) compared to wild-type *F. tularensis* LVS (Fig. S2A and B).

We next tested the sensitivity of the Δ*osrR* mutant to the superoxide-generating compound pyrogallol and compared it with wild-type *F. tularensis* LVS and the *emrA1* mutant. Enhanced killing of the Δ*osrR* mutant was observed upon exposure to increasing concentrations of pyrogallol, at 1 and 3 h postexposure in the spot assay, and bacterial killing assay compared to the wild-type *F. tularensis* LVS. The *emrA1* mutant, as reported earlier, also showed enhanced sensitivity compared to the wild-type *F. tularensis* LVS after one and 3 h of exposure to pyrogallol in the spot and bacterial killing assays (Fig. 2D and E). The sensitivity of the Δ*osrR* mutant to the superoxide-generating compound paraquat, which generates superoxide radicals intracellularly, was also tested. However, the Δ*osrR* mutant did not exhibit any enhanced susceptibility compared to that observed for wild-type *F. tularensis* LVS (Fig. S2C). Similar results were obtained when the sensitivity of the Δ*osrR* mutant was tested against another superoxide-generating compound, menadione (Fig. S2D). Collectively, these results demonstrate that loss of *osrR* is associated with enhanced sensitivity of the Δ*osrR* mutant to oxidants such as H_2O_2, organic peroxides, and pyrogallol compared to wild-type *F. tularensis* LVS. These results also demonstrate that the Δ*osrR* mutant is more sensitive to H_2O_2 and pyrogallol than the *emrA1* mutant.

OsrR regulates the expression of MEP genes under conditions of oxidative stress. Our preceding results demonstrated that the oxidant-sensitive phenotype of the Δ*osrR* mutant mirrored the MEP gene mutant phenotype, specifically the *emrA1* mutant. We hypothesized that the OsrR of *Francisella* might regulate the expression of MEP genes under oxidative stress conditions. Based on our observation that the Δ*osrR* mutant did not exhibit any enhanced sensitivity toward the superoxide-generating compound menadione, we chose menadione to induce the oxidative stress, as both the wild-type and the mutant bacteria exhibit similar menadione sensitivity. Wild-type *F. tularensis* LVS and the Δ*osrR* mutant bacteria were left untreated or treated with 1.25 mM menadione and incubated for 1 h at 37°C. The expression of *emrB*, *emrA1*, and *silC* genes in the Δ*osrR* mutant and the wild-type *F. tularensis* LVS was determined by quantitative real-time PCR (qRT-PCR). No changes in the expression of these genes were observed in the untreated Δ*osrR* mutant, and the levels remained similar to those observed for the wild-type *F. tularensis* LVS. However, upon treatment with menadione, the expression of all the MEP genes was significantly downregulated in the Δ*osrR* mutant compared to the wild-type *F. tularensis* LVS. The expression of these genes was restored to a level similar to those observed for the wild-type *F. tularensis* LVS in the transcomplemented strain (Fig. 3). Collectively, these results demonstrate that OsrR regulates the expression of the Emr MEP genes only under conditions of oxidative stress.

Except for *osrR*, expression of other genes is not significantly altered in the Δ*osrR* mutant in the absence of oxidative stress. We performed RNA sequencing using the RNA isolated from untreated and menadione-treated *F. tularensis* LVS and the Δ*osrR* mutant to determine the differential expression of genes. A total of 38 genes were differentially expressed in the Δ*osrR* mutant in the absence of oxidative stress (Fig. 4A; Fig. S3A and B). The expression of the *osrR* gene (*FTL_0689*) was most significantly downregulated (–0.67 $\log_2$ fold change) (Fig. 4B and Fig. S3A) in the untreated Δ*osrR* mutant compared to the wild-type *F. tularensis* LVS. A total of 87 genes were differentially expressed in the Δ*osrR* mutant compared to *F. tularensis* LVS in the presence

FIG 2 Legend (Continued)
concentration of 16 mM for 1 and 3 h in a spot assay. The results shown are representative of two independent experiments. (C) Bacterial killing assay; equal amounts of the indicated bacterial strains were either left untreated or treated with 1 mM H_2O_2 for 1 and 3 h. The cultures were diluted 10-fold and plated on MH-chocolate agar plates, and the colonies were counted. (D) Wild-type *F. tularensis* LVS, the Δ*osrR* mutant, the Δ*osrR* +p*osrR* strain, and the *emrA1* mutant were exposed to pyrogallol diluted 2-fold with a starting concentration of 8 mM for 1 h and 3 h in a spot assay. The results shown are representative of two independent experiments. (E) A bacterial killing assay was performed using 4 mM pyrogallol as described in panel C. The data for the bacterial killing assay experiments in panels C and E are represented as the mean ± SEM from three independent experiments, each conducted with three technical replicates. The data were analyzed using one-way ANOVA. **, $P < 0.01$; ***, $P < 0.001$.

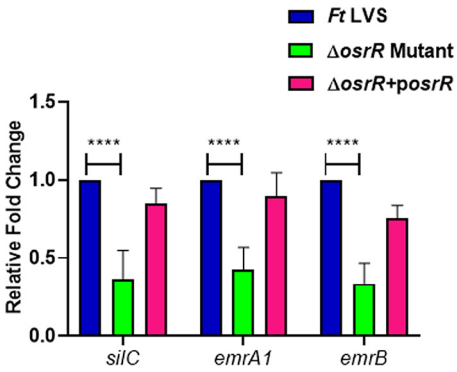

FIG 3 OsrR regulates the expression of Emr multidrug efflux pump (MEP) genes under conditions of oxidative stress. Wild-type *F. tularensis* (*Ft*) LVS, Δ*osrR* mutant, and Δ*osrR*+p*osrR* transcomplemented strains were exposed to 1.25 mM menadione for 1 h to induce oxidative stress. The RNA was isolated and quantitated for the expression of MEP genes *silC*, *emrA1*, and *emrB* by qRT-PCR. The data are represented as the relative fold change compared to *F. tularensis* LVS. The data are represented as the mean ± SEM from three independent experiments, each conducted with three technical replicates and analyzed using one-way ANOVA. ****, $P < 0.0001$.

of oxidative stress induced by menadione. The expression of 33 genes was upregulated, whereas 54 genes were downregulated in the Δ*osrR* mutant (Fig. 4C; Fig. S4 and S5). The expression of the *osrR* gene was downregulated further (–1.08 $\log_2$ fold change) when exposed to oxidative stress caused by menadione compared to the level observed for the untreated Δ*osrR* mutant (Fig. 4D and Fig. S4). These results collectively, together with those observed for the MEP genes *emrB*, *emrA1*, and *silC*, demonstrate that OsrR may not be required for gene regulation of *F. tularensis* under normal growth conditions. However, OsrR does play a regulatory role under oxidative stress conditions. This notion was investigated further.

Exposure of the Δ*osrR* mutant to oxidative stress results in downregulation of genes encoded on the FPI. We next determined the differentially expressed genes in the Δ*osrR* mutant compared to the wild-type *F. tularensis* LVS when the bacteria were exposed to oxidative stress for 1 h. The RNA sequencing results identified five downregulated genes, *pdpE*, *pdpC*, *iglJ*, *iglI*, and *dotU*, encoded within the FPI. The expression of these genes remained unaltered when the Δ*osrR* mutant was not exposed to oxidative stress (Fig. 5A). These FPI genes encode components of the type VI secretion system (T6SS) of *F. tularensis* (28–30). The qRT-PCR analysis confirmed the downregulated expression of two of the most downregulated genes, *pdpC* and *iglJ*, in the Δ*osrR* mutant in the presence of oxidative stress. Transcomplementation of the *osrR* gene in the Δ*osrR* mutant restored the expression levels of both *pdpC* and *iglJ* genes to levels similar to those observed for the wild-type *F. tularensis* LVS in the presence of oxidative stress (Fig. 5B). Overall, these results indicate that OsrR regulates the expression of FPI genes under oxidative stress conditions.

The Δ*osrR* mutant exhibits attenuated intramacrophage growth and virulence in mice. During its intracellular residence, *F. tularensis* is exposed to oxidative stress. We next verified if the downregulated expression of FPI genes in the Δ*osrR* mutant, when exposed to oxidative stress, is associated with its impaired survival in macrophages. We infected murine primary bone marrow-derived macrophages (BMDMs) with the wild-type *F. tularensis* LVS, the Δ*osrR* mutant, and the transcomplemented strain at a multiplicity of infection (MOI) of 100. The BMDMs were lysed at 4 and 24 h postinfection to determine the numbers of bacteria that invaded BMDMs and replicated intracellularly, respectively. The results revealed that all three *Francisella* strains invaded the macrophages with equal efficacy, and identical bacterial numbers were recovered from all the three strains tested at 4 h postinfection. At 24 h, the differences were more prominent. Significantly fewer Δ*osrR* mutant bacteria (5.49 ± 0.3 $\log_{10}$ CFU/ml) were recovered from the infected macrophages than the wild-type *F. tularensis* LVS (7.46 ± 0.1 $\log_{10}$ CFU/ml).

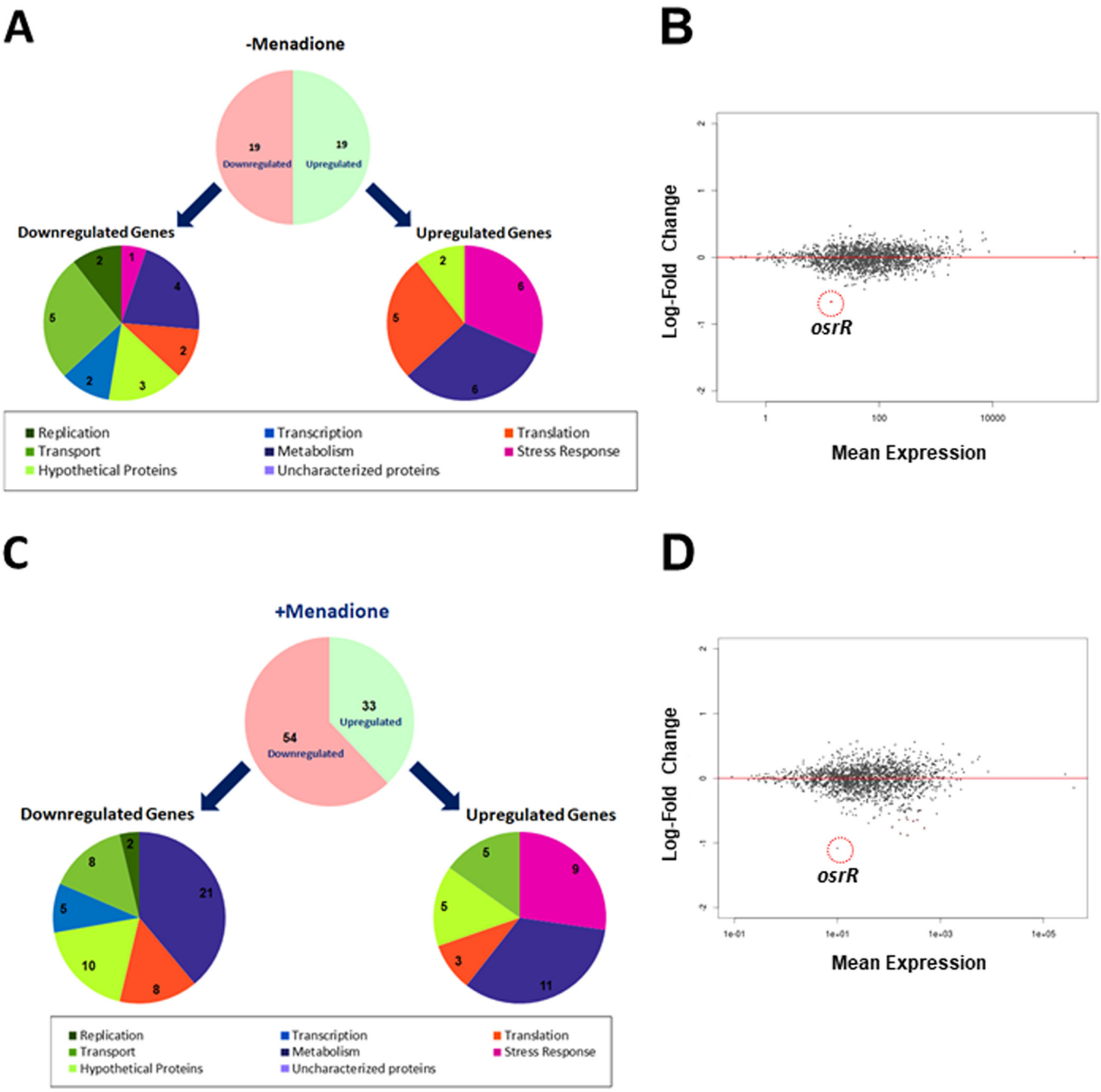

FIG 4 Differential expression of genes in the Δ*osrR* mutant of *F. tularensis* LVS in the absence and presence of oxidative stress induced by menadione. (A and C) Profile of differentially expressed genes involved in various cellular functions in Δ*osrR* mutant compared to *F. tularensis* (*Ft*) LVS as determined by RNA sequence analysis in the absence (A) and presence (C) of oxidative stress induced by exposure to menadione. (B and D) Dot plots of gene expression profiles of differentially expressed genes in the Δ*osrR* mutant compared to those in *F. tularensis* LVS as determined by RNA sequence analysis in the absence (B) and presence (D) of oxidative stress induced by exposure to menadione. The data shown are cumulative of three independent RNA sequencing experiments.

Transcomplementation partially restored the wild-type phenotype with significantly higher numbers (6.89 ± 0.5 $\log_{10}$ CFU/ml) of transcomplemented than Δ*osrR* mutant bacteria recovered from BMDMs 24 h postinfection (Fig. 5C). These results demonstrate that the Δ*osrR* mutant is attenuated for intramacrophage growth and that OsrR contributes to intramacrophage survival of *F. tularensis*.

To further establish that OsrR is required not only for intramacrophage growth and survival but also for virulence in mice, we performed *in vivo* experiments in a mouse model of tularemia. We infected C57BL/6 mice intranasally with incremental doses

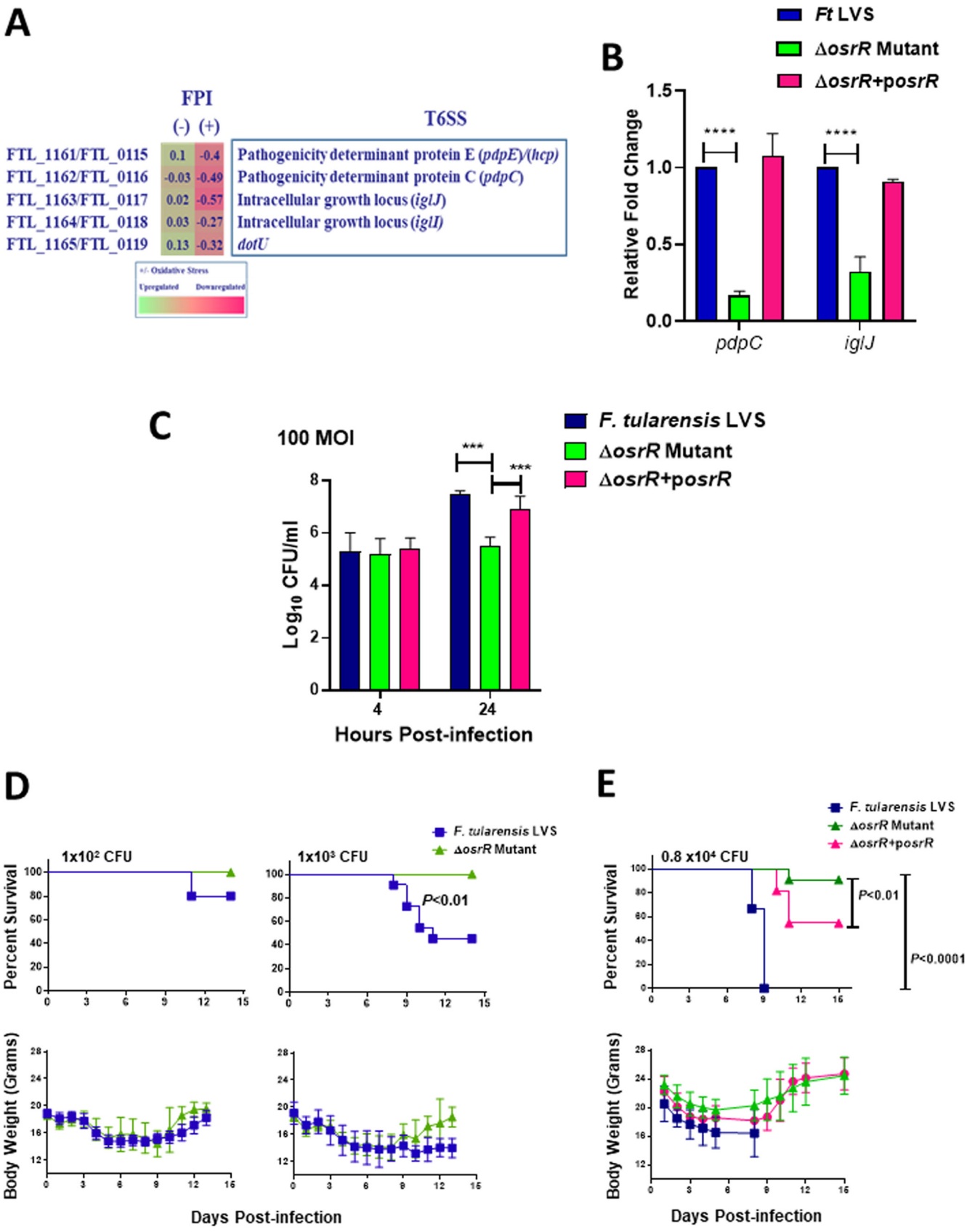

FIG 5 Exposure of the Δ*osrR* mutant to oxidative stress results in downregulation of genes encoded on the *Francisella* pathogenicity island (FPI), attenuated intramacrophage survival, and virulence in mice. (A) Expression profile of FPI genes in the Δ*osrR* mutant in the absence of (–) or upon

(Continued on next page)

(1×10^2 and 1×10^3 CFU) of *F. tularensis* LVS or the Δ*osrR* mutant and monitored their survival and body weights daily for 15 days. It was observed that the Δ*osrR* mutant was attenuated for virulence compared to the wild-type *F. tularensis* LVS. All the mice infected with 1×10^2 and 1×10^3 CFU of the Δ*osrR* mutant survived the infection, whereas 20% of mice infected with 1×10^2 CFU and 40% of those infected with 1×10^3 CFU of the wild-type *F. tularensis* LVS succumbed to infection. Furthermore, all infected mice lost body weight during the first 3 to 7 days and then gradually regained their original body weight, indicating that all mice received the infection (Fig. 5D). In another experiment, C57BL/6 mice were infected intranasally with 0.8×10^4 CFU of *F. tularensis* LVS, the Δ*osrR* mutant, or the transcomplemented Δ*osrR*+p*osrR* strain. They were monitored for their survival and body weights daily for 15 days. All mice infected with 0.8×10^4 CFU of *F. tularensis* LVS succumbed to the infection by day 9 postinfection. However, 90% (1/8) of mice infected with an equal dose of the Δ*osrR* mutant survived the infection. Transcomplementation partially restored the virulence of the Δ*osrR* mutant, and 45% (5/11) of mice infected with the transcomplemented strain succumbed to infection. All mice that survived the infection with either the Δ*osrR* mutant or the transcomplemented strain after an initial loss of their body weight regained their preinfection body weight by day 12 postinfection (Fig. 5E). These results demonstrate that mice infected with the Δ*osrR* mutant survive better than those infected with wild-type *F. tularensis* LVS and that OsrR contributes to the virulence of *F. tularensis*.

Exposure of the Δ*osrR* mutant to oxidative stress results in differential expression of stress response genes. RNA sequencing analysis also revealed downregulated expression of three stress response genes encoded on an operon in the Δ*osrR* mutant treated with menadione. These genes were also marginally downregulated when the Δ*osrR* mutant was not exposed to oxidative stress. These three downregulated genes included *aroA*, a gene encoding a protein of the shikimate pathway required for the synthesis of aromatic amino acids from glucose, a gene encoding a hypothetical protein, and the *rnhA* gene, which encodes RNase H. These findings indicate that OsrR modulates the expression of genes of *F. tularensis* involved in metabolism as well as RNase H abundance under oxidative stress conditions (Fig. 6A).

Interestingly, another gene, *rpoH*, which encodes a sigma factor that shares homology with the σ^{32} heat shock family protein, was also downregulated in the Δ*osrR* mutant irrespective of its exposure to oxidative stress (31). RpoH negatively regulates several genes, including stress response genes *groES*, *gorEL*, *clpB*, *FTL_1957*, *dnaK*, *dnaJ*, and *hsp40* (32). The RNA sequence analysis revealed that all these genes were moderately upregulated in the Δ*osrR* mutant not exposed to oxidative stress (Fig. S3B). However, the expression of these genes was further upregulated upon exposure to oxidative stress in the Δ*osrR* mutant compared to the wild-type *F. tularensis* LVS (Fig. S5). These findings were further confirmed by determining the expression of *groES*, *groEL*, *clpB*, *FTL_1957 dnaK*, *dnaJ*, and *hsp40* genes by qRT-PCR. The expression of all these genes that are primarily involved in heat shock response were significantly upregulated in the Δ*osrR* mutant upon exposure to oxidative stress. Transcomplementation

FIG 5 Legend (Continued)
exposure to (+) menadione compared to the wild-type *F. tularensis* (*Ft*) LVS. The data shown are cumulative of three independent RNA sequencing experiments and expressed as the $\log_2$ fold change. (B) Quantitation of RNA transcripts of *pdpC* and *iglJ* genes in the indicated *Francisella* strains by qRT-PCR. The data are represented as the relative fold change compared to *F. tularensis* LVS. The data are represented as the mean ± SEM from three independent experiments, each conducted with three technical replicates and analyzed using one-way ANOVA. ****, $P < 0.0001$. (C) Attenuation of intramacrophage growth of the Δ*osrR* mutant compared to *F. tularensis* LVS and the transcomplemented Δ*osrR*+p*osrR* strain at the indicated multiplicity of infection at 4 and 24 h postinfection. The data are represented as the mean ± SEM from two independent experiments, each conducted with four technical replicates and analyzed using one-way ANOVA. ***, $P < 0.001$. (D) C57BL/6 mice inoculated intranasally with the indicated CFU of *F. tularensis* LVS or the Δ*osrR* mutant. The morbidity and mortality of infected mice ($n = 5$ mice/group) were recorded for 15 days. (E) C57BL/6 mice inoculated intranasally with the 0.8×10^4 CFU of *F. tularensis* LVS ($n = 6$), the Δ*osrR* mutant ($n = 8$), or the transcomplemented Δ*osrR*+p*osrR* strain ($n = 11$). The morbidity and mortality of infected mice were recorded for 15 days. The data shown are cumulative of two independent experiments. The survival curves shown in the top of panels D and E are plotted as Kaplan-Meier survival curves, and the data were analyzed using the log rank test. Body weights represented as the mean ± SD for the indicated doses of infection are shown in the bottom of panels D and E.

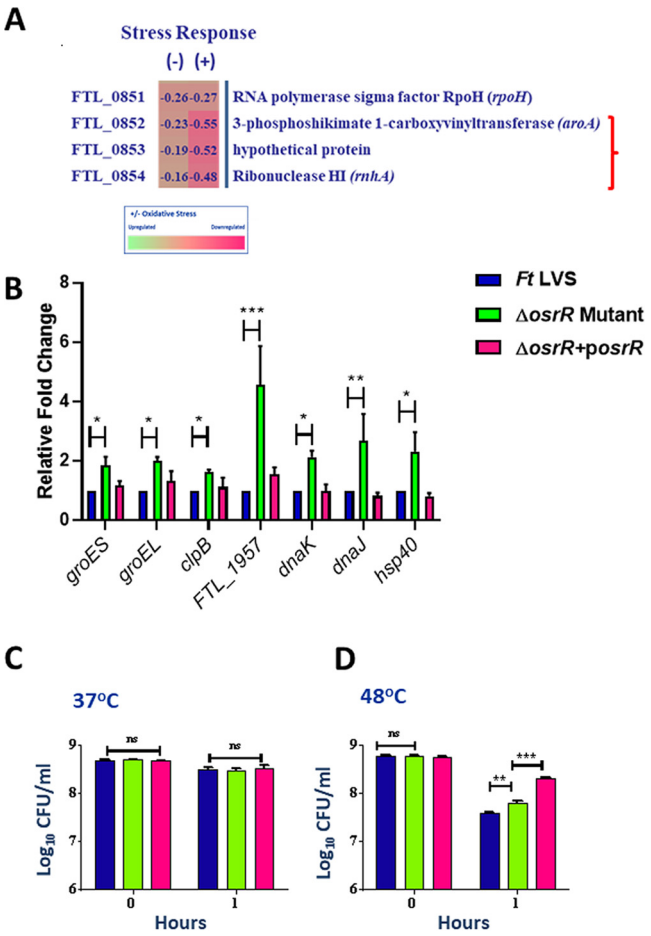

FIG 6 Exposure of the Δ*osrR* mutant to oxidative stress results in differential expression of stress response genes. (A) Expression profile of stress response genes in the Δ*osrR* mutant in the absence of (–) or upon exposure to (+) menadione compared to the wild-type *F. tularensis* (*Ft*) LVS. The data shown are cumulative of three independent RNA sequencing experiments and expressed as the $\log_2$ fold change. (B) Wild type *F. tularensis* LVS, Δ*osrR* mutant, and Δ*osrR*+p*osrR* transcomplemented strains were exposed to 1.25 mM menadione for 1 h to induce oxidative stress. The RNA was isolated and quantitated for the expression of the indicated heat-stress response genes by qRT-PCR. The data are represented as the relative fold change compared to *F. tularensis* LVS. The data are represented as the mean ± SEM from three independent experiments, each conducted with three technical replicates and analyzed using one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C and D) The viability of the indicated strains was determined after exposure to 37 and 48°C for 1 h. The data are represented as the mean ± SEM from three independent experiments, each conducted with three technical replicates. The data were analyzed using one-way ANOVA. **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

restored the expression levels of these genes to levels similar to those observed for the wild-type *F. tularensis* (Fig. 6B).

Further, to verify if OsrR regulates *rpoH*-mediated heat shock response, we conducted a thermal resistance experiment in which bacteria were exposed to a temperature (48°C) higher than the physiological temperature (37°C). The transcomplemented strain was used as a positive control, as the *osrR* gene in the transcomplemented vector is under the control of the GroEL promoter, which is activated at high temperatures. It was observed that the viabilities of all these three *Francisella* strains were not affected when exposed to a temperature of 37°C for 1 h, indicating that at physiological temperature, OsrR does not affect the growth of *F. tularensis* (Fig. 6C).

Next, we induced high-temperature stress by growing the bacteria at 48°C (Fig. 6D). After 1 h of incubation, all three strains exhibited a significant decrease in the viable bacterial count. However, the numbers of the Δ*osrR* mutant bacteria (7.8 ± 0.0 $\log_{10}$

CFU/ml) were significantly higher than those of the wild-type *F. tularensis* LVS (7.5 $\pm$ 0.0 $\log_{10}$ CFU/ml). The transcomplemented strain survived better than the former two *Francisella* strains tested, with significantly higher numbers of bacteria (8.3 $\pm$ 0.0 $\log_{10}$ CFU/ml) remaining viable at the higher temperature. Overall, these results indicate that OsrR of *F. tularensis* LVS regulates the expression of heat shock proteins negatively and facilitates survival under extreme stress conditions.

OsrR of *F. tularensis* positively regulates key components of the TCA cycle during oxidative stress. Exposure of the Δ*osrR* mutant to menadione downregulated expression of genes involved in metabolism encoded on four different operons. One of these operons encodes the enzyme pyruvate dehydrogenase subunits, E1 (*aceE*), E2 (*aceF*), and E3 (*lpd*), required for the biosynthesis of acetyl coenzyme A (acetyl-CoA), an enzyme that initiates the tricarboxylic acid (TCA) cycle (33). Among the three subunits, E3 or dihydrolipoyl dehydrogenase encoded by *lpd* was downregulated more in the Δ*osrR* mutant exposed to oxidative stress compared to the wild-type *F. tularensis* LVS. The other two subunits, E1 pyruvate dehydrogenase (encoded by *aceE*) and E2 dihydrolipoyl transacetylase (encoded by *aceF*), were also downregulated in the Δ*osrR* mutant. These results indicated that OsrR potentially contributes to the activation of the TCA cycle in *F. tularensis* under oxidative stress conditions (Fig. 7A).

The expression of genes encoded on another operon was downregulated in the Δ*osrR* mutant. This operon comprises the genes required for the biosynthesis of pantothenate (vitamin B_5), which is the central component of CoA. The RNA sequencing data analysis revealed that the *coaX* gene encoding type III pantothenate kinase was downregulated in the Δ*osrR* mutant compared to the wild-type *F. tularensis* LVS when subjected to oxidative stress. The analysis also exhibited downregulation of the aspartate-1-decarboxylase (*panD*) gene, required for the formation of β-alanine from L-aspartate, and the *panC* gene encoding the pantothenate synthase that employs β-alanine and L-pantoate to produce pantothenate. The expression levels of the *panB* (ketopantoate hydroxymethyltransferase) and *panG* (ketopantoate reductase) genes were also downregulated in the menadione-treated Δ*osrR* mutant compared to wild-type *F. tularensis* LVS. Enzymes, PanB and PanG, catalyze the conversion of 2-oxoisovalerate to 2-dehydropantoate and pantoate, respectively. Altogether, these results indicate that, under oxidative stress, OsrR of *F. tularensis* positively regulates the pantothenate biosynthesis pathway required for CoA formation to initiate the TCA cycle (Fig. 7A).

Furthermore, exposing the Δ*osrR* mutant to oxidative stress altered the expression of genes encoded on an additional metabolic operon. This operon harbors three genes—*pgk*, *pyk*, and *fba*. The *pgk* gene encodes phosphoglycerate kinase, an enzyme that catalyzes the formation of ATP from ADP. The *pyk* gene encodes pyruvate kinase involved in the production of ATP and pyruvate, which is crucial for several metabolic pathways, including the TCA cycle (34). Moreover, the *fba* gene was downregulated to a greater extent in the Δ*osrR* mutant post-exposure to menadione. This gene encodes fructose-bisphosphate aldolase class II (FBA), which is required for the formation of fructose 1,6-bisphosphate (FBP) through gluconeogenesis and glycolysis pathways in *Francisella* (35). FBA is required for bacterial virulence and is essential for intracellular survival, indicating the significance of these metabolic pathways in bacterial pathogenesis (35) (Fig. 7A).

In addition to the genes described above, RNA expression levels of a set of metabolic genes, *sucA* and *sucB*, were significantly altered in the Δ*osrR* mutant when treated with menadione. These genes encode oxoglutarate dehydrogenase (OGDH) subunits E1 and E2, respectively, which are the key enzymes of the TCA cycle (Fig. 7A). Collectively, the RNA sequencing data analysis indicated that inducing oxidative stress in the Δ*osrR* mutant resulted in differential expression of genes encoding key elements of the metabolic pathways, TCA cycle, and energy production.

To validate the RNA sequencing results, the expressions of *lpd*, *coaX*, *fba*, and *sucA* genes were determined by qRT-PCR analysis in the absence or the presence of oxidative stress induced by menadione. The expression profile of all these genes remained unaltered in the absence of oxidative stress in wild-type *F. tularensis* LVS, the Δ*osrR* mutant, and the transcomplemented strain (Fig. 7B). However, in the presence of

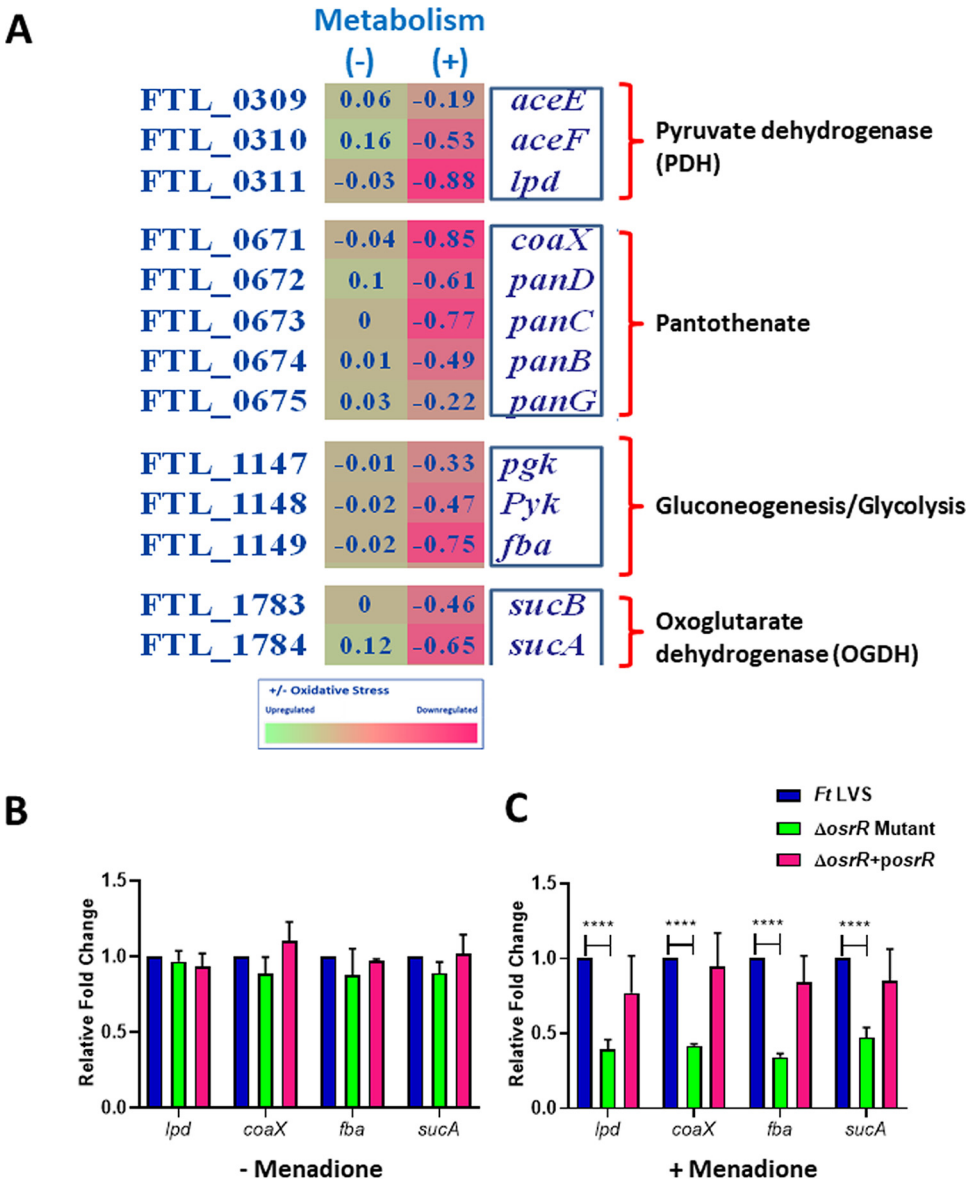

FIG 7 OsrR of *F. tularensis* positively regulates key components of the TCA cycle during oxidative stress. (A) Expression profile of genes of the indicated metabolic pathways encoded as operons in the Δ*osrR* mutant in the absence of (–) or upon exposure to (+) menadione compared to the wild-type *F. tularensis* (*Ft*) LVS. The data shown are cumulative of three independent RNA sequencing experiments and expressed as the $\log_2$ fold change. (B and C) Quantitation of transcripts of *lpd*, *coaX*, *fba*, and *sucA* genes in the indicated *Francisella* strains by qRT-PCR in the absence (B) or the presence (C) of oxidative stress induced by menadione. The data are represented as relative the fold change compared to *F. tularensis* LVS. The data are represented as the mean ± SEM from three independent experiments, each conducted with four technical replicates and analyzed using one-way ANOVA. ****, $P < 0.0001$.

oxidative stress, the expression of all these genes was significantly downregulated in the Δ*osrR* mutant compared to the wild-type *F. tularensis* LVS. Transcomplementation restored the expression of these genes to levels similar to those observed for the wild-type strain (Fig. 7C). Together, these results demonstrate that OsrR of *F. tularensis* positively regulates components of the TCA cycle, pantothenate, and glucose metabolic pathways during oxidative stress to sustain intracellular survival of *F. tularensis*.

Expression of regulatory proteins and several transcription units is downregulated in the Δ*osrR* mutant exposed to oxidative stress. We identified an additional set of genes that were differentially expressed in the Δ*osrR* mutant compared to the wild-type *F. tularensis* LVS after induction of oxidative stress. The expression level of two genes,

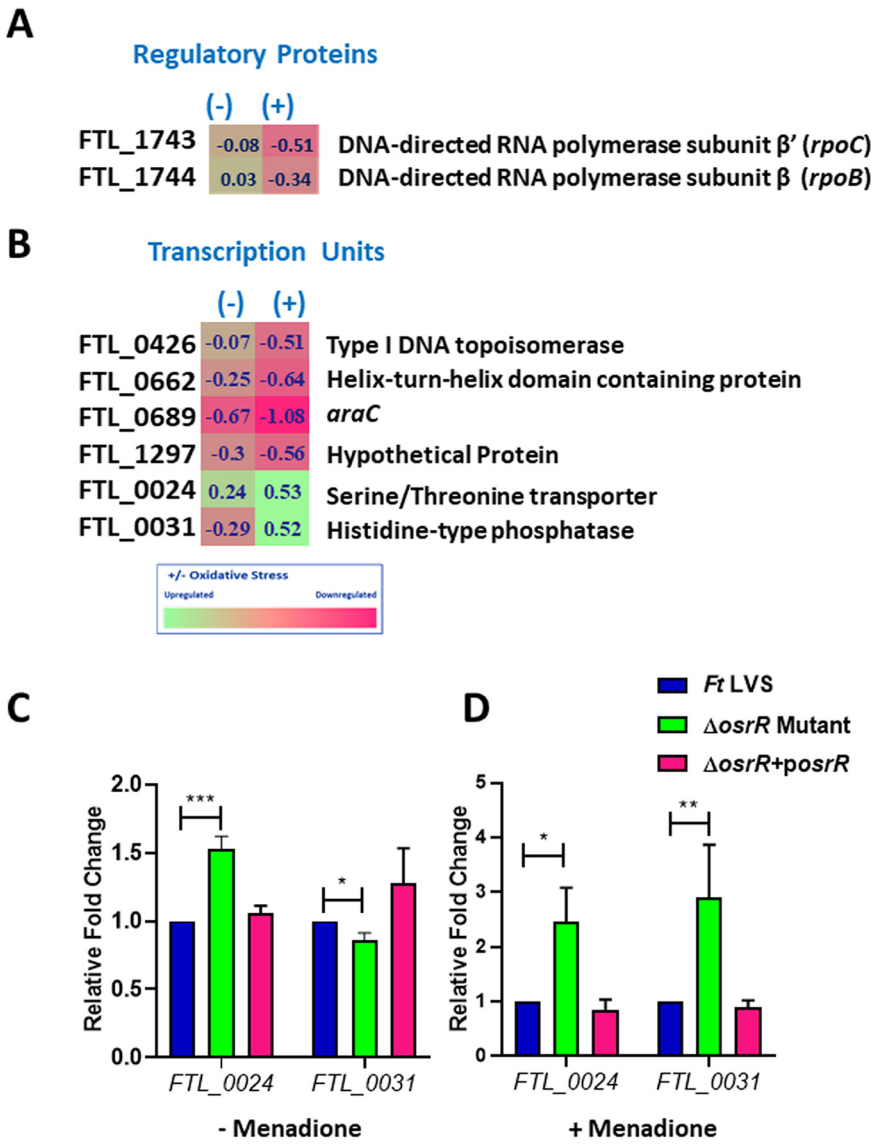

FIG 8 Differential expression of regulatory proteins and several transcription units in the Δ*osrR* mutant exposed to oxidative stress. (A and B) Expression profile of regulatory genes (A) and transcription units (B) in the Δ*osrR* mutant in the absence of (–) or upon exposure to (+) menadione compared to the wild-type *F. tularensis* (*Ft*) LVS. The data shown are cumulative of three independent RNA sequencing experiments and expressed as the $\log_2$ fold change. (C and D) Quantitation of *FTL_0024* and *FTL_0031* gene transcripts in the indicated *Francisella* strains by qRT-PCR in the absence (C) or the presence (D) of oxidative stress induced by menadione. The data are represented as the relative fold change compared to *F. tularensis* LVS. The data are represented as the mean ± SEM from three independent experiments, each conducted with three technical replicates and analyzed using one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

rpoB and *rpoC*, which encoded an operon, was significantly downregulated in the menadione-treated Δ*osrR* mutant. These genes encode two of the RNA polymerase core proteins, the β (RpoB) and β' (RpoC) subunits (31) (Fig. 8A), indicating that OsrR is also involved in regulating the *F. tularensis* transcriptional process during oxidative stress.

Besides regulating the RNA expression levels of genes encoded on operons, OsrR of *F. tularensis* also exhibited a significant regulatory effect on a few transcriptional units during oxidative stress. The *osrR* gene, which is transcribed as a single transcription unit, remained downregulated to the greatest extent irrespective of the treatment with menadione in the Δ*osrR* mutant. The *pyrH* gene that encodes the uridylate kinase

enzyme to catalyze pyrimidine synthesis was also downregulated, suggesting that OsrR is also involved in nucleotide damage repair response during oxidative stress. Another gene that was largely affected in the Δ*osrR* mutant by menadione treatment was *FTL_0426* coding for type I DNA topoisomerase. This enzyme is required for DNA supercoil repair, indicating that OsrR is also involved in regulating DNA repair mechanisms in *F. tularensis* during oxidative stress.

Furthermore, two genes, *FTL_0024* and *FTL_0031*, were significantly upregulated in the Δ*osrR* mutant compared to the wild-type strain. These genes encode serine/threonine transporter and acid phosphatase, respectively, indicating that OsrR negatively regulates genes required to transport amino acids (serine and threonine) during the conditions of oxidative stress (Fig. 8B). qRT-PCR analysis validated the transcription profile of both *FTL_0024* and *FTL_0031* obtained by RNA sequencing in the absence or presence of oxidative stress induced by menadione (Fig. 8C and D). These results indicate that, since acid phosphatase is essential for glucose and G6P phosphorylation and dephosphorylation mechanisms in bacteria, OsrR of *F. tularensis* is possibly involved in the negative regulation of G6P to suppress the glucose metabolism.

DISCUSSION

The AraC/XylS family is one of the largest families of bacterial transcriptional regulators (25). Members of the AraC/XylS family can alter gene expression, mostly positively (36). In rare cases, gene expression can be either negatively altered by family members such as the CelD of *Y. pestis* (37) or both positively and negatively by the AraC of *E. coli* and YbtA of *Y. pestis* (38). AraC/XylS transcriptional regulators are additionally classified into two categories based on the regulation of their own transcription (25). The first category has signal receptors within the same protein that respond to the availability of substrates to activate or repress gene expression. Examples of this type of transcriptional regulators are AraC, XylS, RhaR, and UreR (25, 39, 40). The second category requires the activity of another regulator (activator or repressor) to control gene expression in a two-component regulatory system manner, such as MarA, SoxS, and TcpN (41, 42). The AraC/XylS family proteins regulate three unique processes—regulation of virulence factors, stress response, and metabolism of carbons (25). The present study characterized the role of OsrR, a member of the AraC/Xyls family, in oxidative stress response, intramacrophage survival, and virulence of *F. tularensis*.

Sugar utilization experiments revealed that OsrR transcriptional regulator, unlike its reported role in other bacteria, is not involved in L-arabinose utilization in *F. tularensis* LVS. The genomic organization of the Emr type MEP of *F. tularensis* is unique, comprising three adjacent genes, *emrB*, *emrA1*, and *silC*, that constitute an operon (26, 27). The location of the *osrR* gene in proximity to and its transcription divergent from *emrB*, *emrA1*, and *silC* genes suggested a possible involvement of OsrR in the regulation of these MEP genes. A similar genomic organization of the MEP genes is also present in *Pseudomonas aeruginosa*, with the MexR transcriptional regulator transcribed divergently from the *mexAB-oprM* multidrug efflux system (43). In response to oxidative stress, the MexR transcriptional regulator detaches from the promoter and functions as an activator to initiate the MEP gene expression. Moreover, *Campylobacter jejuni* possesses a *cmeGH* multidrug efflux pump that is controlled by a Fur repressor transcribed divergently upstream of the operon (44). Downregulated expression of the MEP genes observed in this study suggests that the OsrR controls the functioning of the Emr MEP only under conditions of oxidative stress. These findings were further confirmed by the enhanced sensitivity of the Δ*osrR* mutant to peroxides and superoxide-generating compounds compared to those observed for the *emrA* mutant.

The role of the OsrR in oxidative stress response is further cemented by RNA sequencing results. In the absence of oxidative stress, the transcription profiles of both the Δ*osrR* mutant and the wild type *F. tularensis* LVS, except for the *osrR* gene expression, were more or less identical, indicating that OsrR does not exert its regulatory role when *Francisella* is not exposed to oxidative stress. A differential expression of several

genes in the Δ*osrR* mutant indicated that OsrR functions when the bacteria are exposed to oxidative stress conditions. It has been reported that SoxS, an AraC/XylS family transcriptional regulator of *E. coli*, is induced by oxidants such as paraquat and menadione to activate the transcription of the SoxRS regulon (25, 41). However, SoxRS homologs are absent in *F. tularensis*. The transcriptome analysis of the Δ*osrR* mutant treated with menadione does confirm the notion that OsrR is activated in the presence of an oxidant to exert its regulatory effect. Thus, it is probable that OsrR of *F. tularensis* functions similarly to the SoxRS regulon present in *E. coli* and other bacterial pathogens.

Our results demonstrate that oxidative stress induced by menadione resulted in the differential expression of a multitude of genes in the Δ*osrR* mutant of *F. tularensis* transcribed as operons as well as transcriptional units. The results from this study demonstrate the involvement of OsrR in the positive regulation of the FPI genes *pdpE*, *pdpC*, *iglJ*, *iglI*, and *dotC* in response to oxidative stress. These genes constitute the components of the type VI secretion system of *F. tularensis*. Thus far, several studies have published the fundamental role of these FPI genes in the intramacrophage virulence of *F. tularensis* (12, 13, 45, 46). Specifically, the *pdpC* gene encodes a pathogenicity determinant protein that does not contribute to intracellular survival but is required for virulence in mice (47). On the other hand, *pdpE* is required for neither virulence nor intracellular survival. The *iglJ* gene product is required for intracellular growth, cytopathogenicity, and virulence in mice, whereas *iglI* plays an important role in the phagosomal escape, cytopathogenicity, and virulence in mice (48). The *dotU* gene product is an essential structural component of the T6SS of *F. tularensis* and is required for the functioning of T6SS. The Δ*osrR* mutant of *F. tularensis* also exhibits defective intramacrophage survival and attenuated virulence in mice.

The transcriptome analysis of the Δ*osrR* mutant revealed the involvement of OsrR of *F. tularensis* LVS in the regulation of two genes, *aroA*, and *rnhA*, that are a part of the heat stress-response-related operon. The AroA is involved in the shikimate pathway that catalyzes the synthesis of aromatic amino acids from glucose (49). OsrR of *F. tularensis* positively regulated the *rnhA* gene that encodes RNase H. In *Yersinia*, *Salmonella*, *Helicobacter pylori*, *Shigella flexneri*, and *Mycobacterium smegmatis* ribonucleases are known to function as virulence factors (50) and protect bacteria against oxidative stress (51). We observed that the expression level of the *rpoH* gene was not altered in the Δ*osrR* mutant in the absence or the presence of oxidative stress and remained marginally downregulated. RpoH negatively regulates the expression of several genes, including heat shock response genes (32). The RNA sequence analysis revealed that expression of *groEL*, *groES*, *clpB*, *FTL_1957*, *dnaK*, *dnaJ*, and *hsp40* were moderately upregulated in the Δ*osrR* mutant not exposed to oxidative stress and were further upregulated upon exposure to oxidative stress. These findings were also substantiated by qRT-PCR analysis. Collectively, these results indicate a broader role for OsrR in the regulation of oxidative as well as overall stress response of *F. tularensis*.

The results from this study further demonstrated the involvement of OsrR in the regulation of the components of the TCA cycle in *F. tularensis* during oxidative stress. Several studies have linked the TCA cycle with the virulence of *F. tularensis* during stress conditions (35, 52, 53). We observed the downregulated expression of key TCA cycle enzymes in the Δ*osrR* mutant after menadione treatment. The operon encoding pyruvate dehydrogenase enzyme was significantly downregulated. The pyruvate dehydrogenase is an enzyme that catalyzes the synthesis of the first enzyme, acetyl-CoA, of the TCA cycle (33). It has three subunits, E1 (pyruvate dehydrogenase [AceE]), E2 (dihydrolipoamide transacetylase [AceF]), and E3 (dihydrolipoamide dehydrogenase [Lpd]) (52). We also observed the downregulation of another enzyme, oxoglutarate dehydrogenase, encoded on another operon. The oxoglutarate dehydrogenase promotes the conversion of α-ketoglutarate (or 2-oxoglutarate) into succinyl-CoA in the TCA cycle. Oxoglutarate dehydrogenase is also composed of three subunits, E1 (2-oxoglutarate dehydrogenase [SucA]), E2 (dihydrolipoamide succinyltransferase [SucB]), and E3

(lipoamide dehydrogenase [Lpd]). In *F. tularensis* subsp. *novicida*, a study has shown the requirement of the E1 (AceE) subunit of the pyruvate dehydrogenase enzyme in intramacrophage growth (54). In addition, another study has demonstrated the contribution of the E3 (Lpd) subunit to *F. tularensis* LVS virulence (55). Collectively, these results demonstrate that the expression of key TCA cycle enzymes of *F. tularensis* LVS, especially under oxidative stress conditions, is controlled by OsrR.

Results from this study also demonstrate that OsrR regulates the expression of genes involved in pantothenate metabolism when *Francisella* is exposed to oxidative stress. Pantothenate, also known as vitamin B_5, is an essential central metabolic component of the CoA of the TCA cycle (56). In *E. coli* and *S. enterica* serovar Typhimurium, the *de novo* synthesis of pantothenate molecules relies on their ketopantoate reductase genes (57). The ketopantoate reductase genes in *F. tularensis* are composed of a group of five genes (*coaX*, *panD*, *panC*, *panB*, and *panG*) that promote the enzymatic reaction of pantothenate synthesis. *F. novicida* requires the *coaX* gene for the successful dissemination into mouse organs (58). It was observed that all of the five ketopantoate reductase genes were significantly downregulated in the Δ*osrR* mutant exposed to menadione, indicating a key role of OsrR in the pantothenate pathway, which subsequently is required for the formation of CoA enzyme and the initiation of the TCA cycle.

Our observations further indicate that OsrR is also involved in the positive regulation of glucose metabolism in *F. tularensis* LVS. Three genes, *pgk*, *pyk*, and *fba*, were significantly downregulated in the Δ*osrR* mutant after menadione treatment. Phosphoglycerate kinase (PGK) is an essential enzyme for the breakdown of glucose into pyruvate and, subsequently, the release of high energy (59). Pyruvate kinase (PYK) activation is promoted by fructose 1,6-bisphosphate (34), which is then metabolized by the enzyme fructose-bisphosphate aldolase class II (FBA). A study has shown that the FBA plays an important role in *Francisella* cytosolic replication only if gluconeogenesis substrate is available but is not required for the bacterial escape from the phagosome (35). These results suggest that OsrR plays a regulatory role in gluconeogenesis and glycolysis pathways in *F. tularensis*.

The results from this study additionally reveal that OsrR positively regulates the expression of other regulatory genes, *rpoB*, and *rpoC*. These genes encode two key components, the β (RpoB) and β' (RpoC), that constitute the catalytic core of RNA polymerase (60). In addition to the role of OsrR in regulating the expression of genes transcribed as operons, the results from whole-transcriptome analysis reveal OsrR-dependent expression of genes transcribed as single transcriptional units. The most prominent ones are type I DNA topoisomerase, required for the repair of damaged DNA and removal of supercoiling when exposed to oxidative stress. Further, in addition to the downregulated expression, several genes were also upregulated in the Δ*osrR* mutant when exposed to oxidative stress. The most prominently upregulated genes in the Δ*osrR* mutant were serine/threonine transporter and histidine-type phosphatase, indicating additional amino acid requirements due to the loss of OsrR under oxidative stress conditions.

Collectively, these results provide ample evidence of the regulatory role of the OsrR. However, the mechanism(s) through which OsrR exerts its regulatory role during oxidative stress remained elusive in this study. The notion that the expression of all known transcriptional regulators of *Francisella* remained unaltered in untreated or menadione-treated Δ*osrR* mutant compared to wild-type *F. tularensis* LVS indicates that OsrR may have a direct rather than an indirect role in gene regulation.

To conclude, as described for other bacterial pathogens, OsrR annotated as AraC in the *Francisella* genome is not required for L-arabinose utilization. Instead, the results obtained from the present study demonstrate that the OsrR transcriptional regulator exerts a global regulatory role on several key pathways of *F. tularensis* LVS. The OsrR contributes to the oxidative stress response by regulating the expression of Emr MEP genes involved in oxidative stress resistance. OsrR regulates the expression of FPI

genes when *Francisella* is exposed to oxidative stress and contributes to intramacrophage survival and virulence. OsrR also regulates the expression of genes involved in metabolic and other key pathways required by *Francisella* to overcome oxidative stress. Altogether, this study demonstrates that OsrR belonging to the AraC/XylS family of transcriptional regulators plays an essential role in the oxidative stress response of *F. tularensis* LVS and assigns a prominent role to a relatively unknown transcriptional regulator in tularemia pathogenesis.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The wild-type *F. tularensis* LVS used in this study was obtained from BEI Resources, Manassas, VA. The deletion mutant of the *osrR* (*FTL_0689*) gene (Δ*osrR*) of *F. tularensis* LVS and its transcomplemented strain (Δ*osrR*+p*osrR*) were generated in this study. A transposon insertion mutant in the *emrA1* gene (*FTL_0687*) of *F. tularensis* LVS available in our laboratory and used in previously published works (27, 61) was also used in this study. The *E. coli* DH-5α and S-17 strains were obtained from Invitrogen.

The *Francisella* strains were cultured on Mueller-Hinton (MH)–chocolate agar plates (BD Life Sciences) and incubated for 48 h at 37°C and 5% CO_2. Individual colonies were selected and inoculated in MH broth (MHB) enhanced with Isovitalex (BD Biosciences), ferric pyrophosphate, glucose, anhydrous calcium chloride, and hydrous magnesium chloride. The bacterial cultures were incubated at 37°C with constant shaking until the bacterial growth reached the mid-log phase. Bacterial aliquots were snap-frozen in liquid nitrogen and stored at −80°C. The *emrA1* mutant was grown on MH chocolate agar plates or MHB supplemented with kanamycin (10 μg/ml). For the transcomplemented strain (Δ*osrR*+p*osrR*), hygromycin (100 μg/ml) was added to the medium instead of kanamycin. All the work was performed under biosafety containment level 2 (BSL2).

Generation of the Δ*osrR* mutant and the transcomplemented strains of *F. tularensis* LVS. The plasmid and primers sequences used for generation and confirmation of Δ*osrR* mutant and the transcomplemented strain of *F. tularensis* LVS are listed in Table 1. The pJC84 suicide vector that allows for SacB-dependent allele replacement was used as reported earlier (62) for the generation of the in-frame gene deletion mutant of the *osrR* gene (*FTL_0689*) in *F. tularensis* LVS. For the deletion of the *osrR* gene, a 5′ fragment composed of a 1,152-bp sequence upstream of the start codon of the *FTL_0689* and the start codon was amplified by PCR using the MP135 and MP137 end primers. In addition, a 3′ fragment of a 2,081-bp downstream sequence of the *FTL_0689* and the stop codon was also amplified by PCR using MP139 and MP140 primers. By overlapping extension PCR using primers MP135 and MP140, both fragments were fused to generate a larger fragment (2,233 bp). The fused fragment was cloned into the BamHI and SalI sites of the pJC84 to generate pMM03. After that, PCR confirmation was performed and followed by pMM03 electroporation into the *F. tularensis* LVS. The colonies were then selected on MH-chocolate agar plates containing 25 μg/ml kanamycin. Following that, sucrose counterselection was performed by plating bacteria on MH-chocolate agar supplemented with 8% sucrose and incubated at 37°C with 5% CO_2 for 48 to 72 h. The sucrose-resistant and kanamycin-sensitive clones were screened for the loss of the *osrR* gene using PCR. For *osrR* gene deletion confirmation, a duplex colony PCR was performed using the following primer sets *osrR* gene-specific (MP192/MP193) and internal control-*sodB* gene (MP037/MP038). The in-frame gene deletion of *osrR* was confirmed by DNA sequencing of the PCR-positive clones.

For the generation of the transcomplemented strain (Δ*osrR* +p*osrR*), the *F. tularensis osrR* gene was amplified using the primers MP372 and MP373. BamHI and XhoI restriction enzymes were used to digest the amplified sequence and then cloned into *E. coli-Francisella* shuttle vector pMP822 (63) to generate pMM014. This plasmid was then confirmed using PCR and DNA sequencing and was electroporated into the Δ*osrR* mutant. To confirm the transcomplementation of the *osrR* gene, the bacteria were selected in MH-chocolate agar plates containing 200 μg/ml hygromycin. The hygromycin-resistant colonies were screened with PCR.

Determination of the role of OsrR in sugar utilization. Wild-type *F. tularensis* LVS or the Δ*osrR* mutant bacteria were streaked on MH chocolate agar plates supplemented with 1% glucose or 1% arabinose and incubated at 37°C with 5% CO_2 for 48 h. The bacterial growth pattern of the Δ*osrR* mutant was compared to that of the wild-type *F. tularensis* LVS and the transcomplemented strain. *E. coli* DH-5α and *E. coli* S-17 were used as positive controls.

Defined Chamberlain's medium (CDM) was prepared at three different compositions—containing glucose (4%) and no arabinose, containing arabinose (4%) and no glucose, and containing neither glucose nor arabinose. *F. tularensis* LVS or the Δ*osrR* mutant bacteria adjusted to a 0.2 OD_{600}, which corresponded to 1×10^9 CFU/ml, was inoculated into these CDM formulations and incubated at 37°C with shaking. The OD_{600} was recorded every 4 h, and growth curves were generated using GraphPad Prism 7 software.

Determination of the role of OsrR in oxidative stress resistance. The sensitivity of the Δ*osrR* mutant to oxidants was determined by spot assays and bacterial killing assays in the presence or the absence of oxidants as described previously (20, 26, 64, 65). Briefly, peroxides (16 mM H_2O_2, 1 mM tert-butyl hydroquinone [TBH], and 0.7 mM cumene hydroperoxide [CHP]) or superoxide-generating compounds (1.25 mM menadione, 8 mM pyrogallol, 50 mg/ml paraquat) were serially diluted in a 96 well-plate using MHB. Wild-type *F. tularensis* LVS, the Δ*osrR* mutant, and the Δ*osrR*+p*osrR* cultures were adjusted to 0.2 OD_{600} (equivalent to 1×10^9 CFU/ml), and equal numbers of bacteria were added to each well containing

TABLE 1 List of bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description	Source or reference
Strains		
F. tularensis LVS	Wild-type strain	ATCC
F. tularensis Δ*osrR* mutant	Deletion mutant of *osrR* gene of *F. tularensis* LVS	This study
F. tularensis emrA1 mutant	LVS FTL_0687::Tn*5* Kanr	27
F. tularensis osrR transcomplemented strain (Δ*osrR*+p*osrR*)	LVS, Δ*osrR*, pMM09 (pMP822+*osrR*), Hyg^r	This study
E. coli	F– ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF)U169 recA1 endA1 hsdR17* (r_K- m_K^+) *phoA supE44* λ- *thi-1 gyrA96 relA1*	Invitrogen
Plasmids		
pMP822	*E. coli-Francisella* shuttle vector, Hyg^r	63
pJC84	*E. coli-Francisella* suicide vector, Kan^r	62
pMM03	pJC84 + fused flanking fragment of *osrR* gene, Kan^r	This study
pMM014	pMP822 + *osrR*, Hyg^r	This study
Primers for *osrR* gene deletion		
MP135	5′-CAAGGATCCAAATAGATTGTGTTAGCATTGCAC-3′	
MP137	5′-TTTATCTGTACTCTTTCACTAGAGCATACTTTGTCCTTTTTTCACCA-3′	
MP139	5′-TGGTGAAAAAAGGACAAAGTATGCTCTAGTGAAAGAGTACAGATAAA-3′	
MP140	5′-TGATGTCGACGCAATACTCAAGTGGAACAACTGG-3′	
Primers for confirmation of *osrR* gene deletion		
MP037	5′-CCGGATCCATGAAATTTGAATTACCAAAAC-3′	
MP038	5′-CGCTGCAGCTAATCAGCGAATTGCTCAGAAAC-3′	
MP192	5′-ATGGGTGTTGCCATCAAATAGG-3′	
MP193	5′-TGTTGTCGCCAACGTGAAA-3′	
Primers for transcomplementation		
MP372	5′-CAAGGTTCCATGATACGGGAAGAGATCACATAT-3′	
MP373	5′-TGATCTCGAGTCAGTTCTTATAAATATTTTTTATC-3′	
Primers for qRT-PCR		
silC	MP015 5′-AGCCAAGTTAGTGCTGCATATTT-3′	
	MP016 5′-AAGCCAAGTTAGTGCTGCATATTT-3′	
emrA1	MP017 5′-GTGCATCTTGTAAAGAGCCAGCATC-3′	
	MP018 5′-CAGCCAAACTAAGCGCACAGTCAT-3′	
emrB	MP019 5′-TCCTAATCCCTGAATAGCCGTTGT-3′	
	MP020 5′-AGGTGTTGCCGCTATTATTGGTGC-3′	
groES	MP280 5′-ACCCATGATATCTTCTTCTCTC-3′	
	MP281 5′-AGAGTATTAGTTCGTCGTGCAG-3′	
groEL	MP282 5′-TCTTCAAAGCCTTTGCCTTC-3′	
	MP283 5′-TGTCACTGCAGGTATGAATCC-3′	
clpB	MP284 5′-TTTCATAATCCTGACCTTGCA-3′	
	MP285 5′-GGCGGCTATCTAACTGAACAT-3′	
FTL_1957	MP286 5′-AGATATAACAGAAGATGAAGCTGC-3′	
	MP287 5′-TAGGGATGTTCAAGCTTAGTAC-3′	
dnaK	MP446 5′-TATGACCTAGGTGGTGGTACATTC-3′	
	MP447 5′-TTCTCAGCAGCCTCTCTAACTCTT-3′	
dnaJ	MP448 5′-TGATGGTACAGGCTCTAAATCCAG-3′	
	MP449 5′-ACAAATCACCGTTCATAGCACC-3′	
hsp40	MP450 5′-TTTGGTGGTTTCTCGCAAAGT-3′	
	MP451 5′-CCCATTGCTGATGGTATTTTAAC-3′	
pdpC	MP654 5′-GCCTGAGTCATTGCTTGATCTAAA-3′	
	MP655 5′-AATAACCCCAAGGATCACTCAA-3′	
FTL_1163	*MP656 5′-TGGCTATTGAAATACTTGGATACGG-3′*	
	MP657 5′-TGTTGTGAATTTGTGCAGTGCTT-3′	
lpd	MP658 5′-CACGGTTGTTGAGTTTGCTGA-3′	
	MP659 5′-GCAGGATGATCGCCTTCCATA-3′	
coaX	MP660 5′-GCAGATCGTGTTGCAAACTCA-3′	
	MP661 5′-AGCACCGCCGATATAGGTTT-3′	
fba	MP662 5′-TGAGGGTGAGTTAGGTTGCC-3′	
	MP663 5′-GCACCATGCGAAGTACCGA-3′	

(Continued on next page)

TABLE 1 (Continued)

Strain, plasmid, or primer	Description	Source or reference
sucA	MP664 5′-TGAAATGAAGACGCCACCTG-3′	
	MP665 5′-TCTCTTCTAATTGCGCACGAG-3′	
FTL_0024	MP666 5′-ACTGCAAAAGAGCATTGAGTACAA-3′	
	MP667 5′-ACGGCTAAAGAAGAGATAATCGGT-3′	
FTL_0031	MP668 5′-ACTCACCAGAGTGGCAAAACA-3′	
	MP669 5′-ACCATGTGCTTGAGCAACAATC-3′	

the serially diluted compounds. The 96-well plate was incubated for 1 and 3 h at 37°C and 5% CO_2, and then the bacteria were spotted on MH-chocolate agar plates. In another approach, bacterial suspensions were exposed to 1 mM H_2O_2 for 1 and 3 h and to 4 mM pyrogallol for 1 h with shaking at 180 rpm and 37°C. Bacteria were serially diluted (10-fold) and plated on MH chocolate agar and incubated for 48 h at 37°C and 5% CO_2. Bacterial colonies were counted and plotted as $\log_{10}$ CFU/ml.

Macrophage cell culture assay. Primary murine BMDMs were isolated from 6- to 8-week old C57BL/6 mice purchased from Jackson Laboratory (Bar Harbor, ME) as previously described (66). Bone marrow cells were differentiated into macrophages by culturing in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% L-glutamine, 1% sodium pyruvate, 1% HEPES, and 20% medium conditioned with L929 cells. The BMDMs were infected with *F. tularensis* LVS, the Δ*osrR* mutant, or the transcomplemented strain at a multiplicity of infection (MOI) of 100. Cells were centrifuged for 10 min at 1,000 rpm to synchronize bacterial infection and incubated at 37°C and 5% CO_2 for 2 h. Then, 2 h postinfection, the medium from the infected cells was replaced with DMEM containing gentamicin (250 μg/ml) to kill extracellular bacteria and was then replaced with DMEM without any antibiotics after 1 h of incubation. The BMDMs were lysed with 0.1% sodium deoxycholate at 4 and 24 h postinfection, and then a 10-fold serial dilution of the lysed cells was spotted on MH chocolate agar plates. The plates were incubated at 37°C and 5% CO_2 for 48 h, bacterial colonies were counted, and the results were expressed as CFU per milliliter.

Mouse survival studies. The role of OsrR in virulence was investigated by infecting wild-type C57BL/6 mice with *F. tularensis* LVS, the Δ*osrR* mutant, or the Δ*osrR*+p*osrR* transcomplemented strain intranasally after anesthetizing them through intraperitoneal injection of ketamine and xylazine. Mice were inoculated with 1×10^2 and 1×10^3 CFU of *F. tularensis* LVS and the Δ*osrR* mutant or with 0.8×10^4 CFU of *F. tularensis* LVS, the Δ*osrR* mutant, and the transcomplemented strain in a volume of 20 μl (10 μl/naris). The morbidity and mortality of the infected mice were observed for 15 days. Body weight and survival were recorded daily. Survival was expressed as the Kaplan-Meier survival curve, and the data were analyzed statistically by the log rank test. All the protocols were approved by the IACUC of New York Medical College.

RNA sequencing. To determine the role of OsrR as a global transcriptional regulator, we profiled the whole transcriptome of the Δ*osrR* mutant in the absence or the presence of oxidative stress. The superoxide-generating compound, menadione (final concentration of 1.25 mM), was used as an oxidative stress inducer. This concentration of menadione equally affects the viability of wild-type *F. tularensis* LVS or the Δ*osrR* mutant. For untreated control, an equal volume of MHB was added, and bacteria were incubated for 1 h at 37°C with 5% CO_2. Bacteria were then centrifuged at 10,000 rpm for 10 min at room temperature, and pellets were resuspended in 990 μl lysis buffer (from Purelink RNA minikit/Invitrogen) supplemented with 10 μl 2-mercaptoethanol, and RNA was purified following the manufacturer's protocol. RNA samples were treated with DNase using Invitrogen's Turbo DNA-free kit.

Purified RNA samples were sent to the Genomics Core Laboratory at the New York Medical College for sequencing. Differentially expressed genes were evaluated based on $\log_2$ (fold change) value. The differential expression of *F. tularensis* LVS and Δ*osrR* mutant genes was analyzed based on the $\log_2$ fold change in the expression of genes in the Δ*osrR* mutant compared to those in wild-type *F. tularensis* LVS in the untreated and the menadione-treated samples. For the downregulated genes, the expression of the *osrR* gene, which has been deleted in the Δ*osrR* mutant, was taken as 100%. Any gene expressed lower or higher than 50% of the $\log_2$ fold change of the *osrR* gene was considered down- or upregulated, respectively. To further narrow the number of differentially expressed genes in the Δ*osrR* mutant, the adjusted *P* values of less than 0.05 were considered statistically significant. The adjusted *P* values were calculated using the DESeq2 package in R Bioconductor, and the Benjamini-Hochberg algorithm was used for the *P* value adjustment and controlling the false-discovery rate.

Quantitative real-time PCR (qRT-PCR). The qRT-PCR was used to verify the differential expression of *F. tularensis* LVS and Δ*osrR* mutant genes in the untreated and the menadione-treated samples. The gene-specific primers used for qRT-PCR are listed in Table 1. The results were expressed as the relative fold change in gene expression of the Δ*osrR* mutant compared to the *F. tularensis* LVS.

Statistical analysis. All results were analyzed using GraphPad Prism 7 software and were represented as the mean ± standard deviation (SD) or standard error of the mean (SEM). Comparisons among experimental groups were made using one-way analysis of variance (ANOVA). Survival data were plotted as the Kaplan-Meier survival curves, and significance was statistically calculated by the log rank test. A *P* value of <0.05 was considered statistically significant.

Data availability. The RNA-sequencing data are available at the NCBI GEO repository under the accession number GSE183001.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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