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Elucidation of a Mechanism of Oxidative Stress Regulation in *Francisella tularensis* Live Vaccine Strain

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Summary

Francisella tularensis causes a lethal human disease known as tularemia. As an intracellular pathogen, *Francisella* survives and replicates in phagocytic cells, such as macrophages. However, to establish an intracellular niche, *Francisella* must overcome the oxidative stress posed by the reactive oxygen species (ROS) produced by the infected macrophages. OxyR and SoxR/S are two well-characterized transcriptional regulators of oxidative stress responses in several bacterial pathogens. Only the OxyR homolog is present in *F. tularensis*, while the SoxR homologs are absent. The functional role of OxyR has not been established in *F. tularensis*. We demonstrate that OxyR regulates oxidative stress responses and provides resistance against ROS, thereby contributing to the survival of the *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) in macrophages and epithelial cells and contributing to virulence in mice. Proteomic analysis reveals the differential production of 128 proteins in the *oxyR* gene deletion mutant, indicating its global regulatory role in the oxidative stress response of *F. tularensis*. Moreover, OxyR regulates the transcription of the primary antioxidant enzyme genes by binding directly to their putative promoter regions. This study demonstrates that OxyR is an important virulence factor and transcriptional regulator of the oxidative stress response of the *F. tularensis* LVS.

Graphical Abstract

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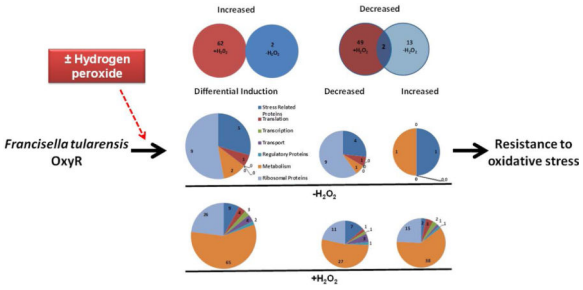
Author Contributions

MM and CSB conceived and designed the experiments. ZM, VCR, SMR, YJ, SV and CSB conducted the experiments. ZM, CSB and MM analyzed the data. CSB and MM wrote the manuscript.



Introduction

Francisella tularensis is a gram-negative, facultative intracellular bacterium that causes a zoonotic disease known as tularemia. *Francisella* is classified into four subspecies: *F. tularensis* subspecies *tularensis*, *F. tularensis* subspecies *holarctica*, *F. tularensis* subspecies *mediasiatica*, and *F. tularensis* subspecies *novicida*. Of these four subspecies, *F. tularensis* subspecies *tularensis* (type A) and subspecies *holarctica* (type B) are associated with human disease. *F. tularensis* subspecies *tularensis* is highly virulent in humans; as low as 10 colony forming units (CFUs) can cause fatal disease (Pechous *et al.*, 2009). Due to its extremely high virulence and ability to cause extensive mortality, *Francisella* has been used in bioweapon programs in the past (Dennis *et al.*, 2001). The Centers for Disease Control has classified *Francisella* as a Tier 1 Category A Select Agent due to its potential to be used as a bioterror agent. The live vaccine strain (LVS) is derived from *F. tularensis* subspecies *holarctica*. The LVS has attenuated virulence in humans but retains its virulence in mice and is widely used as a model to study tularemia pathogenesis (Fortier *et al.*, 1991).

As an intracellular pathogen, *F. tularensis* survives and replicates inside neutrophils, dendritic cells, and macrophages (Sjostedt, 2006; Santic *et al.*, 2006; Allen, 2006; Chase *et al.*, 2009). To establish an intracellular niche, *Francisella* has to overcome reactive oxygen and nitrogen species (ROS/RNS) generated following respiratory burst in these phagocytic cells. The ROS include highly microbicidal superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radicals (HO^-) produced from H_2O_2 via the Fenton reaction (Storz and Imlay, 1999). As a primary defense mechanism against ROS generated by the host's phagocytic cells, *Francisella* produces antioxidant enzymes such as Fe-containing superoxide dismutase (SodB) and Cu-Zn containing superoxide dismutase (SodC) to dismutate O_2^- into H_2O_2 as well as a catalase (KatG) and alkyl-hydro-peroxide reductase (AhpC) to convert H_2O_2 into water and oxygen. SodB and SodC contribute to the oxidative stress resistance and virulence of the *F. tularensis* LVS (Bakshi *et al.*, 2006; Melillo *et al.*, 2009; Melillo *et al.*, 2010; Lindgren *et al.*, 2007; Binesse *et al.*, 2015); however, their role against resistance to oxidative stress and the virulence of *F. tularensis* SchuS4 remains unknown. The catalase activity in *F. tularensis* strains does not correlate with their virulence (Vi-Dor and Yaniv, 1952); accordingly, KatG has been shown to be required for the virulence of the *F. tularensis* LVS, but not for the SchuS4 strain (Lindgren *et al.*, 2007). In addition to these primary antioxidant enzymes, MoxR-like ATPase (Dieppedale *et al.*, 2011) and proteins with sequence similarities to the *ohr* gene product of *Xanthomonas campestris* have also been shown to resist oxidative stress in *F. tularensis* (Meireles *et al.*, 2014;

Llewellyn *et al.*, 2011). The antioxidant enzymes KatG, SodC, and AhpC of *F. tularensis* are induced in response to oxidative stress (Wehrly *et al.*, 2009) and are secreted into the cytosol of the infected macrophages (Lee *et al.*, 2006). Our previous study has demonstrated that a fusion protein of the multidrug efflux pump EmrA1 confers oxidative stress resistance by affecting the secretion of KatG and SodB (Ma *et al.*, 2014). The expression of these primary antioxidant enzymes starts immediately upon phagocytosis and remains significantly upregulated during the phagosomal and cytosolic phases of growth (Wehrly *et al.*, 2009), indicating that their expression is highly regulated in these distinct intracellular locations of *F. tularensis*.

OxyR, SoxR, PerR and RNA polymerase of stationary phase (RpoS) serve as primary regulators of oxidative stress in bacteria (Chiang and Schellhorn, 2012). Additionally, the regulators NorR and IscR are also induced in response to oxidative stress to mitigate RNS and the formation of iron-sulfur (Fe-S) clusters (Gardner *et al.*, 2003). OxyR belongs to the LysR family of transcriptional regulators, consisting of an N-terminal DNA-binding domain with a winged helix-turn-helix motif and a C-terminal regulatory domain (Schell, 1993a). OxyR in *E. coli* regulates the expression of genes required for protection against H_2O_2 toxicity, heat stress, oxidant-mediated cell damage, and phagocyte-mediated killing (Staudinger *et al.*, 2002). The activation of OxyR occurs via the oxidation of two conserved cysteine residues (Cys199 and Cys208) in response to H_2O_2-induced oxidative stress (Lee *et al.*, 2004). In contrast, SoxR protects against O_2^- radicals and is induced in response to O_2^--generating compounds, NO or high levels of H_2O_2 (Nunoshiba *et al.*, 1992). Sequence analysis of the *F. tularensis* genome reveals the presence of OxyR and RpoS, while the SoxR/SoxS regulons are absent (Larsson *et al.*, 2005).

The mechanisms of regulation of oxidative stress responses in *F. tularensis* are not known. This study investigated the role of OxyR of the *F. tularensis* LVS in tularemia pathogenesis and the regulation of oxidative stress responses. The results from this study demonstrate that OxyR of *F. tularensis* regulates oxidative stress responses to promote resistance against ROS, thereby contributing to its intracellular survival and promoting its virulence in mice. Proteomic analysis reveals that OxyR modulates the level of 128 proteins, indicating a broader regulatory role of OxyR in overcoming oxidative stress. Moreover, OxyR regulates the transcription of the primary antioxidant enzyme genes *ahpC* and *katG* by binding directly to their putative promoter regions. These results indicate that OxyR is an important regulator of the oxidative stress response and renders *F. tularensis* a pathoadaptive advantage to establish an intracellular niche. An understanding of these unique pathogenic mechanisms is essential for the development of effective therapeutics and prophylactics against this important biothreat agent.

Results

Genomic organization and generation of an oxyR gene deletion mutant of the *F. tularensis* LVS

The 289-amino-acid, 34.6 kDa OxyR protein in the *F. tularensis* LVS is encoded by the *FTL_1014* (*oxyR*) gene. The *oxyR* gene in *F. tularensis* is located upstream of and transcribed divergently from the gene encoding AhpC. A similar genomic organization is

also present in the virulent *F. tularensis* SchuS4 strain and in *Mycobacterium tuberculosis* (Fig. 1A). However, the *oxyR* gene in *Yersinia pestis*, *B. pseudomallei*, and *E. coli* K-12 is bigger in size and has neighboring genes other than *ahpC* (Fig. 1A). The sequence analysis of the *oxyR* gene reveals a conserved N-terminal (1–60 amino acid) helix-turn-helix domain, which serves as a sequence-specific DNA binding domain, found in the LysR family of transcriptional regulators. The C-terminal domain is a Type 2 periplasmic binding superfamily domain and is required for the uptake of many substrates. The OxyR protein of *F. tularensis* has two redox-sensitive cysteine residues (Cys199 and Cys208) that are highly conserved and are required for its oxidant-dependent activation. Sequence alignment analyses show that OxyR of the *F. tularensis* LVS exhibits 99% identity with the OxyR of *F. tularensis* SchuS4, 36% identity with *E. coli* and *Yersinia pestis*, and 34% identity with that of *B. pseudomallei* (Fig. S1).

To investigate the role of OxyR in the pathogenesis of tularemia and the transcriptional regulation of the genes involved in the oxidative stress response, we generated an in-frame *oxyR* gene deletion mutant (Δ*oxyR* mutant) and a transcomplemented strain (Δ*oxyR* + p*oxyR*) of the *F. tularensis* LVS. The *oxyR* gene deletion and transcomplementation was confirmed by PCR using *oxyR* gene-specific primers (Fig. 1B). *sodB* gene primers were used as internal controls. The in-frame gene deletion of the *oxyR* gene in the Δ*oxyR* mutant was also confirmed by DNA sequencing (data not shown).

OxyR of *F. tularensis* is not essential for cell viability but is required for growth under acidic conditions

Loss of the *oxyR* gene in several bacterial pathogens is associated with a viability-deficient phenotype known as “plating defect” (Jiang *et al.*, 2014; Shi *et al.*, 2015; Gonzalez-Flecha and Demple, 1997). We investigated whether the Δ*oxyR* mutant of the *F. tularensis* LVS exhibits a similar phenotype. Cultures of the wild-type *F. tularensis* LVS, the Δ*oxyR* mutant and the transcomplemented strain grown to mid-log phase were collected, centrifuged, diluted 10-fold and plated on chocolate agar plates to determine the differences in bacterial viability. No differences were observed in the number of colonies recovered from these bacterial strains, indicating that the loss of the *oxyR* gene is not associated with decreased viability (Fig. 2A). We also determined whether the loss of *oxyR* results in enhanced sensitivity towards stressors such as SDS, sodium deoxycholate, Triton X-100 or ethidium bromide by performing disc diffusion assays. Identical zones of inhibition were obtained for all the three bacterial strains against these stressors, indicating that OxyR does not contribute to bacterial membrane integrity (Fig. S2). We further investigated whether the Δ*oxyR* mutant exhibits growth defects when grown aerobically in liquid culture. No differences in growth were observed between the *F. tularensis* LVS, the Δ*oxyR* mutant or the transcomplemented strain grown aerobically in Mueller Hinton broth (MHB) at a pH of 6.8. However, when these bacterial strains were grown in MHB adjusted to a pH of 5.5, the growth of the Δ*oxyR* mutant was severely impaired. On the other hand, the growth of the *F. tularensis* LVS or the transcomplemented strain remained unaltered (Fig. 2B). Collectively, these results demonstrate that OxyR is not required for the viability or structural integrity of *F. tularensis*; however, it does play a role in the survival of *Francisella* at an acidic pH.

OxyR protects *F. tularensis* from the growth-inhibitory effects of superoxide radicals, hydrogen peroxide and organic peroxides

We next determined the role of OxyR in providing resistance against oxidants using disc diffusion assays, growth curves and bacterial killing assays. In disc diffusion assays, the Δ*oxyR* mutant showed enhanced sensitivity towards the superoxide-generating compound menadione and the organic peroxides TBH and CHP, as indicated by significantly larger zones of inhibition (20.0±1.3 mm, 18.2±2.2 mm, and 12.0±1.8 mm for menadione, TBH and CHP, respectively) than those observed for the wild-type *F. tularensis* LVS (15.4±1.4, 12.4±0.9, and 8.8±0.7 mm for menadione, TBH and CHP, respectively) or the transcomplemented strain (15.4±1.23, 12.6±1.86, and 9.1±0.4 mm for menadione, TBH and CHP, respectively). Similarly, when growth curves were generated in the presence of these oxidants, the growth of the Δ*oxyR* mutant was found to be severely impaired (Fig. 2C, D and E). The Δ*oxyR* mutant showed extreme sensitivity towards H_2O_2 compared to the wild-type *F. tularensis* LVS or the transcomplemented strain and failed to grow in the presence of 500 μM or 1 mM concentrations of H_2O_2 (Fig. 2F). The sensitivity of the Δ*oxyR* mutant towards H_2O_2 was also tested using a bacterial killing assay. Significantly fewer viable Δ*oxyR* mutant bacteria were recovered at 1 and 3 hrs post-exposure to H_2O_2 compared to those observed for the wild-type *F. tularensis* LVS or the transcomplemented strain (Fig. 2G). Collectively, these results demonstrate that OxyR of *F. tularensis* mediates resistance against oxidative stress generated by superoxide radicals, organic peroxides and H_2O_2.

OxyR of *F. tularensis* is required for intracellular survival and virulence in mice

The role of OxyR of *F. tularensis* in intramacrophage survival was determined by performing gentamicin protection assays in the RAW macrophage cell line and in primary bone marrow derived macrophages (BMDMs) derived from wild-type or gp91*phox*$^{-/-}$ C57BL/6 mice. Equal numbers of the *F. tularensis* LVS or the Δ*oxyR* mutant bacteria were recovered from the infected RAW macrophages after 4 hrs of infection indicating not only an equivalent uptake but also an equivalent bacterial survival in early phagosomes. However, ten-fold fewer Δ*oxyR* mutant bacteria compared to the wild-type *F. tularensis* LVS or the transcomplemented strain were recovered from the infected macrophages 24 hrs post-infection (Fig. 3A). We further investigated whether the Δ*oxyR* mutant is sensitive to physiological levels of ROS in the host cell cytosol by performing a gentamicin protection assay in the A549 lung epithelial cell line. Similar to RAW macrophages, significantly fewer Δ*oxyR* mutant bacteria were recovered at 12 and 24 hrs post-infection from epithelial cells than those from the wild-type *F. tularensis* LVS or the transcomplemented strain infected cells (Fig. 3B). Similar results were observed in BMDMs derived from wild-type C57BL/6 mice, indicating that the Δ*oxyR* mutant is deficient for intracellular survival. However, the growth of the Δ*oxyR* mutant was restored to the wild-type *F. tularensis* LVS levels in BMDMs derived from gp91*phox*$^{-/-}$ mice (Fig. 3C). We further examined the localization of the Δ*oxyR* mutant in macrophages using transmission electron microscopy (TEM) at 1 and 6 hrs post-infection. The majority of the wild-type *F. tularensis* LVS and the Δ*oxyR* mutant bacteria (>90%) were localized in the phagosomes 1 hr post-infection (not shown). However, at 6 hrs post-infection, only 10% wild-type *F. tularensis* LVS bacteria remained localized to the phagosomes; while 90% bacteria had escaped the phagosomes and were present in the macrophage cytosol. In contrast, 65% of the Δ*oxyR* mutant bacteria were still

trapped inside the phagosomes and only 35% bacteria were present in the macrophage cytosol (Fig. 3D, E). Collectively, these results demonstrate that the Δ*oxyR* mutant has attenuated intracellular growth. Furthermore, these results also indicate that the Δ*oxyR* mutant is sensitive to NADPH oxidase-dependent ROS, which may reduce its fitness to escape phagosomes and therefore its ability to replicate in the cytosol.

The role of OxyR in virulence was determined by infecting wild-type or gp91*phox*$^{-/-}$ C57BL/6 mice intranasally with 1×10^4 (equivalent to $1\times LD_{100}$) *F. tularensis* LVS or 1×10^4 or 1×10^5 CFUs of the Δ*oxyR* mutant. The infected mice were observed for survival for a period of 21 days. One hundred percent of the wild-type and gp91*phox*$^{-/-}$ C57BL/6 mice infected with the *F. tularensis* LVS succumbed to infection by days 6 and 8 post-infection, respectively (Fig. 3F). In contrast, 80 and 60% of the wild-type C57BL/6 mice inoculated with 1×10^4 or 1×10^5 CFUs of the Δ*oxyR* mutant, respectively, survived the infection. However, 100% of the gp91*phox*$^{-/-}$ mice inoculated with similar doses of the Δ*oxyR* mutant succumbed to infection by days 10–11 post-infection (Fig. 3G). Collectively, these results demonstrate that OxyR of *F. tularensis* plays an important role in intracellular survival as well as in *F. tularensis* virulence in mice and that OxyR contributes to virulence by overcoming the oxidative stress generated by NADPH oxidase.

OxyR is a global regulator of the oxidative stress response in *F. tularensis*

Our preceding results demonstrated that the Δ*oxyR* mutant is highly sensitive to oxidants, deficient for intramacrophage growth, and attenuated for virulence in mice. We next investigated the mechanisms through which OxyR regulates the oxidative stress response of *F. tularensis*. We took a broad proteomic approach by performing iTRAQ analysis to profile differentially induced proteins in the Δ*oxyR* mutant compared to those observed in the wild-type *F. tularensis* LVS. The data analyses were performed using the Paragon algorithm against the database within ProteinPilot V4.5 software. For a protein to be designated as being differentially induced, it must have been quantified in at least three spectra (allowing generation of a *P*-value), had a fold change of more than 1.3 or less than 0.7 from two independent experiments performed with two biological replicates each, and had a *P*-value of <0.05. A $\log_2$ ratio of the fold change in protein levels (Δ*oxyR* mutant/*F. tularensis* LVS) of +0.11 or higher indicated an increase, while a ratio less than −0.15 indicated a decrease in protein levels.

A total of 128 out of 252 proteins identified in the iTRAQ analysis were differentially induced in the Δ*oxyR* mutant (Table S1). These included proteins involved in oxidative/general stress resistance, translation, transcription, transport, metabolism, regulatory, and ribosomal functions. Of these, 2 proteins were elevated and 15 were reduced in the Δ*oxyR* mutant not exposed to H_2O_2. Exposure of the Δ*oxyR* mutant to H_2O_2 resulted in increased levels of 62 proteins, while 51 proteins were decreased. Two proteins, AhpC and SodB, remained suppressed in the Δ*oxyR* mutant irrespective of its exposure to H_2O_2 (Fig. 4A). The 15 proteins that were suppressed in the Δ*oxyR* mutant not exposed to H_2O_2 included 4 proteins involved in stress resistance, 1 in translation, 1 in general metabolism, and 9 ribosomal proteins (Fig. 4B). The 51 proteins that were suppressed in the Δ*oxyR* mutant upon exposure to H_2O_2 included 27 proteins involved in metabolism, 11 ribosomal proteins,

7 stress resistance proteins, 3 transport proteins, and 1 protein each involved in transcription, translation and gene regulation (Fig. 4C). The two proteins that were increased in the Δ*oxyR* mutant not exposed to H_2O_2 were ClpB and glucosamine fructose-6-phosphate aminotransferase, while a total of 62 proteins were increased when the Δ*oxyR* mutant was exposed to H_2O_2. A majority of these proteins were those involved in metabolism and transport function and ribosomal proteins (Fig. 4B, C). Collectively, these results indicate that the loss of OxyR results in the differential induction of several proteins in the Δ*oxyR* mutant of *F. tularensis*.

We categorized proteins that were less abundant in the Δ*oxyR* mutant when exposed to H_2O_2 into four categories: Category I included proteins required for oxidant and general stress resistance, such as AhpC (FTL_1015), SodB (FTL_1791), thioredoxin reductase (FTL_1571), the chaperonin protein GroES (FTL_1715), cold shock (FTL_0457) and universal stress (FTL_0166) proteins, and alarmone (p)ppGpp synthase (FTL_1413) (Fig. 5A). Category II included proteins involved in transcription, translation and transport functions. The predominantly less abundant proteins in this category included the translation initiation factor IF-1 (FTL_1236), the transcription termination factor rho (FTL_0610), ABC transporter (FTL_1870), ABC binding protein (FTL_1065), and polyamine transporter protein (FTL_0679) (Fig. 5B). The ribosomal proteins that were prominently suppressed in the Δ*oxyR* mutant were categorized as Category III proteins (Fig. 5C). Category IV included proteins involved in metabolism and cellular function, as well as hypothetical proteins. The prominently suppressed proteins were glucosamine fructose-6-phosphate aminotransferase (FTL_0454), ATP synthase subunit gamma (FTL_1800), adenosine tRNA methylthiotransferase (FTL_0886), and the hypothetical protein FTL_1678 (Fig. 5D). However, our iTRAQ results did not reveal the differential induction of KatG in the Δ*oxyR* mutant compared to the wild-type *F. tularensis* LVS. In several bacterial pathogens, it has been reported that OxyR regulates the expression of *katG* (Ieva *et al.*, 2008;Jagielski *et al.*, 2015;Kim *et al.*, 2015;Kim and Holmes, 2012). To determine the effect of OxyR on the induction of KatG, we performed western blot analysis to determine the levels of KatG in the wild-type *F. tularensis* LVS and the Δ*oxyR* mutant. Significantly lower levels of KatG protein were observed in the Δ*oxyR* mutant compared to the *F. tularensis* LVS (Fig. 5E). Collectively, these results indicate that OxyR in *F. tularensis* serves as a positive regulator of the oxidative stress response.

We next investigated whether decreased levels of the aforementioned proteins in the Δ*oxyR* mutant are due to differences originating at the level of transcription. We focused our attention on the genes encoding the primary antioxidant enzymes *ahpC*, *sodB* and *katG* of *F. tularensis*. An additional gene encoding *ahpC* (*FTL_0996*) has also been found in *F. tularensis*. Although the protein levels of FTL_0996 and another primary antioxidant enzyme, SodC, remained unaltered in the iTRAQ analysis, we also included these two primary antioxidant genes as controls. We conducted transcriptional analysis of the *ahpC, sodB, katG, sodC*, and *FTL_0996* genes by qRT-PCR in the wild-type *F. tularensis* LVS and the Δ*oxyR* mutant with or without exposure to H_2O_2. The transcript levels of *ahpC and katG* were nearly 4- and 2-fold higher, respectively, when the *F. tularensis* LVS was exposed to H_2O_2; however, the transcript levels of *sodB, sodC* and *FTL_0996* remained unaltered. In the Δ*oxyR* mutant, irrespective of its treatment with H_2O_2, the expression of these genes

either remained downregulated (*ahpC, katG* and *sodB*) or remained unchanged (*sodC, FTL_0996*) (Fig. 5F). These results demonstrate that OxyR positively regulates the oxidative stress response, and the regulation occurs at the level of transcription.

Several proteins were found to be increased in the Δ*oxyR* mutant upon exposure to H_2O_2 and were also grouped into four categories. The category I general stress response proteins included DnaK (FTL_1191), ClpB (FTL_0094), and heat shock protein (FTL_1957) (Fig. 6A). The category II proteins involved in translation, transcription, and transport function included: elongation factors G (FTL_0234), Ts (FTL_0225), and TU and G family protein (FTL_0768), the transcription factor NusA (FTL_1810), the ABC transporter ATP binding protein (FTL_1229) and Sigma 54 modulation protein (FTL_1179) (Fig. 6B). A number of hypothetical proteins, ribosomal proteins, and proteins involved in metabolism were also produced at higher levels in the Δ*oxyR* mutant when exposed to H_2O_2 (Fig. 6C, D and E). Transcriptional analysis by qRT-PCR confirmed the upregulated expression of a representative group of the abundant proteins (data not shown). Collectively, these results demonstrate that OxyR serves as a global regulator of the oxidative stress response in *F. tularensis* and regulates the oxidative stress response proteins involved in a multitude of functions, both positively and negatively.

OxyR regulates the transcription of primary antioxidant enzymes by physically interacting with their putative promoter regions

To investigate the mechanism of the transcriptional regulation of the primary antioxidant enzyme genes *ahpC*, *katG* and *sodB* by OxyR, we generated an *F. tularensis* LVS strain expressing an epitope-tagged version of OxyR by fusing the vesicular stomatitis virus glycoprotein (VSV-G) epitope to its C-terminus (OxyR-VSV-G). This allowed the expression of OxyR-VSV-G from the native *oxyR* locus. The *oxyR-VSV-G* strain was similar to the wild-type *F. tularensis* LVS with respect to its growth attributes and sensitivity to oxidants (data not shown). The *oxyR-VSV-G* strain was used to detect the *in vivo* binding of OxyR-VSV-G to the promoters of the *ahpC*, *katG* and *sodB* genes by performing ChIP followed by qRT-PCR analysis. We observed nearly 15-fold enrichment of Region 2, the stretch of DNA immediately upstream of the *ahpC* gene. We did not observe enrichment of either Region 1 (the region within the *oxyR* gene) or Region 3 (the region within the *ahpC* gene). These results indicate that OxyR binds specifically to the upstream promoter region of the *ahpC* gene. The Region 2 DNA sequence upstream of the *ahpC* gene also contains a putative OxyR-binding domain, ATAG-N_7-AAAT-N_7-ATGT, and additional conserved A-N_{11}-T residues that show significant homology to the conserved OxyR-binding domain of *E. coli*, ATAG-N_7-CTAT-N_7-ATAG-N_7-CTAT (Schell, 1993b;Wei *et al.*, 2012) (Fig. 7A). Similarly, nearly 10-fold enrichment of Region 4, immediately upstream of the *katG* gene, which also contains conserved OxyR-binding motifs, was observed (Fig. 7B). The region upstream of the *sodB* gene also contains a conserved OxyR binding domain; however, the binding of OxyR to the *sodB* promoter region (Region 2) did not appear to be as strong as that observed for either the *ahpC* or the *katG* genes, and only 5-fold-enrichment of this region was observed by ChIP analysis (Fig. 7C). No enrichment of the regions upstream of either the *sodC* or *FTL_0996* genes was observed, indicating that OxyR does not bind to the promoter regions of these genes (Fig. 7D and E). The promoter regions of these genes also

lacked a putative OxyR-binding domain. Collectively, these results demonstrate that OxyR regulates the expression of the *ahpC, katG* and *sodB* genes by binding to the upstream promoter regions of these genes.

Next, we performed an electrophoretic mobility shift assay (EMSA) to confirm the binding of OxyR to the promoter regions of the *ahpC, katG*, and *sodB* genes. We used biotin-labeled DNA probes from the regions identified in Fig. 7 and whole-cell lysates from the wild-type *F. tularensis* LVS, the Δ*oxyR* mutant and the transcomplemented strains for EMSA. The unlabeled fragments from the same regions were used as competitor DNA. The activity of the Δ*oxyR* mutant lysate was confirmed by the binding of an OxyR-independent transcriptional regulator, PmrA1, to its cognate *pmrA* promoter region (Fig. S3A and B). Consistent with our ChIP results, we observed a mobility shift as a result of the binding of OxyR to the promoter regions of the *ahpC* and *katG* genes in lysates from the wild-type *F. tularensis* LVS or the transcomplemented strain, but not in lysates from the Δ*oxyR* mutant. The addition of unlabeled competitor DNA to the *F. tularensis* LVS or the transcomplemented strain lysates prevented the mobility shift and confirmed the specificity of the binding of OxyR to the *ahpC* and *katG* gene promoter regions (Fig. 8A and B). However, we failed to detect any mobility shift when EMSA was performed with the binding region identified for the *sodB* gene (Fig. 8C). Similarly, no binding of OxyR to the upstream promoter regions of either the *sodC* or *FTL_0996* genes was observed (Fig. 8D and E). Collectively, these results demonstrate that OxyR regulates the transcription of the *ahpC* and *katG* genes by binding to their promoter regions.

Discussion

OxyR, SoxR, PerR and RopS serve as primary regulators of oxidative stress in bacteria (Chiang and Schellhorn, 2012). Additionally, the regulators NorR and IscR are also induced in response to oxidative stress to mitigate RNS and the formation of iron-sulfur (Fe-S) clusters (Gardner *et al.*, 2003). The activation of these regulators also results in the induction and activation of other oxidative stress regulators, such as Fur and Zur, which are involved in the homeostatic control of intracellular iron and zinc levels, respectively, to minimize oxidative stress (Imlay, 2015). OxyR, a member of the LysR family of transcriptional regulators (Schell, 1993a), regulates the expression of genes required for protection against H_2O_2 toxicity, heat stress, oxidation-mediated cell damage, and phagocyte-mediated killing (Staudinger *et al.*, 2002). Most bacteria, with the exception of *Deinococcus radiodurans*, possess a single copy of the *oxyR* gene (Yin *et al.*, 2010). OxyR positively regulates the expression of the *catalase* and *ahpC* genes in gamma-proteobacteria, such as *E. coli*, *Haemophilus*, *Pseudomonas*, *Salmonella* and *Yersinia*. In beta-proteobacteria, such as *Burkholderia* and *Neisseria*, the *catalase* gene is regulated both positively and negatively by OxyR (Loprasert *et al.*, 2003; Tseng *et al.*, 2003). In addition to OxyR, PerR also serves as a modulator of the oxidative stress response induced by H_2O_2 (Rea *et al.*, 2005). SoxR, on the other hand, protects against O_2^- radicals and is induced in response to O_2^--generating compounds, NO, or high levels of H_2O_2 (Nunoshiba *et al.*, 1992; Manchado *et al.*, 2000). SoxR homologs are present in alpha- and gamma-proteobacteria and serve as regulators of efflux pump genes and Sods. However, SoxR homologs are absent in *Bacteroidetes* and *Actinobacteria* (Ohara *et al.*, 2006). OxyR- and SoxR-dependent responses are induced

during the exponential phase, whereas RpoS-dependent oxidative stress responses are induced during the stationary phase of bacterial growth (Eisenstark *et al.*, 1995).

This study investigated the role of OxyR of *F. tularensis* in tularemia pathogenesis and the regulation of oxidative stress responses. As observed for other bacterial pathogens, sequence analysis of *F. tularensis* OxyR reveals two conserved cysteine residues (Cys199 and Cys208) that are required for the oxidant-dependent activation of OxyR. However, it exhibits only 36% homology with the well-characterized OxyR of *E. coli*. The *oxyR* gene in *F. tularensis* is transcribed divergently from the *ahpC* gene. Our results demonstrate that OxyR regulates the genes encoding the primary antioxidant enzymes *katG, ahpC* and *sodB* in response to H_2O_2-induced oxidative stress in *F. tularensis*. These results also indicate that similar to *Bacteroidetes* and *Actinobacteria*, in the absence of SoxR homologs, OxyR regulates the expression of SodB in *F. tularensis*. Furthermore, despite an identical genomic organization of the *oxyR* gene between *Francisella* and *M. tuberculosis*, OxyR does not regulate catalase or the *ahpC* gene due to mutations in the OxyR protein in *M. tuberculosis* (Deretic *et al.*, 1995; Sherman *et al.*, 1995)..

A previous study identified an *oxyR* mutant by screening transposon mutants of *F. novicida* in *Drosophila melanogaster*. It was reported that the *oxyR* mutant of *F. novicida* is highly sensitive to H_2O_2 and had attenuated virulence in flies; however, the virulence of the *oxyR* mutant is restored in flies that cannot synthesize melanin and therefore fail to produce ROS (Moule *et al.*, 2010). The results from our study extend this observation in *F. tularensis* and demonstrate that OxyR is required to resist oxidative stress caused by superoxide-generating compounds, organic peroxides, and H_2O_2 under acellular growth conditions. The loss of *oxyR* does not result in the loss of viability of the Δ*oxyR* mutant grown in the absence of oxidative stress, but it does contribute to its survival at acidic pH. Exposure of the bacterial cells to acidic pH induces highly toxic ROS, including OH^- radicals by Fenton chemistry (Mols and Abee, 2011). Our results suggest that OxyR protects *F. tularensis* against acid stress, primarily by regulating the production of the antioxidant enzymes involved in the neutralization of ROS. During macrophage infection, the *Francisella*-containing phagosomes are not acidified, and thus OxyR may not be required to overcome acidic pH in macrophages. However, *F. tularensis* causes gastrointestinal (GI) infections and must survive the acidic pH of the stomach. We speculate that OxyR may contribute to the survival of *F. tularensis* in the GI tract. An acid-sensitive *oxyR* mutant of *Klebsiella pneumoniae* deficient in GI tract colonization has been reported (Hennequin and Forestier, 2009).

The results of this study demonstrate that the Δ*oxyR* mutant of *F. tularensis* is deficient in survival and growth in macrophages and A549 lung epithelial cells. However, the intramacrophage replication and virulence of the Δ*oxyR* mutant is rescued in *gp91phox*$^{-/-}$ BMDMs or mice, indicating a role for NADPH-oxidase-dependent ROS in diminishing its fitness. The NADPH-oxidase-dependent bacterial killing in the phagosomes is rapid and accomplished quickly following bacterial uptake (Vazquez-Torres *et al.*, 2000). Nearly equal numbers of viable wild-type *F. tularensis* LVS or Δ*oxyR* mutant bacteria were recovered from the infected macrophages after 4 hrs of infection. These results indicate that NADPH-oxidase-dependent killing of the Δ*oxyR* mutant bacteria may not occur during its phagosomal residence. However, the numbers of the Δ*oxyR* mutant bacteria recovered at 24

hrs were similar to those observed after 4 hrs of infection. These observations suggest that the enhanced sensitivity of the Δ*oxyR* mutant to oxidative stress makes it susceptible to low levels of NADPH-oxidase-dependent ROS in the phagosomes, thereby reducing its fitness and ability to escape from the phagosomes. Furthermore, the reduced phagosomal escape may be associated with reduced growth in the presence of physiological levels of ROS in the macrophage/epithelial cell cytosol. Indeed, our TEM results demonstrate that the Δ*oxyR* mutant remains trapped in the phagosomes for up to 6 hrs post-infection, indicating its reduced fitness to escape the phagosomes. The attenuated growth of the Δ*oxyR* mutant in A549 lung epithelial cells further supports the notion that the Δ*oxyR* mutant is indeed sensitive to physiological levels of ROS encountered in the host cell cytoplasm.

The mechanism(s) of regulation of oxidative stress responses in *F. tularensis* remains unknown to date. In *Francisella*, very few factors regulate gene expression, and reported evidence suggests that gene regulation occurs at the level of transcription. The well-characterized transcriptional regulators in *Francisella* include *mglA* (Charity *et al.*, 2007; Guina *et al.*, 2007), *fevR/pigR* (Wehrly *et al.*, 2009; Charity *et al.*, 2007; Brotcke and Monack, 2008; Brotcke *et al.*, 2006), response regulator *pmrA* (Sammons-Jackson *et al.*, 2008; Bell *et al.*, 2010), *Fur* (Ramakrishnan *et al.*, 2008; Deng *et al.*, 2006; Kiss *et al.*, 2008), *qseC* (Weiss *et al.*, 2007), the sensor kinase *kdpD*, and the sigma factors *rpoD* and *rpoS* (Grall *et al.*, 2009). Initially, it was speculated that H_2O_2-induced oxidative stress responses in *F. tularensis* are regulated by the *Francisella* Pathogenicity Island protein IglC (Lenco *et al.*, 2005). However, the Δ*iglC* mutant did not show sensitivity to H_2O_2, and with the exception of AhpC, did not reveal differential induction of oxidative stress response proteins when exposed to H_2O_2. These results do not support the notion that IglC is a regulator of oxidative stress responses (Andersson *et al.*, 2006). The role of the transcriptional regulator MglA has been widely studied in both *F. novicida* and the *F. tularensis* LVS. In addition to the regulation of the genes encoded on the FPI, it has been reported that MglA also regulates several stress response proteins involved in heat shock and oxidative stress resistance. Moreover, the *mglA* mutant exhibits increased sensitivity towards the superoxide-generating compound paraquat; however, it is resistant to H_2O_2 (Guina *et al.*, 2007). The enhanced H_2O_2 resistance has been attributed to increased amounts of KatG in the *mglA* mutant (Guina *et al.*, 2007). A later study investigated the mechanism of the resistance of *mglA* mutant to H_2O_2. It was reported that increased oxidation in the *mglA* mutant under aerobic growth conditions results in a compensatory increase in KatG activity that ultimately leads to H_2O_2 resistance. However, under microaerobic conditions, the *mglA* mutant is as susceptible to H_2O_2 as the wild-type *F. tularensis* LVS (Honn *et al.*, 2012). Additionally, H_2O_2 resistance in the *mglA* mutant was suggested to be due to a concomitant upregulation of KatG and downregulation of the genes involved in iron sequestration (Honn *et al.*, 2012). The exact mechanism through which MglA regulates the expression of KatG remains elusive. Recently, it has been demonstrated that MglA and stringent starvation protein A (SspA) form a complex and work in concert with the transcriptional activator PigR. The MglA-SspA complex interacts with all the *F. tularensis* promoters; however, a sequence-specific motif recognized by PigR determines their expression (Ramsey *et al.*, 2015). Although the MglA-SspA complex binds to the *oxyR* promoter, it is not regulated by the MglA-SspA-PigR complex (Ramsey *et al.*, 2015) ruling out the possibility that MglA may

drive the OxyR-dependent transcription of the oxidative stress response genes, including *katG*. Considering these observations and the results obtained from the present study, it is possible that MglA may not have a direct role in the regulation of oxidative stress response genes *per se*. However, an exaggerated oxidative environment in the *mglA* mutant may result in the activation of OxyR, which in turn may upregulate the expression of KatG and AhpC observed in the *mglA* mutant. Clarification of this notion will require further studies.

This study established a mechanism of regulation of the classical antioxidant enzyme genes of *F. tularensis*. The OxyR targets *ahpC* and *katG* are positively regulated by the direct binding of OxyR to their putative upstream promoter regions, as revealed by *in vivo* ChIP and *in vitro* EMSA. However, despite a smaller magnitude of binding of OxyR to the promoter region of the *sodB* gene in the ChIP assay, the EMSA failed to validate this finding. This variation may be due to differences in the amounts of oxidized OxyR required, or due to a lack of additional regulatory components required for optimal binding to the *sodB* gene promoter of *F. tularensis*. Most importantly, the results of this study demonstrate OxyR as a global regulator of oxidative stress response in *F. tularensis*. A differential induction of the proteins involved in oxidative stress resistance, transcription, translation, transport, metabolism, and other cellular functions was observed in the Δ*oxyR* mutant subjected to oxidative stress conditions induced by H_2O_2. The differential expression of proteins involved in a multitude of cellular functions indicates that the OxyR-dependent oxidative stress response is mediated both by suppressing as well as elevating the levels of proteins involved in transcription, translation, transport functions, and metabolism. The suppression of proteins involved in cellular functions apparently help *Francisella* to slow down metabolically intensive processes to minimize the oxidative stress caused by aerobic respiration and rapid bacterial growth. In contrast, OxyR facilitates the efficient utilization of the limited resources available to *Francisella* during the conditions of oxidative stress by activating crucial cellular mechanisms, including the primary antioxidant enzymes. The OxyR function, especially in *E. coli*, is intimately linked with the activation of iron utilization genes, such as those belonging to the *fur* operon (Zheng *et al.*, 1999). Surprisingly, none of the proteins involved in iron utilization were differentially expressed in the Δ*oxyR* mutant, indicating that the regulation of the *fur* operon may be independent of OxyR in *F. tularensis*. A similar observation has also been recorded for *Pseudomonas aeruginosa*, where OxyR has not been shown to be required for the expression of *fur* genes (Wei *et al.*, 2012). The mechanism of regulation of the *sodC* gene of *F. tularensis* remained unanswered in this study. However, based on the observations from other bacterial pathogens, it is possible that the transcription of *sodC* may be regulated by RpoS. It has been reported that RpoS-dependent expression of *sodC* in *E. coli* and *Salmonella* occurs in response to oxidative stress during the stationary phase of growth (Gort *et al.*, 1999). Moreover, the *oxyR* regulon is not functionally characterized in *F. tularensis* SchuS4. Previous studies have reported differences in the magnitude of oxidant resistance between the *F. tularensis* LVS and SchuS4 strains (Lindgren *et al.*, 2007). However, the identical genomic organization as well as nucleotide and amino acid sequences of the *oxyR* gene in the *F. tularensis* LVS and SchuS4 strains suggests a similar regulatory role for OxyR in the latter strain.

In conclusion, this study describes OxyR as an important virulence factor of *F. tularensis*. Most importantly, this study provides unique insights into a mechanism of OxyR-dependent regulation of the oxidative stress response in *F. tularensis*. An understanding of these unique pathogenic mechanisms is essential for the development of effective therapeutics and prophylactics against this important biothreat agent.

Experimental Procedures

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. The *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) (ATCC 29684; American Type Culture Collection, Rockville, MD) was obtained from BEI Resources, Manassas, VA. *F. tularensis* cultures were grown on Mueller Hinton (MH) chocolate agar plates (BD Biosciences, San Jose, CA) or modified MH-chocolate agar (MMH) (Bakshi *et al.*, 2006) supplemented with IsoVitaleX at 37°C with 5% CO_2 or in MH broth (BD Biosciences, San Jose, CA) supplemented with ferric pyrophosphate and IsoVitaleX (BD Biosciences, San Jose, CA) at 37°C with shaking (160 rpm). Active mid-log-phase bacteria grown in MH broth were harvested and stored at −80°C. The *Escherichia coli* DH5-α strain was used for cloning experiments. *E. coli* cultures were grown in Luria-Bertani (LB) broth or on LB agar plates. When necessary, kanamycin (25 μg/mL) or hygromycin (200 μg/mL) was included in the broth and agar media for the selection of transformants, mutants or transcomplemented strains.

Construction of *F. tularensis* deletion mutants and transcomplementation strains

The plasmids and primers used for the generation of mutants and transcomplemented strains in this study are listed in Table 1. To generate in-frame gene deletion mutants of *F. tularensis*, a suicide vector, pJC84 (Wehrly *et al.*, 2009), allowing for SacB-assisted allelic replacement in *Francisella* was used. For *F. tularensis oxyR* gene deletion, a 5' 1214 bp fragment upstream of the start codon of the *oxyR* (*FTL_1014*) gene and its first 5 codons, as well as a 3' 1272 bp fragment containing the *oxyR* gene stop codon and its downstream region were amplified by PCR. These 5' and 3' fragments were fused by overlapping extension PCR, and fused fragments were cloned into the pJC84 vector at the *BamH*I and *Sal*I sites, resulting in the pMM05 plasmid. pMM05 was used to transform the *F. tularensis* LVS by electroporation, as published previously (Maier *et al.*, 2004). The kanamycin-resistant colonies obtained after 3–5 days of incubation were serially diluted and plated on MMH agar with 8% sucrose and incubated at 37°C with 5% CO_2 for 2–3 days. Sucrose-resistant clones were re-plated on kanamycin-containing MMH agar plates to verify the loss of kanamycin resistance. The sucrose-resistant and kanamycin-sensitive clones were screened for *oxyR* gene deletion using *oxyR* gene-specific primers or primer pairs flanking the *oxyR* gene (Table 1). To further confirm *oxyR* gene deletion, a duplex colony PCR was performed using primers specific for the *oxyR* gene. *sodB* gene primers were used as internal controls. This improved gene deletion strategy preserved the frame of the downstream gene(s) and prevented any polar effects resulting from gene deletion. To further confirm, DNA sequencing was performed on the *oxyR* gene deletion mutants (Δ*oxyR*) to verify that the deletion of *oxyR* did not disrupt the reading frame.

To transcomplement the Δ*oxyR* gene deletion mutant, the *oxyR* gene was amplified by PCR. The amplified fragment was digested with the restriction enzymes *BamH*I and *Xho*I and cloned into the *E. coli-Francisella* shuttle vector pMP822 (LoVullo *et al.*, 2009). The resulting plasmid, pMM08, was verified by PCR and DNA sequencing, electroporated into the Δ*oxyR* mutant, and selected on MMH agar supplemented with 200 μg/mL hygromycin. The transcomplementation was confirmed by PCR.

Susceptibility of the ΔoxyR mutant to oxidants

The wild-type *F. tularensis* LVS, the Δ*oxyR* mutant, and the transcomplemented strain were tested for their susceptibilities to detergents, superoxide-generating compounds, and peroxides. For disc diffusion assays, bacterial suspensions adjusted to an OD_{600} of 2.0 were spread uniformly on MH-chocolate agar plates with a spreader. Sterile paper discs with sodium dodecyl sulfate (SDS; 750 μg/disk), sodium deoxycholate (100 μg/disk), Triton X-100 (2.5% solution), ethidium bromide (5 μg/disk), menadione (0.156 μg/mL), tert-butyl hydroperoxide (TBH; 21.9% solution), and cumene hydroperoxide (CHP 1.25% solution) in a 5 μL volume were impregnated on plates. The plates were incubated for 48 hrs, and zones of inhibition around the discs were measured. Growth curves were generated by diluting the bacterial strains grown on MH-chocolate agar plates to an OD_{600} of 0.2 (corresponds to 1×10^9 CFU/mL) and treating with menadione (1.56 μM), TBH (17.48 μM), and hydrogen peroxide (H_2O_2, 500 μM and 1 mM). The OD_{600} was measured at 4 h intervals. The susceptibility of the Δ*oxyR* mutant to H_2O_2 was also confirmed by performing bacterial killing assays. Equal numbers of bacteria (1×10^9 CFU/mL) were exposed to 750 μM H_2O_2. The numbers of viable bacteria were determined after 1 and 3 hrs of incubation by plating serial dilutions on MH-chocolate agar plates. Bacterial colonies were counted after 48 hrs, expressed as CFU/mL and compared with those obtained for the wild-type *F. tularensis* LVS or the transcomplemented strain.

Cell culture assays

A gentamicin protection assay was performed to determine the role of the Δ*oxyR* mutant in intracellular survival (Mahawar *et al.*, 2012). Briefly, the murine macrophage cell line RAW264.7 (from an already existing collection), BMDMs isolated from gp91*Phox*$^{-/-}$ or wild-type C57BL/6 mice (according to approved protocol), and A549 Type II alveolar epithelial cells were infected with the *F. tularensis* LVS, the Δ*oxyR* mutant, or the transcomplemented strain at a multiplicity of infection (MOI) of 100. Two hrs after infection, the macrophages or epithelial cells were treated with gentamicin (100 μg/mL) for 2 hrs to kill all the extracellular bacteria. Medium containing gentamicin was then replaced with medium without antibiotics, followed by incubation at 37°C in 5% CO_2. The macrophages were lysed at 4 and 24 hrs post-infection, while the epithelial cells were lysed at 4, 8, 12 and 24 hrs post-infection with 0.1% sodium deoxycholate. The cell lysates were serially diluted in sterile PBS and plated on MH-chocolate agar plates for bacterial enumeration. The results were expressed as CFU/mL.

Transmission electron microscopy (TEM)

For TEM, RAW264.7 cells were infected with the *F. tularensis* LVS or the Δ*oxyR* mutant at an MOI of 100 for 1 and 6 hrs. The cells were fixed in 2.5% glutaraldehyde, processed

following standard protocol for sectioning, and viewed by Hitachi HT 7700 TEM. For quantitation of the phagosomal and cytosolic bacteria, at least 100 bacteria were counted in randomly selected sections (14–20 independent sections) of the macrophages at 61,000× magnification.

Mouse experiments

All mouse experiments were conducted in the Animal Resource Facility of New York Medical College according to the approved IACUC protocols. Briefly, deeply anesthetized wild-type C57BL/6 or gp91*Phox*$^{-/-}$ mice (Jackson Laboratories) were infected intranasally with 1×10^4 of the *F. tularensis* LVS or 1×10^4 and 1×10^5 CFU of the Δ*oxyR* mutant and observed for morbidity and mortality for a period of 21 days. The results are expressed as Kaplan-Meier survival curves, and statistical significance was determined by log-rank test.

Quantitative proteomic analysis

To investigate the global effects of *oxyR* gene deletion on protein expression, iTRAQ analysis was performed. The wild-type *F. tularensis* LVS and the isogenic Δ*oxyR* mutant were grown to an OD_{600} of 0.6 in 12 mL MH broth. The cells were pelleted by centrifugation, re-suspended in the same volume of MH broth, and divided equally into two tubes. H_2O_2 (1 mM) was added to one tube, while the second tube was kept as an untreated control. Both tubes were incubated for 2 hrs at 37°C. The bacterial cells were centrifuged, washed once with 1× PBS, and resuspended in 200 μl lysis buffer (10 mM Tris-Cl, pH 7.5; 0.1% SDS; 0.5% sodium deoxycholate; 0.5% Triton X-100; 0.5 mM EDTA; 0.1 mM DTT) containing protease inhibitors (Sigma). The bacterial cell lysates were sonicated in a water bath sonicator and were processed for iTraq analysis using ABSCIEX iTraq reagents at the Center for Functional Genomics (CFG), SUNY Albany, as described previously with slight modifications (Ross, Huang et al. 2004; Luo, Ning et al. 2009). Briefly, total protein extracts were precipitated by trichloroacetic acid followed by acetone wash. The resulting protein precipitates were re-suspended in 400 μl of sample preparation buffer (100 mM Tris-HCl; 7 M urea; 2 M thiourea; 0.4% SDS; 5 mM tributylphosphine, pH 8.3) followed by iodoacetamide alkylation. The protein concentrations were determined with a MicroBCA protein assay kit (Pierce, Rockford, IL). The protein mixtures were then diluted 10-fold in 50 mM Tris-HCl, pH 8.5. Modified trypsin (Sigma) was added to a final substrate-to-enzyme ratio of 30:1, and the trypsin digests were incubated overnight at 37°C. The resulting peptides were cleaned up using a Discovery DSC-18 Cartridge (Sigma). Equal amounts (100 μg) of sample were labeled with 8-plex iTRAQ reagent (ABSCIEX) according to the manufacturer's instructions. Briefly, after desalting on a C18 cartridge, the peptide mixtures were lyophilized and re-suspended in 30 μl of 0.5 M triethylammonium bicarbonate (TEAB), pH 8.5. The appropriate iTRAQ reagent (dissolved in 70 μl isopropanol) was added, allowed to react for 2 hrs at room temperature, and then quenched with 10 μl of 1 M Tris, pH 8.5. The iTRAQ-labeled peptide mixtures were then concentrated, mixed and acidified to a total volume of 8.0 mL, followed by an off-line cation exchange chromatography. A total of 30 fractions were collected, and the samples were dried by a speed-vacuum prior to RP-LC-MS/MS analysis. The on-line nano-LC ESI QqTOF MS analysis was conducted as described previously (Luo *et al.*, 2009). The data were analyzed using Paragon Algorithm against the database within ProteinPilot v4.5

software with trypsin as the digesting agent, cysteine alkylation, an ID focus of biological modifications, and other default settings (ABSCIEX). Proteins designated as being significantly differentially produced were quantified in at least three spectra (allowing the generation of a *P*-value), had a fold change of more than 1.3 or less than 0.7 from two independent experiments performed with two biological replicates each, and had a *P*-value of <0.05. A $\log_2$ ratio of the fold change in protein levels (Δ*oxyR* Mutant/*F. tularensis* LVS) of 0.11 or higher indicated an increase, while a ratio of less than −0.15 indicated a decrease in the protein levels.

Transcriptional analysis

The wild-type *F. tularensis* LVS, the Δ*oxyR* mutant and the transcomplemented strain were grown to an OD_{600} of 0.5 (~2.5×10^9 CFU/mL) at 37°C in 10 mL MH broth. The bacterial cultures were divided equally into two tubes. A final concentration of 1 mM H_2O_2 was added to the first tube, while the second tube of culture was left untreated. After incubation for an additional 2 hrs, both the treated and untreated bacterial cells were pelleted, and the total RNA was isolated using a Purelink RNA Mini Kit (Ambion). The contaminating DNA from the RNA preparations was removed using on-Column Purelink DNase treatment. cDNA was synthesized using an iScript cDNA Synthesis Kit (Ambion). Quantitative real-time PCR (qRT-PCR) was performed using iQ SYBR Green Supermix (BioRad) to quantitate the gene transcripts. The amount of target gene amplification was normalized to a *Tul4* internal control. The relative mRNA levels of the target genes are presented as averages of three biological replicates with standard deviation (SD). The primer sequences used for qPCR are shown in Table 1.

Construction of *F. tularensis* oxyR-vesicular stomatitis virus glycoprotein (VSV-G) tagged strain

The *F. tularensis* LVS *oxyR-VSV-G* strain, which expresses an OxyR fusion protein with a C-terminal VSV-G tag, was constructed to determine the *in vivo* binding of OxyR to putative promoter regions of genes coding for antioxidant enzymes. A tag-integration vector, pKL02, containing a cloned RpoC-VSV-G tag protein (Ramsey *et al.*, 2015) was used. A DNA fragment specifying the 5' end of the *oxyR* gene of the *F. tularensis* LVS minus the stop codon engineered to have *Sal*I site and a 3' region engineered to have a *Not*I site was amplified by PCR using the primer combinations shown in Table 1. The PCR fragment was cloned into pKL02 at the *Sal*I and *Not*I sites, replacing the *rpoC* gene in pKL02 and fusing *oxyR* in frame with the VSV-G tag sequence. The resulting vector, pMM012, and the parental vector, pKL02, were used to transform the wild-type *F. tularensis* LVS by electroporation and selected for integration on MH-chocolate agar containing 25 μg/mL kanamycin. The resulting *F. tularensis* LVS strains expressing *oxyR-VSV-G* under the native *oxyR* promoter or expressing *rpoC-VSVG* were verified by PCR and DNA sequencing (Fig. S4).

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed to demonstrate the binding of OxyR to the promoter regions of the antioxidant enzyme genes of *F. tularensis*. Cells of the wild-type, *oxyR-VSV-G*, and *rpoC-VSV-G F. tularensis* strains were grown in 50 mL MH broth at 37°C with shaking. When an

OD_{600} of 0.4 was achieved, cultures of the *rpoC-VSV-G* strain were treated with a final concentration of 50 μg/mL rifampicin (Sigma) for 30 min. All the treated and untreated cultures were then cross-linked with 1% formaldehyde for 30 min, followed by 5 min incubation with 250 mM glycine to prevent further crosslinking. The bacterial cells were washed thrice with 1× PBS, resuspended in lysis buffer (20 mM HEPES pH 7.9; 50 mM KCl; 0.5 mM DTT; 10% glycerol) with protease inhibitors (Sigma), and sonicated in sonicator water bath to lyse the cells and shear their chromosomal DNA. After the cell debris was removed by centrifugation, the supernatant was adjusted for the salt concentration and immunoprecipitated overnight at 4°C using anti-VSV-G-agarose beads (Sigma). A 50 μl aliquot of supernatant was diluted in 200 μL TE + 1% SDS for use as an input control. The immunoprecipitates were washed five times with IPP150 buffer (10 mM Tris HCl, pH 8.0; 150 mM NaCl; 0.1% NP40), twice with TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA, pH 8.0), and subsequently eluted with 150 μL elution buffer (50 mM Tris-HCl, pH 8; 10 mM EDTA; 1% SDS) and 100 μL TE + 1% SDS buffer, respectively. The eluted and input samples were incubated overnight at 65°C to reverse the crosslinking. The DNA was purified using a PCR purification kit (Qiagen). The ChIP and input samples were analyzed for specific DNA fragments by qRT-PCR using the primers detailed in Table 1. The qRT-PCR values were normalized to the inputs and a *fopA* coding region internal control. The ChIP assays were also performed with the wild-type *F. tularensis* LVS (mock) and *rpoC-VSV-G* (positive control) strains for comparison. The results are expressed as relative enrichments of the detected fragments.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific). The promoter DNA probe was generated from wild-type *F. tularensis* LVS genomic DNA by PCR amplification with a 5' biotin-labeled forward primer from IDT (Integrated DNA Technologies) and an unlabeled reverse primer. The competitor DNA was amplified with the same unlabeled primer pairs. Both the biotin-labeled probe and the competitor DNA were purified with a PCR purification kit (Invitrogen) and used in the binding reaction. The primer sequences for the promoters of five genes, including *ahpC* (MP352/MP353, amplifying a 239 bp fragment encompassing −179 to + 60 relative to the start site of the ahpC ORF), *katG* (MP332/MP335, amplifying a 508 bp fragment encompassing −505 to +3 relative to the start site of the *katG* ORF), *sodB* (MP342/MP343, amplifying a 186 bp fragment from −148 to +38b relative to the start site of the *sodB* coding sequence), *sodC* (MP362/MP363, amplifying a 264 bp fragment from –218 to +46 relative to the start site of the *sodC* gene), *FTL_0996* (MP368/MP369, amplifying a 212 bp fragment from −158 to +54 relative to the start site of the FTL_0996 coding sequence) and *pmrA* (*FTL_0552*; MP402/MP403, amplifying a 505 bp fragment covering the entire upstream intergenic region of the *pmrA* gene) are shown in Table 1. The binding of the transcriptional regulator PmrA1 (Sammons-Jackson *et al.*, 2008) to its promoter region was used as a positive control to test the activity of the Δ*oxyR* mutant lysates (Table 1). The primer pairs were similar to those used for ChIP detection, except that the forward primers were labeled with biotin at the 5' end to be used as a probe in the EMSA experiments. The wild-type *F. tularensis* LVS, the Δ*oxyR* mutant, and the transcomplemented strain were grown to an OD_{600} of 0.5 in 10 mL MH broth at 37°C with shaking, centrifuged, washed once with PBS, and resuspended in

250 μl TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0) containing protease inhibitors (Sigma). Bacterial cell suspensions were sonicated in a sonicator water bath to lyse the cells and extract the proteins. The cell lysates were centrifuged for 15 min at 4000×g at 4°C to pellet the insoluble fraction. The supernatants containing soluble proteins were used for the binding assay. The protein concentrations were determined using BioRad reagent. One nanogram of biotin-labeled probe alone or in combination with 30 ng of specific competitor DNA was incubated with 5 μg whole-cell protein extracts for 20 min at room temperature in 20 μl of reaction buffer as per the instructions of the EMSA kit (Thermo Scientific). The reaction mixture was loaded onto 5% TBE non-denaturing Ready Gel (BioRad), electrophoresed at room temperature in 0.5% TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.3), and transferred to a Hybond-N+ nylon membrane (Amersham). The biotin-labeled DNA on the membrane was probed with streptavidin-horseradish peroxidase conjugate and detected by chemiluminescent substrate according to the manufacturer's protocol. The DNA bands were visualized on a Chemidoc XRS system (BioRad), and digital images were captured.

Western blot analysis

The *F. tularensis* LVS and the Δ*oxyR* mutant strain were grown at 37°C with shaking in 10 mL MH broth to an OD_{600} of approximately 0.5. Aliquots were collected and centrifuged at 4000×g for 10 min. The bacterial cell pellets were resuspended in 200 μl lysis buffer [200 mM Tris-HCl, pH 8.0; 320 mM $(NH_4)_2SO_4$; 5 mM $MgCl_2$; 10 mM EDTA; 10 mM EGTA; 20% glycerol; 1 mM dithiothreitol (DTT); protease and phosphatase inhibitors]. The protein concentrations of the cell lysates were determined with BioRad reagent. Five micrograms of protein from each sample was run on a 10% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane (Millipore) and probed with anti-KatG (1:20000) (kindly provided by Dr. Karsten Hazlett, Albany Medical College, Albany NY) and secondary monoclonal antibodies (anti-rabbit immunoglobulin, IgG, 1:5000) conjugated to horseradish peroxidase (Amersham). The protein bands on the membrane were visualized using Supersignal West Pico chemiluminescent substrate (Thermo Scientific) on a Chemidoc XRS system (BioRad) and quantitated.

Statistical analysis

The results are expressed as the means ± S.E.M. or S.D. Statistical significance between the groups was determined by one-way ANOVA followed by Tukey-Kramer and Multiple Comparison tests or by Student's t test. The survival data were analyzed by a log-rank test and presented as Kaplan-Meier survival curves. Differences between the experimental groups were considered statistically significant at a $P < 0.05$ level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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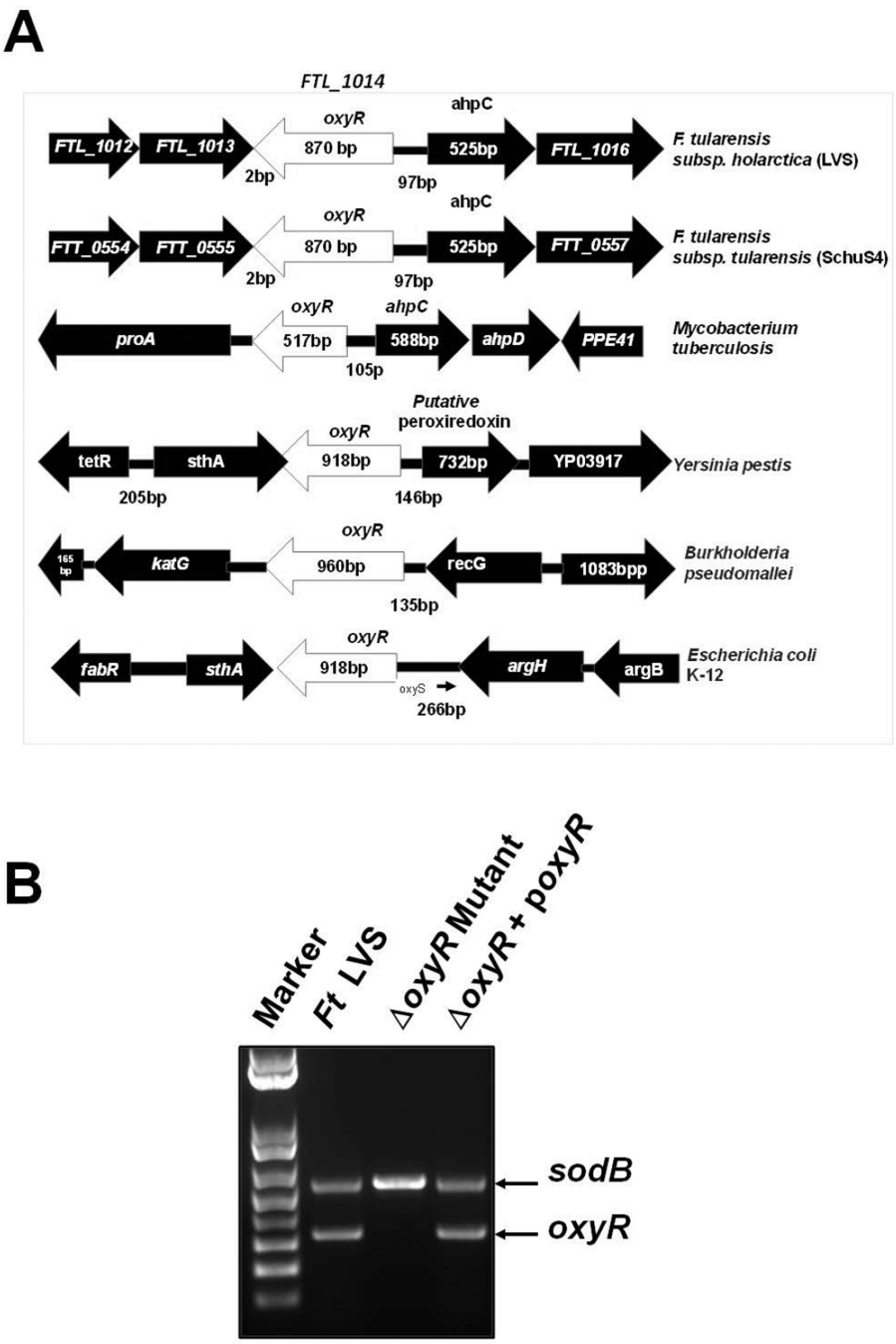

Figure 1. Genomic organization and generation of the oxyR gene deletion mutant of the *F. tularensis* LVS

(A) The genomic organization of the *oxyR* gene of the *F. tularensis* LVS and its comparison with the *oxyR* gene from the indicated bacterial pathogens. (B) Multiplex colony PCR using *oxyR* gene-specific primers and *sodB* gene primers as internal controls. Amplification of the *sodB* gene confirmed the presence of the DNA template in the reaction, whereas the absence of the *oxyR* gene product in the Δ*oxyR* mutant confirmed the deletion of the *oxyR* gene.

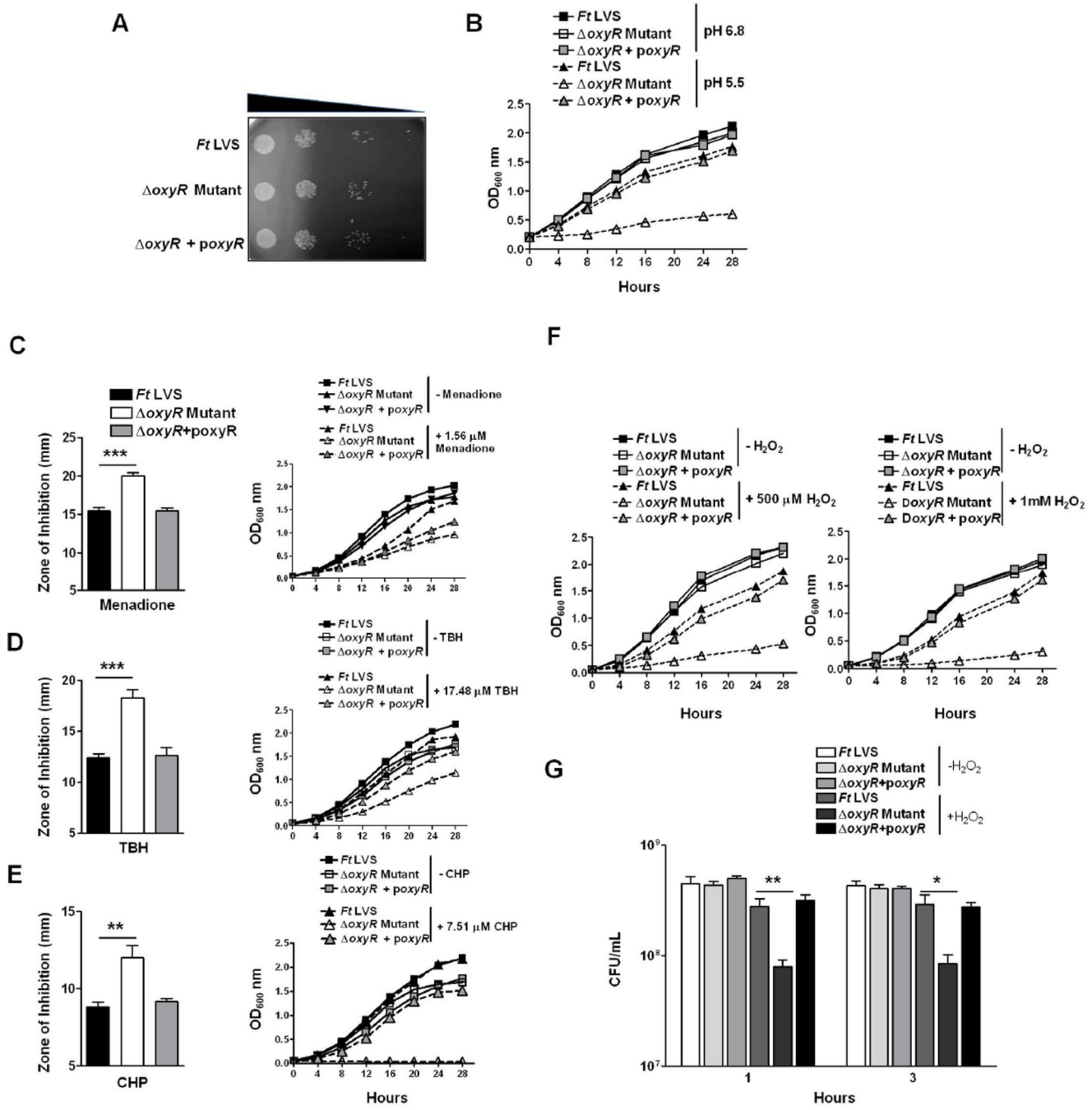

Figure 2. OxyR of *F. tularensis* is not essential for cell viability but is required for growth under acidic conditions and resistance against superoxide radicals, hydrogen peroxide, and organic peroxides

(A) A cell viability assay was performed by growing the indicated bacterial strains aerobically. The cultures were diluted 10-fold and spotted on MH-chocolate agar plates. (B) The *F. tularensis* LVS, the Δ*oxyR* mutant and the Δ*oxyR* + p*oxyR* transcomplemented strains were grown aerobically in MHB at pH 6.8 and pH 5.5, and OD_{600} readings were recorded at 4 hr intervals. (C–E) The susceptibility of the Δ*oxyR* mutant to the superoxide-generating compounds menadione (C), organic peroxide tert-Butyl hydroperoxide (TBH; D)

and cumene hydroperoxide (CHP; E) was tested by disk diffusion assay as described in Experimental Procedures. The plates were incubated for 48–72 hrs, and the zones of inhibition around the discs were measured (left panels). Growth curves of the *F. tularensis* (*Ft*) LVS, the Δ*oxyR* mutant and the transcomplemented strain in the presence of the indicated concentrations of menadione (C), TBH (D) and CHP (E), right panels. (F) Growth curves in the presence of 500 μM and 1 mM concentrations of hydrogen peroxide (H_2O_2). (G) Bacterial killing assay in the presence of 750 μM H_2O_2. The indicated bacterial strains were exposed to H_2O_2 for 1 and 3 hrs, diluted 10-fold and plated on MH-chocolate agar plates to recover viable bacteria. The results are expressed as CFU/mL. The data are representative of at least 4–5 independent experiments and represented as the mean ± SD. The data were analyzed by ANOVA, and the *P* values were recorded. **P*<0.05; ***P*<0.01; ****P*<0.001.

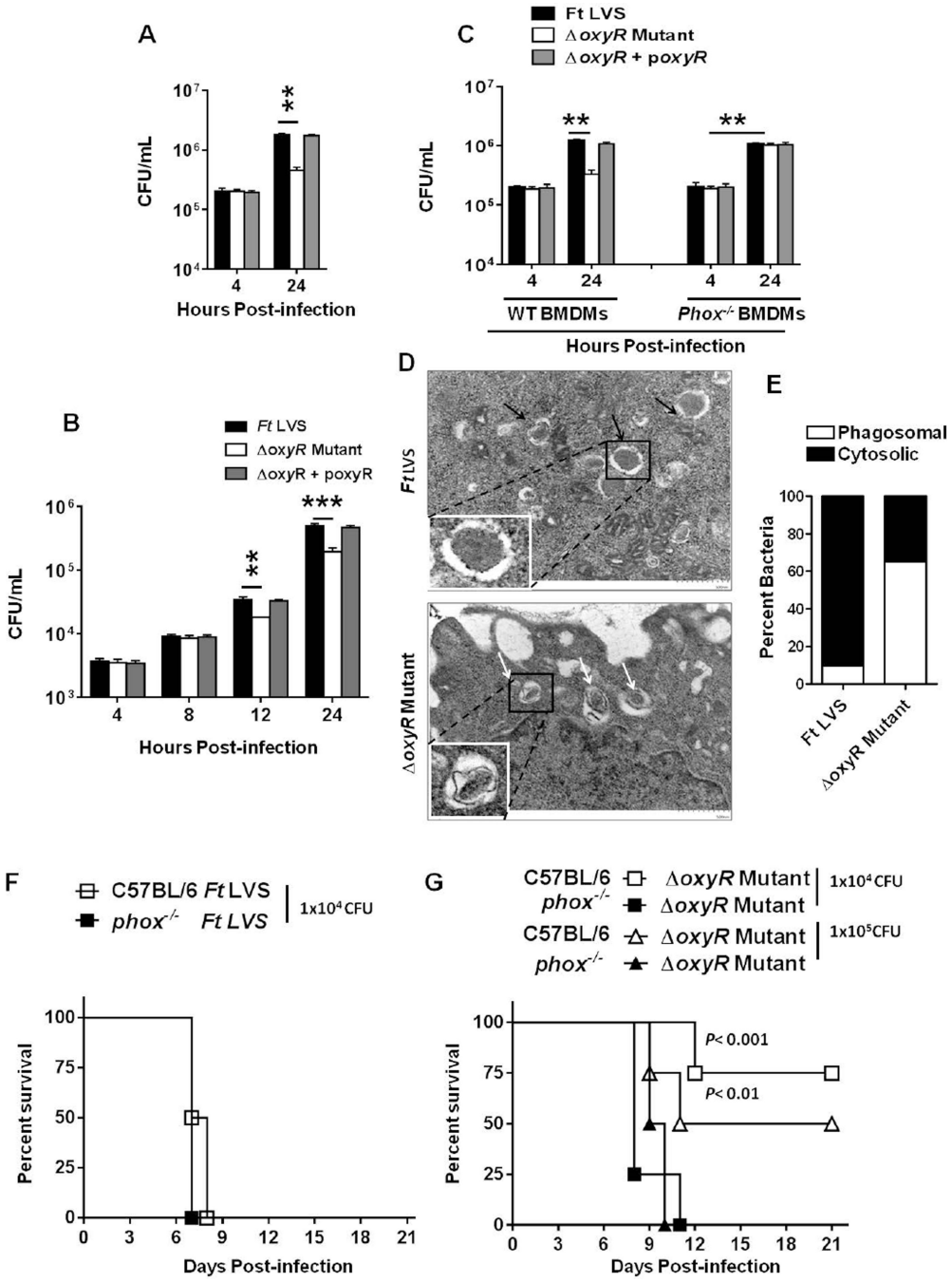

Figure 3. OxyR of *F. tularensis* is required for intracellular survival and virulence in mice

(A) The RAW macrophage cell line, (B) A549 Type II alveolar epithelial cells and (C) primary BMDMs derived from wild-type C57BL/6 or gp91*phox*$^{-/-}$ mice (n=4 biological replicates) were infected with the *F. tularensis* (*Ft*) LVS, the Δ*oxyR* mutant or the transcomplemented strain (Δ*oxyR* + p*oxyR*) at an MOI of 100. The macrophages were lysed after 4 and 24 hrs (A and C); while the epithelial cells were lysed at 4, 8, 12 and 24 hrs post-infection (B), diluted 10-fold and plated on MH-chocolate agar plates for the enumeration of bacterial numbers. The data are representative of three independent experiments conducted

with identical results (A and C) or cumulative results from two independent experiments (B). The data are expressed as CFU/mL and analyzed by ANOVA with a Tukey-Kramer post-test, and comparisons are shown with *Ft* LVS. ***P*<0.01; ****P*<0.001. (D) TEM of RAW macrophages infected with the *F. tularensis* LVS or the Δ*oxyR* mutant at an MOI of 100, imaged 6 hrs post-infection. Black arrows indicate extra-phagosomal bacteria, while the white arrows indicate phagosomal bacteria. (E) Quantitation of phagosomal and cytosolic bacteria was performed by counting at least 100 bacteria in randomly selected sections of the infected macrophages on a Hitachi HT 7700 TE Microscope (61,000× magnification). The data shown are representative of two independent experiments conducted with similar results. (F) Wild-type or gp91*phox*$^{-/-}$ mice on a C57BL/6 background (n=6 mice/ group) were infected intranasally with 1×10^4 CFUs of the *F. tularensis* LVS. (G) Wild-type or gp91*phox*$^{-/-}$ mice on a C57BL/6 background (n=6 mice/ group) were infected intranasally with 1×10^4 or 1×10^5 CFUs of the Δ*oxyR* mutant. The mice were observed for morbidity and mortality over a period of 21 days. The results are represented as Kaplan-Meier survival curves, and the statistical significance was determined by log-rank test.

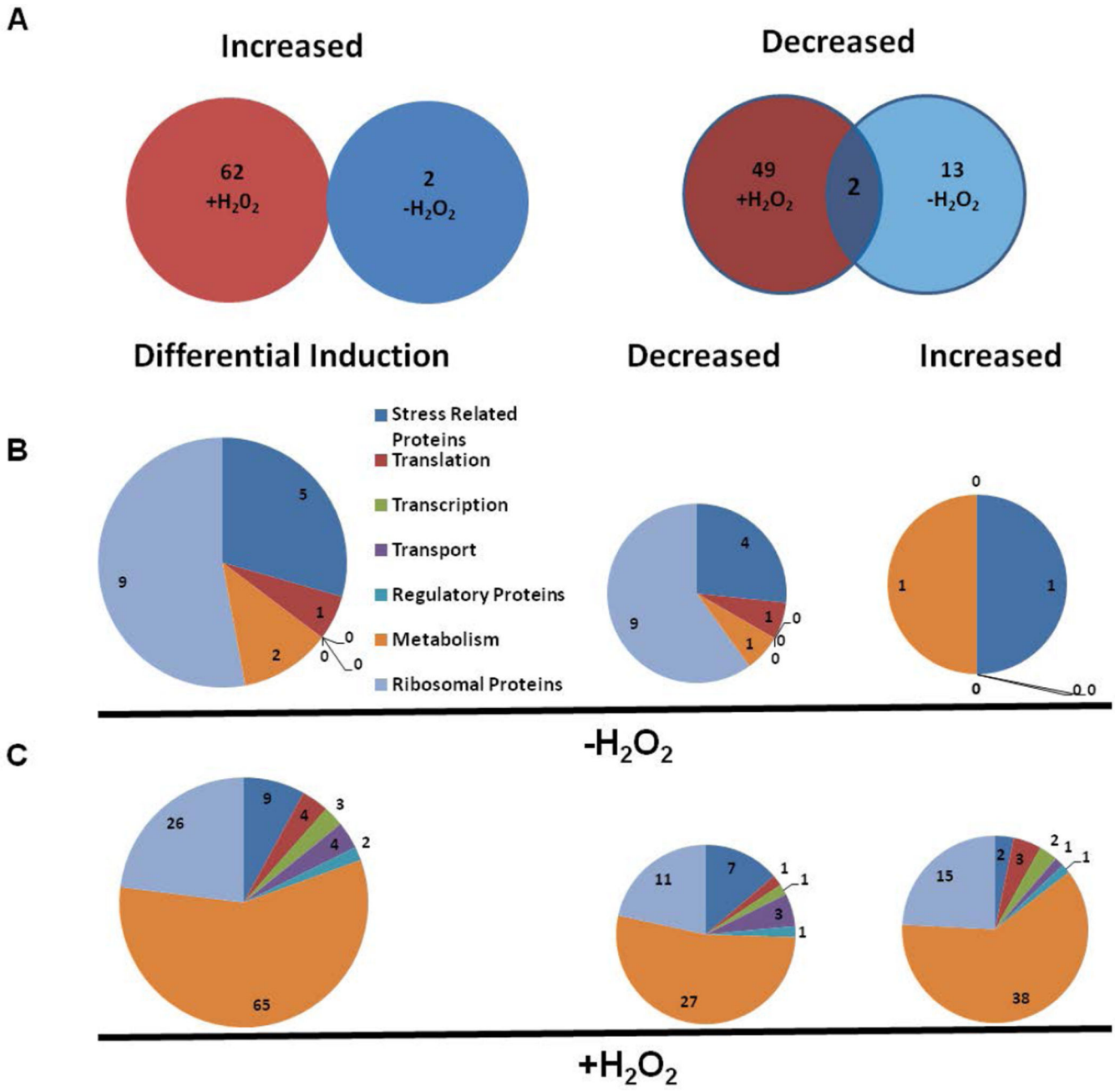

Figure 4. Differential induction of proteins in the ΔoxyR mutant of *F. tularensis* with or without exposure to H_2O_2

(A) Total number of differentially induced proteins in the Δ*oxyR* mutant compared to the wild-type *F. tularensis* LVS. (B, C) Profile of differentially induced proteins involved in various cellular functions in the Δ*oxyR* mutant in the presence or absence of H_2O_2-induced oxidative stress.

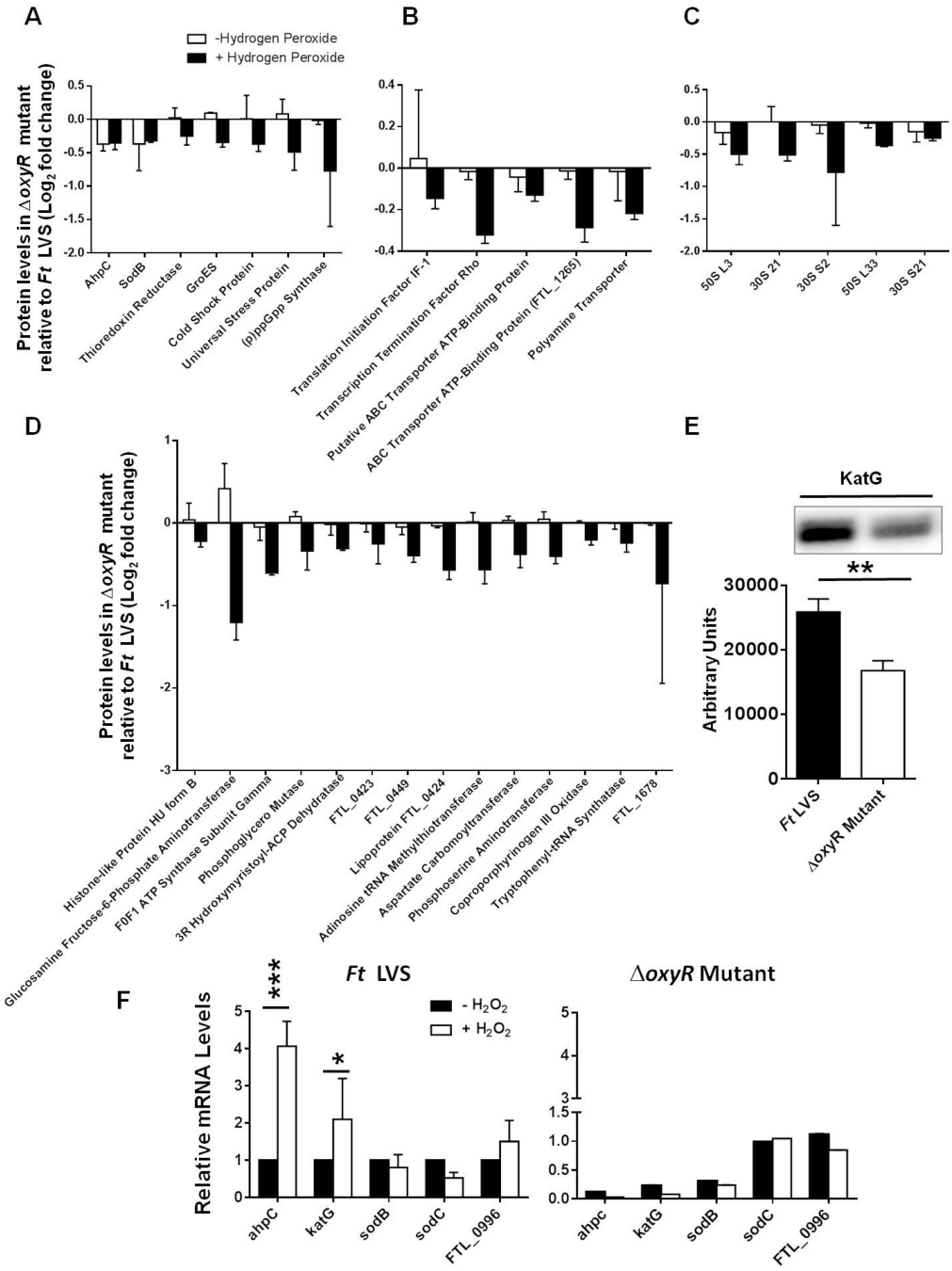

Figure 5. Suppressed proteins in the ΔoxyR mutant of *F. tularensis* with or without exposure to H_2O_2

The most prominently decreased proteins in the Δ*oxyR* mutant compared to the wild-type *F. tularensis* LVS are shown from the top 128 differentially induced proteins in the Δ*oxyR* mutants. The proteins are grouped according to their functional categories. (A) oxidant and general stress resistance (Category I); (B) transcription, translation, and transport function (Category II); (C) ribosomal proteins (Category III); (D) metabolism and hypothetical proteins (Category IV). The data shown are cumulative of two independent iTRAQ experiments, each conducted with duplicate samples. The data are represented as the ratio of

the $\log_2$-fold change in protein levels between the wild-type *F. tularensis* LVS and the Δ*oxyR* mutant. (E) Western blot analysis for the determination of KatG expression in the wild-type *F. tularensis* LVS and the Δ*oxyR* mutant. The bottom panel shows quantitation of the bands (n=3 blots). The statistical analysis was performed by Student's t test. $^{**}P<0.01$. (F) Transcriptional analysis of the indicated antioxidant enzyme genes in the *F. tularensis* (*Ft*) LVS and the Δ*oxyR* mutant in the presence or absence of H_2O_2. The data are representative of three independent experiments with similar results and are presented as relative mRNA levels. The data were analyzed by ANOVA, and the *P* values were recorded. $^{*}P<0.05$; $^{***}P<0.001$.

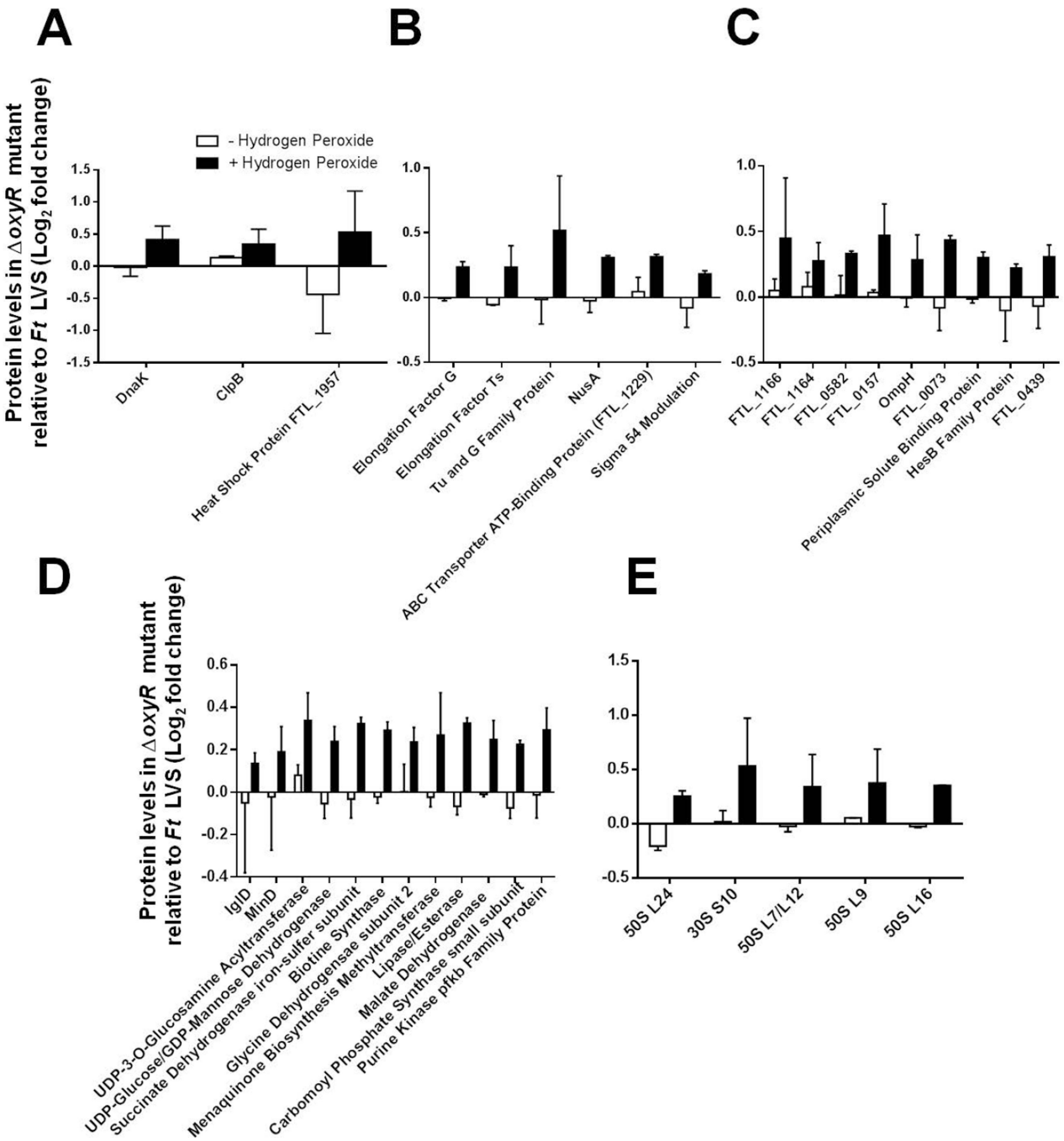

Figure 6. Elevated proteins in the ΔoxyR mutant upon exposure to H_2O_2

The most prominently increased proteins in the Δ*oxyR* mutant compared to the wild-type *F. tularensis* LVS are shown from the top 62 elevated proteins in the Δ*oxyR* mutants exposed to H_2O_2. The proteins are grouped according to their functional categories. (A) oxidant and general stress resistance (Category I); (B) transcription, translation, and transport functions (Category II); (C) hypothetical proteins (Category III); (D) metabolism (Category IV); (E) ribosomal proteins (Category V). The data shown are cumulative of two independent iTRAQ experiments, each conducted with duplicate samples. The data are represented as the ratio of

$\log_2$-fold changes in the protein levels between the wild-type *F. tularensis* LVS and the Δ*oxyR* mutant.

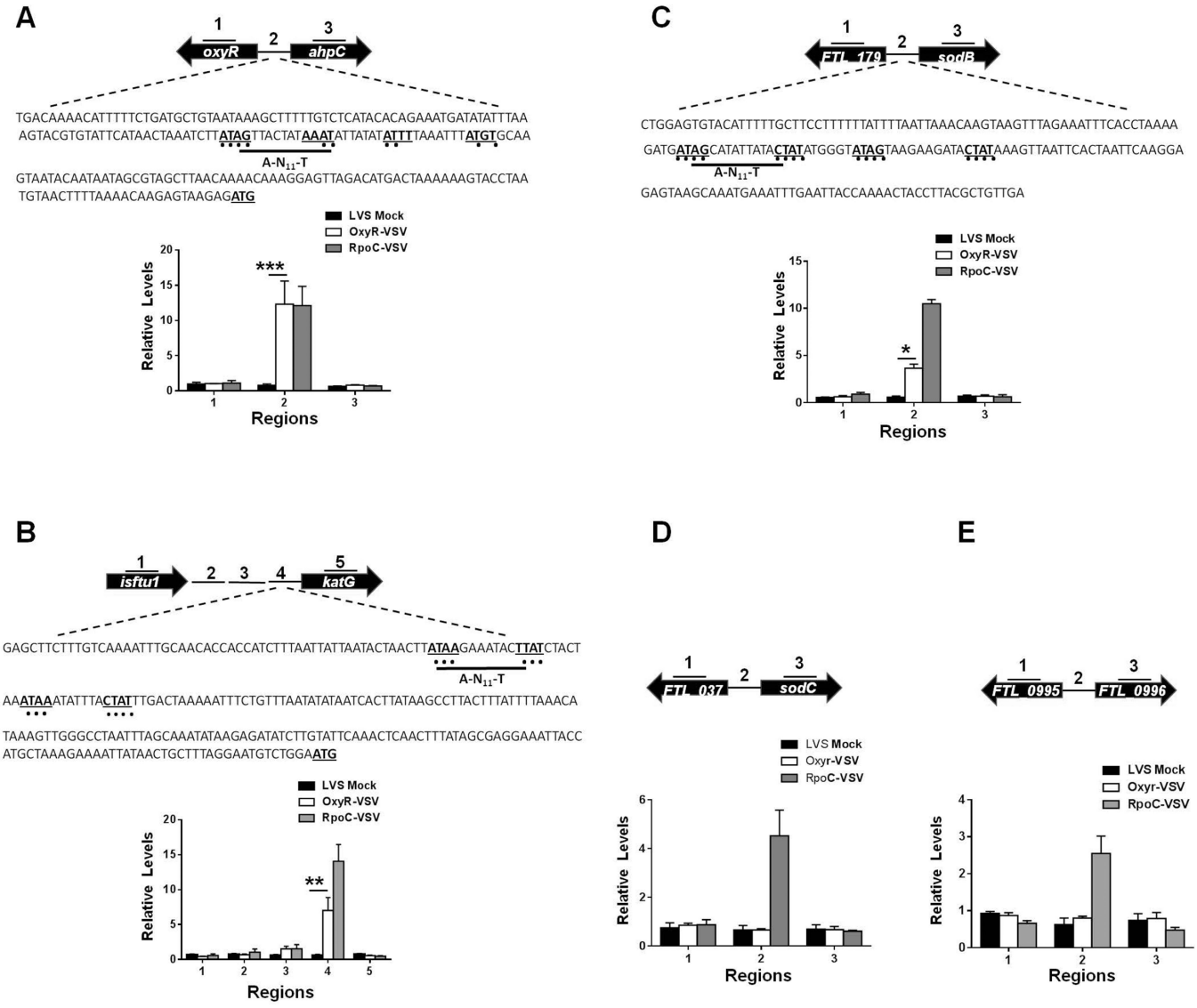

Figure 7. OxyR regulates the transcription of genes encoding primary antioxidant enzymes of *F. tularensis* by physically interacting with their putative promoter regions

Chromatin immunoprecipitation (ChIP) was performed using anti-VSV-G-agarose beads, as described in the Experimental Procedures section. The fragments of intergenic regions covering the putative promoter sequences and the coding regions of the up- and downstream genes (indicated by numbers) in ChIP and input samples of the indicated bacterial strains were analyzed for enrichment by qRT-PCR. The values were normalized to the input and a *fopA* coding region as internal controls. The qRT-PCR results of ChIP from *Ft oxyR-VSVG* along with *Ft* LVS (mock) and *rpoC-VSVG* (positive control) are shown (n=3 biological replicates). (A) Illustrated *ahpC* locus; (B) *katG* locus (C) *sodB* locus; (D) *sodC* locus and (E) *FTL_0996* locus. The data shown are representative of three independent experiments with identical results. In A, B and C, the DNA sequences of the upstream regions of the *ahpC, katG* and *sodB* genes, respectively, with conserved OxyR binding motifs (bold letters

with underlined dots) are shown. The data were analyzed by ANOVA, and the *P* values were recorded. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

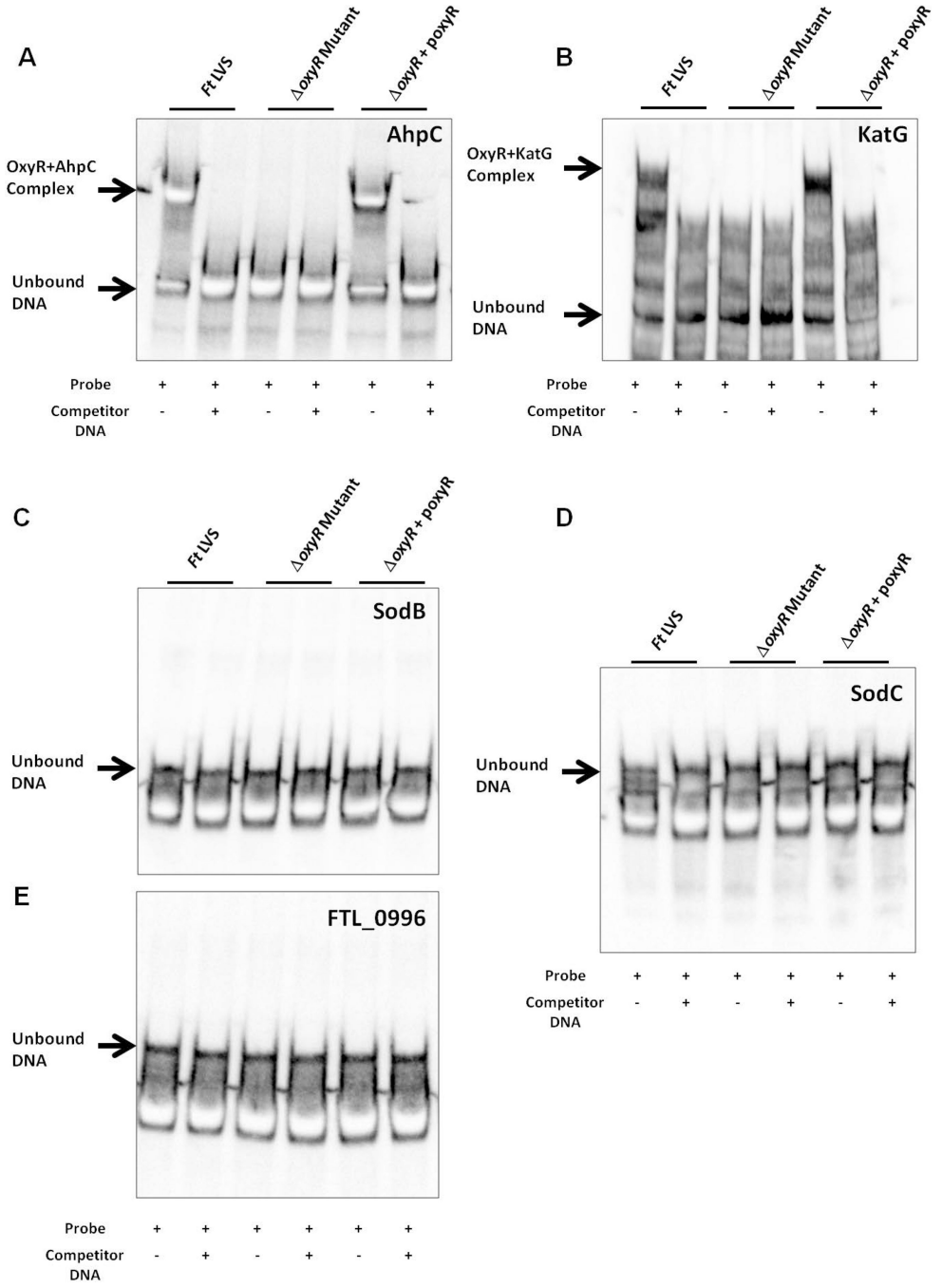

Figure 8. OxyR interacts with the putative promoter regions of primary antioxidant genes of *F. tularensis*

Electrophoretic mobility shift assay (EMSA) with the promoter regions (identified in Fig. 7) for *ahpC* (A); *katG* (B); *sodB* (C); *sodC* (D) and *FTL_0996* (E) of *F. tularensis*. EMSA was performed using bacterial lysates from the *F. tularensis* (*Ft*) LVS, the Δ*oxyR* mutant and the transcomplemented strain (Δ*oxyR*+poxyR) with a LightShift Chemiluminescent EMSA kit, as described in the Experimental Procedures section. Biotinylated promoter regions were

used as the probes, whereas unlabeled promoter regions were used as controls. The results are representative of two independent experiments.

Table 1

List of bacterial strains, plasmid vectors and primers used in this study.

***Francisella* Strains**	**Genotype**	**Source**
F. tularensis LVS	Wild-type strain	ATCC
Δ*oxyR* mutant	Deletion mutant of *F. tularensis* LVS *oxyR* gene	This study
oxyR tag strain	*F. tularensis* LVS, *oxyR-VSV-G* fusion, Kan^r	This study
rpoC tag strain	*F. tularensis* LVS, *rpoC-VSV-G* fusion, Kan^r	This study
oxyR transcomplement (Δ*oxyR* + p*oxyR*)	*F. tularensis* LVS, Δ*oxyR* pMM08(pMP822+*oxyR*), Hygro^r	This study
***E. coli* Strains**		
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
ME004	DH5α with pMP822, Hygro^r	(LoVullo *et al.*, 2009)
ME011	DH5α with pJC84, Kan^r	This Study
ME014	DH5α with pMM05, Kan^r	This Study
ME017	DH5α with pMM08, Hygro^r	This study
ME022	DH5α with pKL02, Kan^r	(Ramsey *et al.*, 2015)
ME024	DH5α with pMM12, Kan^r	This study
Plasmids		
pMP822	*E. coli-Francisella* shuttle vector, Hygro^r	(LoVullo *et al.*, 2006)
pJC84	*E. coli-Francisella* suicide vector, Kan^r	(Wehrly *et al.*, 2009)
pKL02	*rpoC-VSV-G* tag integration vector	(Ramsey *et al.*, 2015)
pMM05	pJC84 + fused flanking fragment of *oxyR* gene, Kan^r	This study
pMM08	pMP822 + *oxyR*, Hygro^r	This study
pMM12	pKL02+*oxyR-VSV-G*, Kan^r	This study
Primers	**Sequence**	**Purpose**
***oxyR* deletion construct**		
oxyR upstream fragment		
MP159	5'-CAAggatccCCAGCTACAGACTTAAGATAAGCA-3'	F-primer with a BamHI site at the 5' end
MP160	5'-AGTACGTGTATTCATAACTAAATC-3'	R-primer
oxyR downstream fragment		
MP162	5' -GATTTAGTTATGAATACACGTACTTAAGCTCACATAAA TATCATCCAA-3'	F-primer
MP163	5'-TGATgtcgacTACCATGTCAGGTTTAGCTGAGGT-3'	R-Primer with a SalI site
oxyR deletion mutant screening		
MP037	5'-CCGGATCCATGAAATTTGAATTACCAAAAC-3'	F-primer for *sodB* as a control
MP038	5'-CGCTGCAGCTAATCAGCGAATTGCTCAGAAAC-3'	R-primer for *sodB* as a control

***Francisella* Strains**	**Genotype**	**Source**
MP233	5’-CGCACAGAGTTTAAGAGTTTGATC-3’	F-primer for *oxyR*
MP234	5’-CTATTGGCGCATTTCCAACT-3’	R-primer for *oxyR*
***oxyR* complementation construct**		
MP253	5’-CAAggatccATGAATACACGTACTCTTAAATAT-3’	F-primer for *oxyR* with a BamHI site
MP254	5’-TGATctcgagTTAATGATTATTTGAAATTATTTT-3’	R-primer for *oxyR* with an XhoI site
Transcriptional analysis		
tul4 (Internal control)		
MP029	5’-TCGCAGGTTTAGCGAGCTGTTCTA-3’	F-primer
MP030	5’-ACAGCAGCAGCTTGCTCAGTAGTA-3’	R-primer
Peroxidase/Catalase (*FTL_1504, katG*)		
MP077	5’-CCTGCCAAATAAAGTTTTGCTC-3’	F-primer
MP078	5’-AGCTCACCAATGGACTCCTAC-3’	R-primer
Superoxide dismutase [Fe](*FTL_1791, sodB*)		
MP101	5’-GGCGGAATATTTAATAACGCTGC-3’	F-primer
MP102	5’-GTGCTCCCAAACATCAAAAG-3’	R-primer
Superoxide dismutase (Cu-Zn) precursor (*FTL_0380, sodC*)		
MP103	5’-TGTCAATACTCATAAAGAGGTTG-3’	F-primer
MP104	5’-AGTTGCTGTACCATCTGCGTTA-3’	R-primer
AhpC/TSA family protein (*FTL_1015, ahpC*)		
MP258	5’-TTGTATTCTCATTACCAGGAGCA-3’	F-primer
MP259	5’-ACAATCATTGCATAGCGCCA-3’	R-primer
FTL_0996		
MP264	5’-CAGCTAAGCTAAAAGAGCTTGGTG-3’	F-primer
MP265	5’-CTACCATTCTGATAACTTCATCCA-3’	R-primer
***oxyR-VSV-G* epitope tag construct**		
MP316	5’-CAA gtcgacTCTCCCTGCTATCAAACAAGAAC-3’	F-primer for *oxyR* with a SalI site
MP317	5’-TGAT gcggccgcATGATTATTTGAAATTATTTTAGCG-3’	R-primer for *oxyR* with a NotI site
MP322	5’-TATGCTTCCGGCTCGTATGTTGTG-3’	Sequencing primer
ChIP Analysis (qPCR)		
***katG* locus**		
MP326	5’-GAAAAGAACATGAAAGGTTGGAG-3’	F- primer for ChIP region 1
MP327	5’-AGTGTTCCTCAAACCATTGATTA-3’	R-primer for ChIP region 1
MP330	5’-TCTTTTGATGCTCTATATCACTG-3’	F- primer for ChIP region 2
MP331	5’-AGCCATAACTAAGGATGTTATGC-3’	R-primer for ChIP region 2

***Francisella* Strains**	**Genotype**	**Source**
MP332	5’-TGATTGATAATAGAACCTACCCCT-3’ ***	F- primer for ChIP region 3
MP333	5’-GCAAATTTTGACAAAGAAGCTC-3’	R-primer for ChIP region 3
MP334	5’-GAGCTTCTTTGTCAAAATTTGCA-3’ ***	F- primer for ChIP region 4
MP335	5’-CATTCCAGACATTCCTAAAGCAG-3’	R-primer for ChIP region 4
MP338	5’-AAACTGGGGACTATCACCTGAAGA-3’	F-primer for ChIP region 5
MP339	5’-TGCTTGCTTGACTTTATCCTCTG-3’	R-primer for ChIP region 5
***sodB* locus**		
MP340	5’-TCGCCAGATTCATTCATTTC-3’	F- primer for ChIP region 1
MP341	5’-ATGCAGCAACGGCAATTAGA-3’	R-primer for ChIP region 1
MP342	5’-CTGGAGTGTACATTTTTGCTTCC-3’ ***	F- primer for ChIP region 2
MP343	5’-TCAACAGCGTAAGGTAGTTTTGG-3’	R-primer for ChIP region 2
MP346	5’-CGCTGCTCAAGTTTTTAATCATAC-3’	F-primer for ChIP region 3
MP347	5’-TCTCTGTTAATGGGCAACCA-3’	R-primer for ChIP region 3
***ahpC* locus**		
MP348	5’-TGGTAGTTTTCAGTAGGAGTTGC-3’	F-primer for ChIP region 1
MP349	5’-TTGCTGAGCTACTCTTAGAGAATG-3’	R-primer for ChIP region 1
MP352	5’-TGACAAAACATTTTTCTGATGCTG-3’ ***	F-primer for ChIP region 2
MP353	5’-GCCAATACCCTCATCTCTTACTCT-3’	R-primer for ChIP region 2
MP356	5’-CGGTAAACGATAGCTTTGTTATGA-3’	F-primer for ChIP region 3
MP357	5’- ACAATCATTGCATAGCGCCA-3’	R-primer for ChIP region 3
***fopA* coding region (internal control)**		
MP358	5’-TGCTGGTTGGGCAAATCTA-3’	F-primer
MP359	5’-TGTAGTCGCACCATTATCCTGA-3’	R-primer
***sodC* locus**		
MP360	5’-TCTGCATGTCTTCTTTAGGGAT-3’	F-primer for ChIP region 1
MP361	5’-GGAATAATCATAGGCAAGGCATC-3’	R-primer for ChIP region 1
MP362	5’-GCGTATCAGCTAAAGTGATAATCG-3’ ***	F-primer for ChIP region 2
MP363	5’-CAAGTGAAAGCATACTCATTCCAC-3’	R-primer for ChIP region 2
MP364	5’-CACCATATATTCATGATGGTAACC-3’	F-primer for ChIP region 3

***Francisella* Strains**	**Genotype**	**Source**
MP365	5’-CCCGCTAGCTCTTCTAGAGAATTA-3’	R-primer for ChIP region 3
***FTL_0996* locus**		
MP366	5’-TGACATTCTCGAGGCTTTAGGTT-3’	F-primer for ChIP region 1
MP367	5’-CAAACACCTAAAATAGCTGCTGA-3’	R-primer for ChIP region 1
MP368	5’-TGCGATAGTACCATCACAATCAA-3’ ***	F-primer for ChIP region 2
MP369	5’-ACCTAAAACAGCTGGTGCATTG-3’	R-primer for ChIP region 2
MP370	5’-CAGCTAAGCTAAAAGAGCTTGGTG-3’	F-primer for ChIP region 3
MP371	5’-CATTAACCACTTGATGACGCAC-3’	R-primer for ChIP region 3
PmrA EMSA		
MP402	5’-GGGAGCTAGTGAGAGGAATTTTT-3’ ***	F- Primer for PmrA promoter
MP403	5’-CCAAATGAAGATCATCTTCAGCC-3’	R- Primer for PmrA promoter

*** These primers were labeled with biotin at the 5’ end for the EMSA experiments.