



Lack of OxyR and KatG Results in Extreme Susceptibility of *Francisella tularensis* LVS to Oxidative Stress and Marked Attenuation *In vivo*

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Francisella tularensis is an intracellular bacterium and as such is expected to encounter a continuous attack by reactive oxygen species (ROS) in its intracellular habitat and efficiently coping with oxidative stress is therefore essential for its survival. The oxidative stress response system of *F. tularensis* is complex and includes multiple antioxidant enzymes and pathways, including the transcriptional regulator OxyR and the H₂O₂-decomposing enzyme catalase, encoded by *katG*. The latter is regulated by OxyR. A deletion of either of these genes, however, does not severely compromise the virulence of *F. tularensis* and we hypothesized that if the bacterium would be deficient of both catalase and OxyR, then the oxidative defense and virulence of *F. tularensis* would become severely hampered. To test this hypothesis, we generated a double deletion mutant, $\Delta oxyR/\Delta katG$, of *F. tularensis* LVS and compared its phenotype to the parental LVS strain and the corresponding single deletion mutants. In accordance with the hypothesis, $\Delta oxyR/\Delta katG$ was distinctly more susceptible than $\Delta oxyR$ and $\Delta katG$ to H₂O₂, ONOO⁻, and O₂⁻, moreover, it hardly grew in mouse-derived BMDM or in mice, whereas $\Delta katG$ and $\Delta oxyR$ grew as well as *F. tularensis* LVS in BMDM and exhibited only slight attenuation in mice. Altogether, the results demonstrate the importance of catalase and OxyR for a robust oxidative stress defense system and that they act cooperatively. The lack of both functions render *F. tularensis* severely crippled to handle oxidative stress and also much attenuated for intracellular growth and virulence.

Keywords: *Francisella tularensis*, OxyR, KatG, oxidative stress, virulence

INTRODUCTION

Francisella tularensis, a Tier 1 select agent and the causative agent of tularemia, is a zoonotic, facultative intracellular bacterium with two clinically relevant subspecies, *tularensis* and *holarctica*, the former of which causes an aggressive disease with high mortality if left untreated (Oyston et al., 2004). Although there is no licensed vaccine against this potential bioterrorism agent, the subspecies *holarctica* live vaccine strain, LVS, is used to vaccinate laboratory workers, and is widely used in *Francisella* research as it is attenuated in humans, but retains its virulence in mice (Sjöstedt, 2006; Conlan, 2011).

Francisella tularensis is capable of infecting numerous cell types, including professional phagocytes, like macrophages. Upon phagocytosis, it transiently resides within the phagosome before escaping into the cytosol to replicate (Bröms et al., 2010; Chong and Celli, 2010). Phagocytes constitute a hostile environment utilizing a wide array of anti-bacterial mechanisms, such as phagosome acidification, disruption of pathogen membrane integrity, removal or sequestration of nutrients, and the production of reactive oxygen species (ROS) (Flanagan et al., 2009) and since *F. tularensis* is an intracellular bacterium, it will encounter a continuous exposure to ROS. Vital macromolecules, such as proteins and DNA, will react with ROS, thereby disrupting their functions (Fridovich, 1998; Schaible and Kaufmann, 2004; Flanagan et al., 2009). There are several ROS with potent antibacterial effects, such as superoxide and H₂O₂. The former is produced at high levels by the phagocyte oxidase (phox) and it rapidly combines with nitric oxide (NO), which is produced at high levels by inducible nitric oxide synthase (iNOS), to form peroxynitrite, a highly reactive compound. H₂O₂ is toxic *per se*, but the damage it exerts can be exacerbated in combination with intracellular ferrous iron, resulting in the formation of hydroxyl radicals (HO•) and hydroxide anions (OH⁻) through the Fenton reaction.

Reactive oxygen species (ROS) are not only formed during host attack, but low levels are also formed as by-products of normal aerobic metabolism. Thus, pathogens, in particular intracellular pathogens, have a pressing need for defense mechanisms to combat the ever present levels of ROS, but even more so to combat the assault of ROS experienced within a host (Betteridge, 2000). The critical roles of ROS and NO for the host defense against tularemia are illustrated by the extreme susceptibility of phox-deficient and iNOS-deficient mice to an *F. tularensis* infection (Lindgren et al., 2004). Moreover, *ex vivo*, it has been demonstrated that the requirements for host protection vary depending on the cell type investigated, since killing of *F. tularensis* by mouse peritoneal cells is NO-dependent, but NO-independent by mouse pulmonary cells (Anthony et al., 1992; Polsinelli et al., 1994; Lindgren et al., 2005).

The oxidative stress defense system of *Escherichia coli* has been extensively studied and includes numerous detoxifying enzymes, such as catalase, superoxide dismutases (SODs), alkyl hydroperoxide reductase (Ahp), and the H₂O₂-activated transcriptional regulator OxyR. The latter combats the effect of H₂O₂ by dual mechanisms, since it regulates the expression of both catalase and the ferric uptake regulator (Fur) (Farr and Kogoma, 1991; Zheng et al., 1998, 1999; Pomposiello and Demple, 2001). Catalase renders H₂O₂ harmless by degrading it to oxygen and water, whilst Fur down-regulates the expression of genes involved in iron uptake, thus limiting the amount of iron with which H₂O₂ can combine in the Fenton reaction (Andrews et al., 2003; Troxell and Hassan, 2013). Catalase, SODs, AhpC and other detoxifying enzymes are employed as oxidative stress defense mechanisms also by *F. tularensis* (Bakshi et al., 2006; Lindgren et al., 2007; Melillo et al., 2009; Binesse et al., 2015). The *F. tularensis* catalase, encoded by *katG*, mediates H₂O₂ tolerance and is known to be important for the virulence of *F. tularensis* LVS (Lindgren et al., 2007). SodB, FeSOD, and SodC, CuZnSOD,

are both known to be important for the dismutation of O₂⁻ in *F. tularensis*, and SodB further acts in the defense against oxidative stress by harnessing iron (Bakshi et al., 2006; Melillo et al., 2009). The *F. tularensis* AhpC enzyme is important for the detoxification of O₂⁻ and peroxynitrite (ONOO⁻), but not of H₂O₂, in the highly virulent SCHU S4 strain (Binesse et al., 2015), but the importance in the LVS strain is yet unknown. *F. tularensis* also encodes an *oxyR* homolog, the role of which has been studied recently (Ma et al., 2016). It was found that the absence of OxyR rendered LVS defective for oxidative stress defense, growth in macrophages and epithelial cells, and virulence in mice. Moreover, it was demonstrated that OxyR regulates the expression of the *ahpC*, *katG*, and *sodB* genes, with the most pronounced regulatory effect exerted on *ahpC*.

A more thorough understanding of the *F. tularensis* antioxidant system will undoubtedly reveal virulence mechanisms of this bacterium, since ROS constitute such an essential threat to the pathogen. As aforementioned, antioxidant enzymes, such as catalase, AhpC, SodC, and SodB, all contribute to the virulence of *F. tularensis* in mice, although each appears to render the bacterium only moderately attenuated and this indicates that the antioxidant system of *F. tularensis* is complex and may in part possess overlapping functions (Lindgren et al., 2007; Ma et al., 2016). Indeed, a double deletion mutant of *katG* and *ahpC* has not been possible to generate in *F. tularensis* (Binesse et al., 2015) and this demonstrates that the cooperative functions of these enzymes are crucial, although either one is not essential. The aim of the present study was to better understand this interconnecting web of antioxidants in *F. tularensis*. To this end, a double deletion mutant, $\Delta oxyR/\Delta katG$, was generated since this mutant, besides lack of catalase activity, should have a repressed expression of OxyR-regulated antioxidant genes, one of which is AhpC (Ma et al., 2016). We hypothesized that the lack of both KatG and OxyR would lead to a severely impaired phenotype of *F. tularensis* LVS. We therefore characterized the phenotypes of single deletion mutants, $\Delta oxyR$ and $\Delta katG$, and a double deletion mutant, $\Delta oxyR/\Delta katG$, in comparison to the parental LVS strain.

MATERIALS AND METHODS

Bacterial Strains

The *F. tularensis* LVS strain was obtained from the *Francisella* strain collection (FSC) at FOI, Swedish Defense Research Agency. The *katG* deletion mutant ($\Delta katG$) has been described previously (Lindgren et al., 2007).

The $\Delta oxyR$ and $\Delta oxyR/\Delta katG$ mutants of the LVS strain were generated by allelic replacement as described previously (Golovliov et al., 2003). Briefly, sequences up- and down-stream of *oxyR* were amplified by PCR. The fragments contained complementary sequences, which were joined together by a second PCR. The resulting fragment was cloned into the pDM4 suicide-vector, which was transformed into *Escherichia coli* S17- λ pir and thereafter transferred to LVS by conjugation. Clones with a successful recombination event were selected on plates supplemented with Cm and polymyxin B. Correct integration was confirmed by PCR. Positive clones were subjected to sucrose

selection to select for a second recombination event and clones were screened by PCR to identify successful deletion mutants. The double deletion mutant $\Delta oxyR/\Delta katG$ was generated using the same procedure, apart from using the pDMK3 plasmid carrying kanamycin resistance. The deletions were verified by sequencing 1500 bp on each side of the deleted region.

Aerobic and Microaerobic Growth

Bacteria were cultivated overnight on plates based on modified GC-agar (MC plates) and then inoculated to an OD₆₀₀ of 0.1 in Chamberlain's chemically defined medium (CDM). All cultures were split into triplicates and were incubated at 37°C and 200 rpm in an aerobic (normal air) or a microaerobic (10% O₂ and 10% CO₂) milieu up to 48 h with monitoring of the OD₆₀₀.

H₂O₂ Susceptibility Assay

Bacteria were cultivated overnight on MC plates, inoculated to an OD₆₀₀ of 0.1 in CDM and H₂O₂ was added to the final concentration of 0.02, 0.1, or 0.5 mM, respectively. Controls were grown without the addition of H₂O₂. All cultures were split into triplicates and were incubated at 37°C and 200 rpm up to 24 h with monitoring of the OD₆₀₀.

Catalase Activity Assay

Catalase degrades H₂O₂ to O₂ and H₂O. H₂O₂ absorbs light at 240 nm and degradation of H₂O₂ can therefore be measured as a reduction of A₂₄₀ nm over time.

Strains were cultivated overnight after being diluted to an OD₆₀₀ of 0.1 in CDM. For each strain, one set of tubes were left untreated and another set of tubes were supplemented with H₂O₂ to a final concentration of 0.02, 0.1, or 0.2 mM. All cultures were split into triplicates and incubated at 37°C, 200 rpm for 2, 4, and 24 h before sampling for evaluation of catalase activity. Depending on the density and growth phase of the culture, a volume of 10–50 µl were withdrawn and diluted in PBS to reach a final volume of 120 µl in UV-clear 96-well plates (Greiner Bio-one, Frickenhausen, Germany). Then, 80 µl 100 mM H₂O₂ in PBS was added to each sample immediately before placing the plate in a Tecan Infinite 200 pro plate reader and measuring the reduction in absorption at 240 nm for 10 min. A molar extinction coefficient of H₂O₂ at 240 nm of 43.6 M⁻¹cm⁻¹ was used to calculate the concentration of H₂O₂ using the Beer-Lambert law, $A = \epsilon cl$. One unit of catalase is defined as the amount that decomposes 1 µmol of H₂O₂ per minute per OD₆₀₀ at 25°C. The catalase units were normalized against the OD of the culture.

Paraquat Susceptibility Assay

Susceptibility of *F. tularensis* strains to O₂⁻ was determined by use of the O₂⁻ generating compound paraquat dichloride hydrate (Sigma-Aldrich, St. Louis, USA) in a disc diffusion assay. Paraquat generates O₂⁻ through reacting with parts of the respiratory chain in bacteria, causing the reduction of O₂ to O₂⁻ (Hassan and Fridovich, 1979). Bacterial strains were cultivated on MC plates overnight, re-suspended in phosphate-buffered saline (PBS) and approximately 3 × 10⁵ CFU were plated onto MC plates. Sterile filter discs (Oxoid Blank Antimicrobial Susceptibility Discs, Thermo Scientific, MA, USA) were placed

in the center the plates once they had dried, and 10 µl of MQ-water, 1.25 mM, 5 mM or 20 mM paraquat solution was added to each disc. The plates were incubated for 4 days at 37°C, 5% CO₂ before the size of the growth inhibition zone surrounding each disc was determined.

Peroxyntirite Susceptibility Assay

3-morpholiniosydnonimine hydrochloride (SIN-1) (Molecular Probes, Oregon, USA) spontaneously releases (NO) and O₂⁻ under physiological conditions, thereby generating peroxyntirite (ONOO⁻). Under physiological conditions 1 mM SIN-1 generates 10 µM ONOO⁻/min (Lindgren et al., 2007).

Strains were cultivated in CDM to logarithmic growth phase and diluted to a density of approximately 2 × 10⁶ bacteria/ml in PBS. The bacterial suspensions were incubated with or without the addition of 0.48 mM SIN-1 with equal amounts of SIN-1 added at the start of the experiment and again after 1.5 h to ensure stable levels of ONOO⁻ (Lindgren et al., 2005). After 3 h samples were collected, diluted and plated on MC plates for determination of viable bacteria.

Analysis of Gene Expression by Real Time PCR

Bacteria were cultivated overnight on MC plates, inoculated to an OD₆₀₀ of 0.1 in CDM and incubated at 37°C, 5% CO₂ for 10 h before sampling. RNA extraction, cDNA synthesis and Real Time PCR (RT-PCR) were all performed as described previously (Honn et al., 2012).

Briefly, RNA was extracted using Trizol reagent (Invitrogen, CA, USA) from pelleted bacteria, 3 × 10⁹ CFU/sample. Contaminating DNA was removed using the DNA-free kit (Ambion, Inc, Austin, TX, USA) and RNA was quantified by Nanodrop (Thermo Fisher Scientific, Wilmington, DE, USA). cDNA was synthesized from 1 µg RNA/sample using iScript (BioRad, Hemel, Hempstead, UK), RT-PCR was performed using the Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the ABI Prism 7900Ht Sequence Detection System (Applied Biosystems) as described (Honn et al., 2012). Trizol, DNA-free, iScript and Power SYBR green were all used in accordance with the instructions provided by the manufacturers. Forward and reverse primers were obtained from Invitrogen and have been published previously for *fslA* (FTL_1832), *fslB* (FTL_1833), *fslC* (FTL_1834), *fslD* (FTL_1835), *fslE* (FTL_1836), *fupA* (FTL_0439), *furA* (FTL_1831), (Lindgren et al., 2009), *tul4*, *iglC* (FTL_0113), (Bröms et al., 2009), *mglA* (FTL_0260), *feoB* (FTL_0133), and *katG* (FTL_1504) (Honn et al., 2012), sequences for, *grxA* (FTL_0985), *grxB* (FTL_1792), *gpx* (FTL_1383), *sspA* (FTL_1606), *ahpC1* (FTL_0542), *ahpC2* (FTL_1191), *sodB* (FTL_0380), *sodC* (FTL_1791), *clpB* (FTL_0094), *groES* (FTL_1715), *groEL* (FTL_1714), and *dnaK* (FTL_1191) are available upon request.

The Ct values of the selected genes were normalized to the Ct value of the house keeping gene *FTT0901* (*lpnA*) and relative copy numbers (RCN) were calculated according to the following equation: $RCN = 2^{-\Delta Ct} \times 100$, where ΔCt is Ct(target)–Ct(*FTT0901*) (Gavrilin et al., 2006). Thus, the copy

number of a given gene is related to the copy number of *FTT0901*. Normalized Ct values were used for statistical evaluation of the data by One way ANOVA followed by Tukey's honest significant difference (HSD).

Preparation and Infection of BMDM

The capacity of LVS and the mutants to proliferate intracellularly were assessed in bone marrow-derived macrophages (BMDMs). BMDMs were generated from C57BL/6 mice essentially as described previously (Bröms et al., 2011).

The day before infection, BMDM cells were seeded at a density of 4×10^5 cells/ml in 24-well tissue-culture plates and incubated at 37°C, 5% CO₂ with or without murine recombinant 1000 U/ml of IFN- γ (PeproTech, Rocky Hill, NJ, USA). The next day, the cells were washed and reconstituted with fresh, pre-warmed culture media. Bacteria were grown overnight on MC plates and re-suspended in PBS to a density of approximately 3×10^9 bacteria/ml. Bacteria were diluted in DMEM and added to each well at multiplicity of infection of 30 and bacterial uptake was allowed to occur for 90 min at 37°C, 5% CO₂. Remaining extracellular bacteria were removed by rinsing the monolayers three times with DMEM and incubating with gentamicin for 45 min followed by rinsing the monolayers three times. This time-point was defined as 0 h. After 0, 4 and 24 h incubation the macrophages were lysed in 0.1% deoxycholate in PBS. The lysate were serially diluted in PBS and plated on MC plates for determination of viable bacteria.

Mouse Experiments

Virulence of the mutant strains was determined by subcutaneous infection of female C57BL/6 mice with 4×10^3 CFU/mouse of LVS, $\Delta oxyR$, $\Delta katG$, and $\Delta oxyR/\Delta katG$. Mice were monitored for signs of illness and were euthanized by inhalation of isoflurane followed by CO₂ asphyxiation after 3 or 6 days, whereupon the number of viable bacteria in spleens and livers were determined by homogenizing the organs in PBS and plating dilutions on MC plates. All animal experiments were approved by the Local Ethical Committee on Laboratory Animals, Umeå, Sweden (no. A 1-09, A 99-11, and A 67-14).

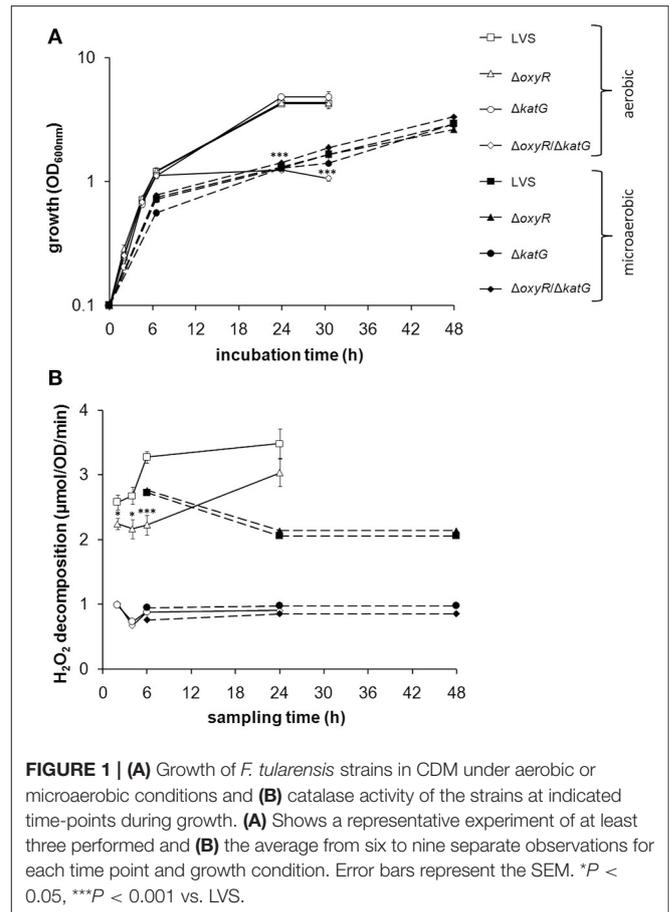
Statistical Analysis

One way ANOVA followed by Tukey's HSD test was used to determine statistical significant difference between groups.

RESULTS

Growth under Aerobic vs. Microaerobic Conditions

CDM effectively supports growth of LVS. We therefore compared growth of the bacterial strains, LVS, $\Delta oxyR$, $\Delta katG$, and $\Delta oxyR/\Delta katG$. The former three strains all replicated to the same extent, whereas $\Delta oxyR/\Delta katG$ showed intact growth to late log phase, but impaired growth thereafter. Therefore, it did not reach as high densities as LVS and the other strains at 24 h ($P < 0.001$; **Figure 1A**). To explore if a reduced oxygen tension could rescue the growth of $\Delta oxyR/\Delta katG$, the strains were cultivated under microaerobic conditions, i.e., 10% O₂ and



10% CO₂. Indeed, $\Delta oxyR/\Delta katG$ grew as well as the other strains and reached an optical density of > 2.0 within 48 h (**Figure 1A**). As noted before (Honn et al., 2012), the growth rate of LVS under microaerobic conditions was reduced compared to aerobic conditions (**Figure 1A**).

Catalase Activity under Aerobic vs. Microaerobic Conditions

The results so far suggested that LVS experienced oxidative stress during growth in an aerobic environment and to handle this stress, required either the function of catalase, or the expression of OxyR-regulated detoxifying mechanisms. OxyR is known to respond to oxidative stress by inducing antioxidant enzymes, such as catalase. As an indicator of oxidative stress and to investigate if catalase is under the regulation of *oxyR* in LVS, we measured the activity of the enzyme during growth of the bacteria in CDM. The catalase activity in LVS gradually increased during the two to 24 h period, whereas the catalase activity in $\Delta oxyR$ was sustained at a constant, but lower level compared to LVS from two to six h ($P < 0.05$ at 2 and 4 h and $P < 0.001$ at 6 h; **Figure 1B**). However, the catalase activity of the two strains was similar at 24 h (**Figure 1B**). In the microaerobic environment, the catalase activity of LVS and $\Delta oxyR$ was similar, but for both lower than in the aerobic environment (**Figure 1B**). The H₂O₂

decomposition in samples containing $\Delta katG$ or $\Delta oxyR/\Delta katG$ was below $1 \mu\text{mol}$, regardless of growth condition and time point, indicating the absence of catalase activity (**Figure 1B**).

In summary, $\Delta oxyR$ demonstrated a basal catalase activity, but did not induce this activity further during the aerobic logarithmic growth phase as LVS did. $\Delta oxyR/\Delta katG$, which lacks this basal catalase activity, failed to grow to high densities under the aerobic condition, but grew as well as LVS in the microaerobic milieu.

H₂O₂ Tolerance

$\Delta oxyR$ and $\Delta katG$ grew as well as LVS in CDM despite the reduced, or lack of catalase activity (**Figure 2A**). To investigate their adaptation to stress, H₂O₂, the substrate of catalase, was added to the cultures. Growth of LVS or $\Delta oxyR$ was not affected by 0.02 mM H₂O₂, whereas, initially, the growth rate of $\Delta katG$ was reduced ($P < 0.01$) and growth of $\Delta oxyR/\Delta katG$ almost completely inhibited ($P < 0.001$; **Figure 2B**). At 0.1 mM of H₂O₂, LVS and $\Delta oxyR$ still grew rapidly, in contrast to $\Delta katG$ and $\Delta oxyR/\Delta katG$ that did not grow at all ($P < 0.001$; **Figure 2C**). Growth of $\Delta oxyR$ was significantly reduced in the presence of 0.5 mM of H₂O₂ compared to LVS ($P < 0.001$; **Figure 2D**). Exposure

of the strains to H₂O₂ did not significantly change their catalase activity (data not shown).

In summary, the mutant strains displayed increased susceptibility to H₂O₂ as compared to LVS, with the effect being most pronounced for $\Delta oxyR/\Delta katG$, followed by $\Delta katG$, and the least affected strain being $\Delta oxyR$.

Susceptibility to Paraquat-Mediated Killing

O₂⁻ is continuously generated as a by-product of the respiratory chain during growth of bacteria. To investigate the capacity of the bacteria to defend against such ROS, LVS, $\Delta oxyR$, $\Delta katG$, and $\Delta oxyR/\Delta katG$ were exposed to paraquat in a disc diffusion assay (**Figure 3**). Paraquat dichloride hydrate generates O₂⁻ through a reaction with parts of the respiratory chain in bacteria, causing the reduction of O₂ to O₂⁻ (Hassan and Fridovich, 1979). $\Delta oxyR$ displayed a significantly larger zone of inhibition than did LVS in the presence of 1.25 and 5 mM paraquat ($P < 0.001$ and 0.01, respectively), but the zones were similar when exposed to 20 mM (**Figure 3**). The zone of inhibition for $\Delta katG$ was larger compared to LVS at 1.25 mM ($P < 0.05$), but similar at the two higher concentrations (**Figure 3**). A significantly larger zone of inhibition was observed for $\Delta oxyR/\Delta katG$ vs. LVS and $\Delta katG$ at

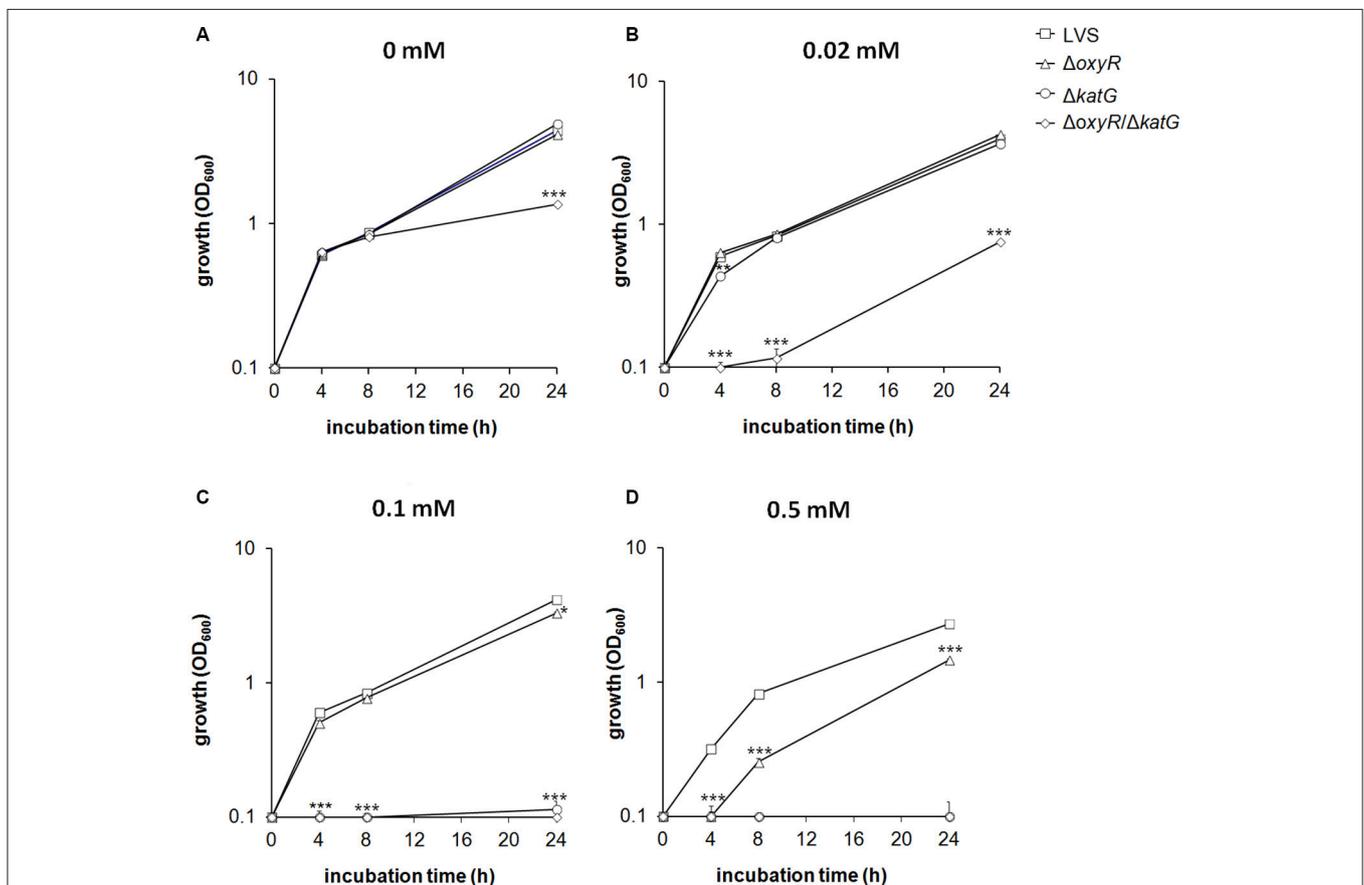


FIGURE 2 | Growth of *F. tularensis* strains in CDM during exposure to various concentrations of H₂O₂ (A) 0 mM, (B) 0.02 mM, (C) 0.1 mM, and (D) 0.5 mM. The results shown illustrate one representative experiment of at least three performed. Each value represents the average for triplicate samples and error bars represent the SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. LVS.

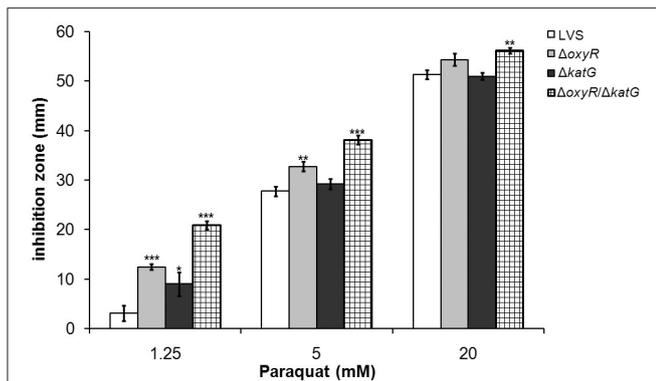


FIGURE 3 | *F. tularensis* strains were exposed to the O_2^- -generating compound paraquat in a disc diffusion assay. Each bar represents the average from three separate experiments with triplicate samples in each and error bars represent the SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. LVS for each concentration.

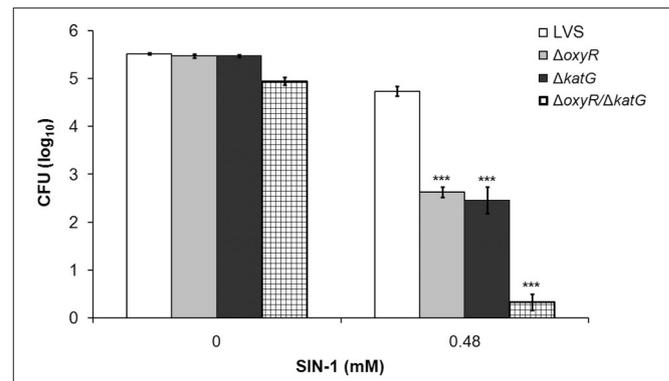


FIGURE 4 | *F. tularensis* strains were exposed to the peroxynitrite generating compound SIN-1 for 3 h. After 1.5 h of incubation, additional SIN-1 was added to the tubes to ensure a constant generation of peroxynitrite during the whole incubation period. Each bar represents the average from three separate experiments with triplicate samples in each and error bars represent the SEM. *** $P < 0.001$ vs. LVS.

all three concentrations of paraquat ($P < 0.001$ for 1.25 and 5 mM and $P < 0.01$ for 20 mM) and also larger compared to $\Delta oxyR$ at 1.25 and 5 mM ($P < 0.01$; **Figure 3**).

In summary, the results demonstrated that $\Delta oxyR$ and $\Delta oxyR/\Delta katG$ were more susceptible to paraquat-mediated killing compared to LVS, with $\Delta oxyR/\Delta katG$ being the most susceptible, whereas $\Delta katG$ was only slightly more susceptible than LVS.

Susceptibility to SIN-1-Mediated Killing

Peroxyntirite ($ONOO^-$) is a highly reactive and bactericidal ROS formed through the reaction between (NO) and O_2^- and it is active against *F. tularensis* in activated macrophages (Lindgren et al., 2005). Experimentally, SIN-1 can be used to mimic a continuous exposure to $ONOO^-$. SIN-1 slowly decomposes, thereby releasing both NO and O_2^- that combine to form $ONOO^-$, which quickly is internalized since it passes through lipid bilayers (Hogg et al., 1992; Murphy et al., 1998).

The exposure to 0.48 mM SIN-1 for 3 h reduced the viability of all strains in comparison to un-treated cultures ($P < 0.001$ for all strains), but affected the mutant strains to a greater extent compared to LVS ($P < 0.001$ vs. LVS for all; **Figure 4**). The viability of LVS decreased approximately 0.8 \log_{10} , of $\Delta oxyR$ 2.8 \log_{10} , of $\Delta katG$ 3.0 \log_{10} , and of $\Delta oxyR/\Delta katG$ 4.6 \log_{10} CFU. The latter was significantly more susceptible than any of the other strains ($P < 0.001$; **Figure 4**).

In summary, all mutant strains displayed increased susceptibility to $ONOO^-$ as compared to LVS, with the effect being similar for $\Delta oxyR$ and $\Delta katG$ and most pronounced for $\Delta oxyR/\Delta katG$.

Gene Expression

$\Delta oxyR/\Delta katG$ did not grow after the late logarithmic growth phase (**Figure 1A**), and we therefore found it of interest to explore the gene expression of the strains at 10 h, i.e., during the late logarithmic growth phase. The analysis was focused on genes expressing proteins influencing the oxidative stress response of

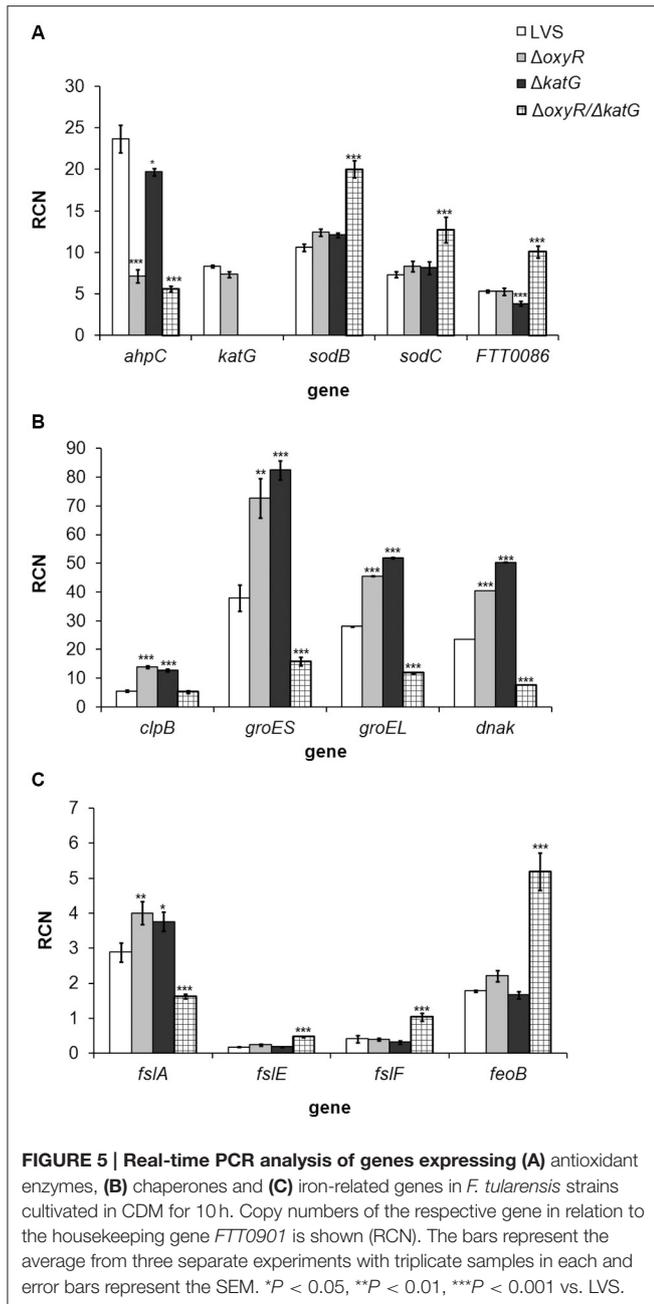
the bacterium, such as antioxidant enzymes, chaperones and iron-related proteins. Genes found to be differentially expressed vs. LVS are shown in **Figure 5**. Of all genes examined, *ahpC* was the sole gene significantly repressed in $\Delta oxyR$ ($P < 0.001$; **Figure 5A**). A similar degree of repression, about 3-fold, was observed in $\Delta oxyR/\Delta katG$, which in addition, had a 1.5 to 2-fold increased expression of *sodB*, *sodC* and *FTT0086* ($P < 0.001$ for all genes; **Figure 5A**). *ahpC* was not repressed in $\Delta katG$ and as expected, *katG* transcripts were not detected in either $\Delta katG$ or in $\Delta oxyR/\Delta katG$ (**Figure 5A**). All chaperone genes examined were upregulated 1.6 to 2.5-fold in $\Delta oxyR$ and $\Delta katG$ ($P < 0.001$ for all genes; **Figure 5B**). In contrast, these genes, except for *clpB*, were suppressed 2.4 to 3.1-fold in $\Delta oxyR/\Delta katG$ ($P < 0.001$ for all genes).

fslA, the first gene of the siderophore operon, was slightly up-regulated in $\Delta oxyR$ and $\Delta katG$, although only about 1.2-fold, whereas the other iron-related genes were expressed at similar levels as in LVS. In contrast, *fslA* was suppressed 1.8-fold in $\Delta oxyR/\Delta katG$ and *fslE*, *fslF* and *feoB* were upregulated 2.5 to 2.9-fold ($P < 0.001$; **Figure 5C**).

In summary, the absence of OxyR resulted in a suppressed expression of *ahpC* and an up-regulated expression of genes encoding chaperone proteins. Except for *ahpC*, the expression profile of $\Delta katG$ was similar to $\Delta oxyR$. In contrast, loss of both *oxyR* and *katG* changed the expression profile and low expression of chaperone-encoding genes was observed in $\Delta oxyR/\Delta katG$, together with high expression of antioxidant genes, except for *ahpC* and *katG*, and an altered expression of genes related to iron-uptake.

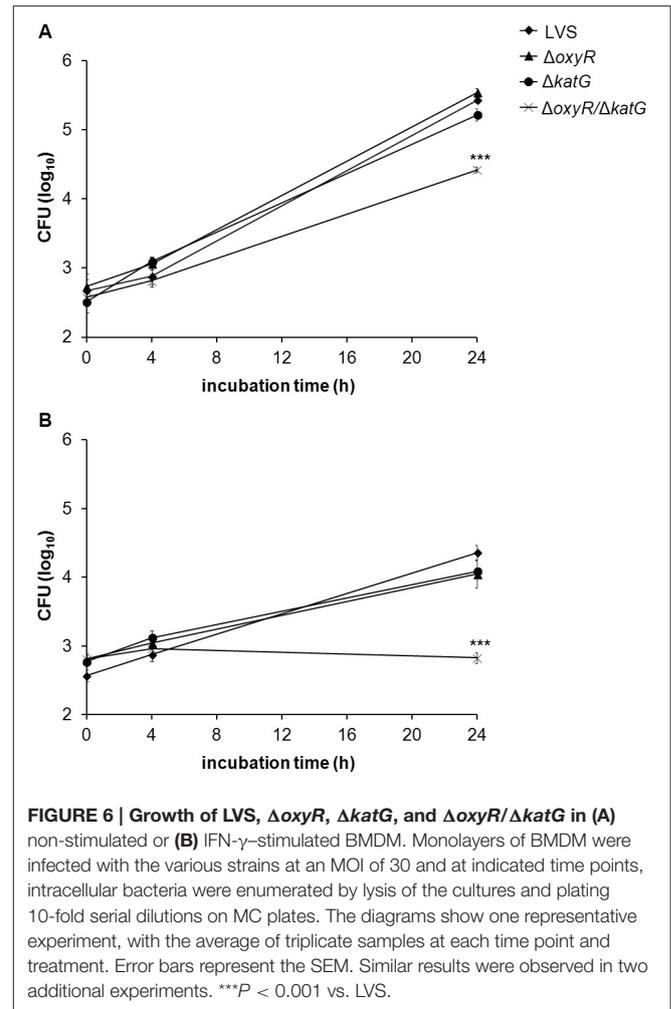
Intracellular Replication in BMDM

Based on the increased susceptibility to various ROS displayed by $\Delta oxyR$, $\Delta katG$, and $\Delta oxyR/\Delta katG$, it was of interest to test whether the strains were defective for replication in professional phagocytes. Non-stimulated or IFN- γ -stimulated BMDMs were infected with LVS, $\Delta oxyR$, $\Delta katG$, or $\Delta oxyR/\Delta katG$ at an MOI



of 30, and the viability of internalized bacteria was determined after 0 h, 4 h, and 24 h. In non-stimulated BMDM, LVS grew from approximately $2.5 \log_{10}$ CFU to more than $5.0 \log_{10}$ CFU within 24 h and also $\Delta oxyR$ and $\Delta katG$ grew to similar extent (Figure 6A). $\Delta oxyR/\Delta katG$ grew in non-stimulated cells, but reached approximately 10-fold lower numbers compared to the other strains after 24 h ($P < 0.001$; Figure 6A).

IFN- γ -stimulation of BMDM prior to infection reduced the numbers of LVS, $\Delta katG$, and $\Delta oxyR$ about 10-fold at 24 h vs. the numbers in non-stimulated cultures ($P < 0.001$; Figures 6A,B). There was no growth of $\Delta oxyR/\Delta katG$ in IFN- γ -stimulated cultures and, thus, significantly lower bacterial

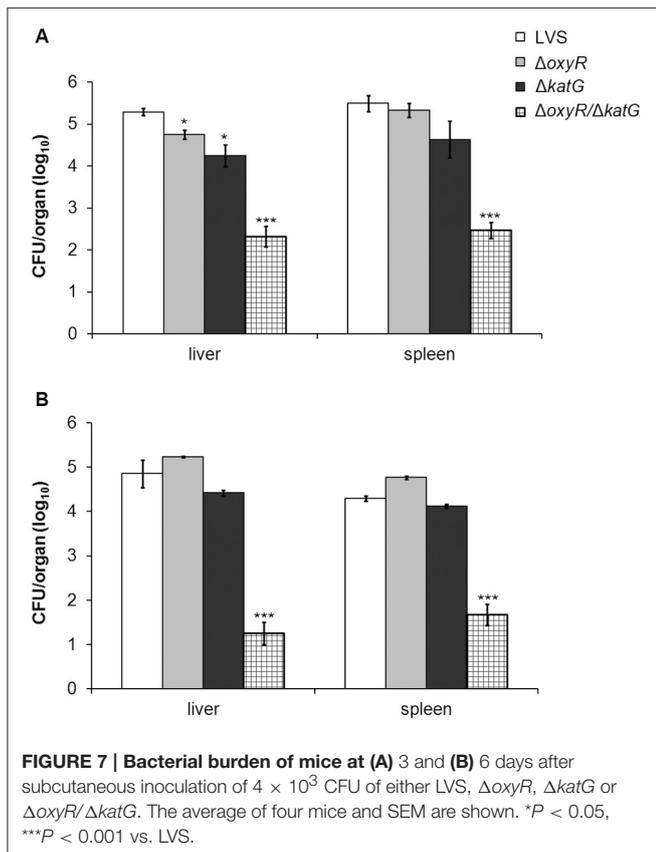


numbers compared to non-stimulated cultures at 24 h ($P < 0.001$; Figures 6A,B) and vs. all the other strains exposed to IFN- γ ($P < 0.001$; Figure 6B).

Thus, the $\Delta oxyR$ and $\Delta katG$ mutants showed intact capacity of intracellular replication, whereas the $\Delta oxyR/\Delta katG$ mutant showed impaired replication in BMDM, both in the presence and absence of IFN- γ .

Virulence in Mice

The virulence of LVS, $\Delta oxyR$, $\Delta katG$, and $\Delta oxyR/\Delta katG$ was determined by subcutaneous infection of C57BL/6 mice with 4×10^3 CFU/mouse, a non-lethal dose, and enumeration of viable bacteria in spleen and liver on day 3 and 6 of infection. Compared to LVS, there were lower numbers of both $\Delta oxyR$ and $\Delta katG$ on day 3 in the liver of the mice ($P < 0.05$; Figure 7A), whereas there were no differences between these strains in either the liver or spleen at the other time points (Figures 7A,B). Numbers of $\Delta oxyR/\Delta katG$ in both organs were at least 100-fold lower vs. all other strains at both time points ($P < 0.001$). Thus, both $\Delta oxyR$ and $\Delta katG$ showed slight attenuation in mice, whereas $\Delta oxyR/\Delta katG$ was highly attenuated.



DISCUSSION

Francisella tularensis is a versatile bacterium capable of surviving in many different hosts, vectors and in various cell types, including the normally bactericidal macrophages. Upon phagocytosis, *F. tularensis* is encased in a phagosome, a membrane-bound compartment designed for the annihilation of phagocytosed microbes, which is rich in antimicrobial molecules, such as reactive oxygen and nitrogen species. Although *F. tularensis* only transiently resides in this compartment, it must still muster defenses against highly reactive species in order to survive and escape to the cytosol, where it proceeds to replicate. By entering the cytosol, *F. tularensis* gains access to a nutrient-rich, protected niche in which it multiplies. As survival and replication in the intracellular niche is essential for the life cycle of *F. tularensis*, a thorough understanding of how the bacterium survives intracellularly is essential to fully grasp its defense mechanisms against oxidative stress. To this end, the study focused on understanding the interplay between catalase and OxyR, the latter being important for the expression of several antioxidant enzymes, in the defense against ROS and their impact on the survival of the bacterium in professional phagocytes.

To investigate if OxyR is involved in the oxidative stress response of LVS, we constructed an in-frame deletion of *oxyR*. A similar investigation has been performed recently by Ma et al. which studied the role of OxyR in LVS (Ma et al., 2016).

It was found that OxyR controlled transcription of *katG* and the findings agree with the reduced catalase activity of $\Delta oxyR$ observed in the present study. Nevertheless, our study revealed that even in the absence of OxyR, there was still prominent catalase activity. Overall, it appears that OxyR, as expected, regulates *katG* in the LVS strain, however, the regulation does not completely abolish its expression as is the case observed for various other bacterial species, e.g., *E. coli* (Michán et al., 1999), *Salmonella enterica* (Morgan et al., 1986), *Haemophilus influenza* (Whitby et al., 2012), or *Moraxella catarrhalis* (Hoopman et al., 2011). In both the present study, and in the previous study, it was observed that the lack of OxyR led to marked suppression of *ahpC2* (Ma et al., 2016). In addition Ma et al. demonstrated suppressed expression of both *katG* and *sodB* in $\Delta oxyR$ by real-time PCR and demonstrated that OxyR binds to the upstream promoter regions of each gene. In contrast, there was no down-regulation of *katG* or *sodB* observed in the present study. Likely, this is a consequence of the rapid on/off switch of the promoter binding capacity of OxyR in response to the oxidative levels in the bacteria leading to a limited window when elevated mRNA levels can be detected (Wei et al., 2012).

Besides antioxidant genes, our study revealed an aberrant expression of genes encoding chaperone proteins of the mutants. Such proteins are induced in response to various stresses, including oxidative stress (Hartl et al., 2011). Thus, the induced expression of these genes in $\Delta oxyR$ and in $\Delta katG$, also observed by Ma et al. (2016), likely is a reflection of oxidative stress encountered by the mutants. The chaperone network likely helps the bacterium to handle this stress through unfolding and/or degradation of mis-folded/damaged proteins. The reason behind the suppressed expression of multiple chaperone genes in $\Delta oxyR/\Delta katG$ is obscure, but should lead to an accumulation of damaged or mis-folded proteins and may explain why it was so impaired for growth in broth. The intact growth of $\Delta oxyR/\Delta katG$ under microaerobic conditions likely reflects that reduced levels of ROS are formed and therefore that antioxidant defenses are less important. The aberrant expression of genes related to iron-uptake did not result in a skewed iron content of $\Delta oxyR/\Delta katG$ (data not shown) and it is therefore not obvious that this would influence the susceptibility of the strain to various ROS.

The *F. tularensis* *ahpC2* gene is divergently transcribed from the *oxyR* promoter, a feature commonly seen for genes transcriptionally regulated by OxyR (Hahn et al., 2002; Maddocks and Oyston, 2008). AhpC belongs to the peroxiredoxin family, which is ubiquitously found in nature (Rhee et al., 2005) and is known to be involved in defenses against peroxides in *E. coli* (Storz et al., 1989), and both peroxides and peroxynitrite in, e.g., *Salmonella typhimurium* (Bryk et al., 2000), and in the defense against superoxide and peroxynitrite in the virulent SCHU S4 strain of *F. tularensis* subsp. *tularensis* (Binesse et al., 2015). In agreement with this, and in view of the reduced expression of AhpC in $\Delta oxyR$, this mutant was also highly susceptible to ONOO⁻. $\Delta katG$ was as susceptible as $\Delta oxyR$ to ONOO⁻ and in view of the substantial catalase activity remaining in $\Delta oxyR$, this result implies that the function of catalase overlaps with other OxyR-regulated detoxifying mechanisms, presumably AhpC, to

protect against ONOO⁻. Further corroborating the importance of AhpC and catalase was the failure to generate a *katG* and *ahpC* double deletion mutant and even an *ahpC* mutant in LVS. Hence, AhpC seems indispensable to LVS, which is in stark contrast to SCHU S4, where deletion of *ahpC* resulted in only slight attenuation (Binesse et al., 2015). This indicates that there is a disparity regarding the importance of the enzyme between the SCHU S4 and LVS strains, possibly a factor that to some extent explains the difference in virulence between the strains, since it implies that the detoxifying mechanisms of SCHU S4 are much more elaborate. Nevertheless, as for LVS, it has not been possible to generate a *katG* and *ahpC* double deletion mutant of SCHU S4 (Kadzhaev et al., 2009; Binesse et al., 2015). Collectively, this indicates that the mechanisms of protection conferred by these enzymes may be overlapping and the lack of both is detrimental to the survival of both LVS and highly virulent *F. tularensis* strains.

Based on the failure to generate a *katG* and *ahpC* double deletion mutant and the marked suppression of *ahpC* in the Δ *oxyR* mutant, we hypothesized that the absence of OxyR together with the absence of catalase would severely disarm the capability of the bacterium to handle ROS. Indeed, we observed that the Δ *oxyR*/ Δ *katG* mutant was hyper-susceptible to H₂O₂, ONOO⁻, and O₂⁻; much more so than either Δ *oxyR* or Δ *katG*. Collectively, the results demonstrate that the roles of OxyR-regulated antioxidant enzymes and catalase overlap to protect LVS against various ROS. We find it likely that the reduced activity of catalase and expression of *ahpC* observed in *oxyR* contributed to the increased susceptibility of the mutant to H₂O₂, O₂⁻, and ONOO⁻ through the increase of both Fenton-mediated toxicity and direct O₂⁻- and ONOO⁻-mediated damage. We further suggest that the reduced levels of AhpC together with the lack of catalase in the Δ *oxyR*/ Δ *katG* strain, despite an increased expression of *sodB*, *sodC* and *FTT0086*, resulted in enhanced Fenton-mediated toxicity and ONOO⁻-mediated damage, which likely account for the extreme susceptibility of the double mutant to O₂⁻, H₂O₂, and ONOO⁻. Our findings concur with those of Ma et al. (2016), and, in addition, demonstrate that the combined activity of catalase and OxyR-regulated detoxifying mechanisms are critical for ROS detoxification by *F. tularensis*.

Despite the enhanced susceptibility of both Δ *oxyR* and Δ *katG* to various ROS, the strains replicated as efficiently as LVS in mouse BMDM, but importantly, the capacity to replicate in professional phagocytes required either OxyR or catalase, since Δ *oxyR*/ Δ *katG* failed to replicate. IFN- γ -activation of BMDM restricted growth of LVS, Δ *katG*, and Δ *oxyR* to a similar degree and completely blocked the growth of Δ *oxyR*/ Δ *katG*. The majority of *F. tularensis* LVS escapes the phagosome of IFN- γ -activated macrophages (Lindgren et al., 2004), but the mechanism of growth inhibition appears to vary depending on the cell model used (Edwards et al., 2010). IFN- γ -mediated inhibition of intracellular growth of *F. novicida* is dependent on the expression of IRGB10 and various guanylate-binding proteins (Meunier et al., 2015; Man et al., 2016), however, the role of this pathway is unknown for other *F. tularensis* species.

Our results reveal elaborate interconnecting roles between OxyR-regulated ROS-detoxifying mechanisms and catalase and demonstrate that either needs to be intact for the bacterium to thrive in professional phagocytes. The roles of the anti-oxidative mechanisms could be to protect the bacterium from direct damage by various ROS, such as ONOO⁻, which has been demonstrated to be crucial for killing of *F. tularensis* in peritoneal cells (Lindgren et al., 2005). Alternatively, or additionally, the antioxidants may restrict macrophage activation through their ability to preserve phosphatase activity required for kinase signaling and proinflammatory cytokine production (Melillo et al., 2010).

Our finding that Δ *oxyR* replicated as efficiently as LVS in BMDM is in contrast to findings in a previous study, which reported that an *oxyR* mutant of LVS was markedly impaired with regard to escape from the phagosome, replication in professional phagocytes, and virulence in the mouse model (Ma et al., 2016). Notably, the LVS strain used by Ma et al. replicated less than 10-fold during 24 h in C57BL/6 BMDM, whereas the LVS strain used in the present study replicated about 500-fold. Isolates of LVS with different virulence are used in the research community (Griffin et al., 2015) and the distinct differences in the intracellular growth of these two LVS strains are additional examples of such distinct phenotypes. The phenotypic differences between the two LVS strains likely explain the discrepant findings of the two studies. The observation in the present study of the intact growth of the single mutants in BMDM was corroborated by findings *in vivo*, since the Δ *oxyR* and Δ *katG* mutants showed essentially intact growth in organs of mice, whereas Δ *oxyR*/ Δ *katG* hardly grew at all. Despite their effective growth in the organs, a previous study demonstrated a more distinct growth defect of the Δ *katG* mutant, most likely because a 100-fold higher dose was given (Lindgren et al., 2007). Moreover, by the intranasal route, Δ *oxyR* was demonstrated to be moderately attenuated (Ma et al.). Based on these collective findings, it can be concluded that both OxyR and KatG contribute to the virulence of *F. tularensis* LVS and that the concomitant loss is detrimental to the virulence of the bacterium.

Altogether, the results presented in this study clearly demonstrate the mutual importance of catalase and OxyR for a robust oxidative stress defense system and that either of these systems is vital for the intracellular replication of *F. tularensis* and for its virulence.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AS, MH, and HL. Performed the experiments: MH, HL, and GB. Analyzed the data: AS, MH, HL, and GB. Wrote the paper: HL, MH, and AS.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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