

Roles of Reactive Oxygen Species-Degrading Enzymes of *Francisella tularensis* SCHU S4

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***Francisella tularensis* is a facultative intracellular bacterium utilizing macrophages as its primary intracellular habitat and is therefore highly capable of resisting the effects of reactive oxygen species (ROS), potent mediators of the bactericidal activity of macrophages. We investigated the roles of enzymes presumed to be important for protection against ROS. Four mutants of the highly virulent SCHU S4 strain with deletions of the genes encoding catalase (*katG*), glutathione peroxidase (*gpx*), a DyP-type peroxidase (*FTT0086*), or double deletion of *FTT0086* and *katG* showed much increased susceptibility to hydrogen peroxide (H₂O₂) and slightly increased susceptibility to paraquat but not to peroxynitrite (ONOO⁻) and displayed intact intramacrophage replication. Nevertheless, mice infected with the double deletion mutant showed significantly longer survival than SCHU S4-infected mice. Unlike the aforementioned mutants, deletion of the gene coding for alkyl-hydroperoxide reductase subunit C (*ahpC*) generated a mutant much more susceptible to paraquat and ONOO⁻ but not to H₂O₂. It showed intact replication in J774 cells but impaired replication in bone marrow-derived macrophages and in internal organs of mice. The live vaccine strain, LVS, is more susceptible than virulent strains to ROS-mediated killing and possesses a truncated form of *FTT0086*. Expression of the SCHU S4 *FTT0086* gene rendered LVS more resistant to H₂O₂, which demonstrates that the SCHU S4 strain possesses additional detoxifying mechanisms. Collectively, the results demonstrate that SCHU S4 ROS-detoxifying enzymes have overlapping functions, and therefore, deletion of one or the other does not critically impair the intracellular replication or virulence, although *AhpC* appears to have a unique function.**

Francisella tularensis is a highly virulent facultative intracellular pathogen that causes tularemia in humans and many other mammalian species. Two *F. tularensis* subspecies are of clinical importance: *tularensis* (type A) and *holarctica* (type B). Strains of both subspecies are highly infectious since inoculation of as few as 10 CFU is sufficient to cause disease in humans (1); however, strains of *F. tularensis* subsp. *tularensis* show distinctly higher virulence for humans and cause high mortality if the infection is untreated. Therefore, the bacterium is classified as a tier 1 select agent (i.e., a category containing the most likely bioterrorist agents) (2). Experimentally, the live vaccine strain (LVS) of *F. tularensis* is widely used as a model for virulent strains of the species. It was derived from a type B strain in the former Soviet Union during the 1950s (3). Although widely used, the mechanisms behind its attenuation are still not fully understood.

The ability to survive or suppress the normally antibacterial activities of host macrophages is considered to be critical for the virulence of intracellular pathogens like *F. tularensis*, and both LVS and fully virulent strains proliferate effectively within resting macrophages. Gamma interferon (IFN- γ) activation of macrophages enhances their bactericidal activity, and this effectively restricts the multiplication of *F. tularensis*. The bactericidal ability of macrophages that control LVS is conferred by mechanisms involving both reactive oxygen and nitrogen species (ROS and RNS, respectively), whereas their roles are less evident with regard to virulent strains, such as the prototypic SCHU S4 strain of *F. tularensis* subsp. *tularensis* (4–8). In fact, the mechanisms mediating the bactericidal activity of IFN- γ -activated macrophages toward virulent *F. tularensis* strains are still unclear (8).

Like other intracellular pathogens of professional phagocytes, *F. tularensis* expresses an array of enzymes that metabolize and neutralize ROS and RNS, such as superoxide dismutases (SODs),

catalase (KatG), glutathione peroxidase (Gpx), organic hydroperoxides, alkyl-hydroperoxide reductase (AhpC), and MoxR ATPase (9–13), and all of the published *F. tularensis* genomes contain genes encoding each of these enzymes, most of which are highly conserved. They have overlapping catalytic activities and, among other things, catalyze reduction of hydrogen peroxide (H₂O₂), organic hydroperoxides, and peroxynitrite (ONOO⁻) (14–16). Previously, we observed that LVS is more susceptible than virulent strains, such as SCHU S4, to killing mediated by H₂O₂ or ONOO⁻ (17). KatG has a pivotal role for resistance to H₂O₂ *in vitro*, but *in vivo*, KatG was demonstrated to be essential only for the full virulence of LVS but played a less important role for SCHU S4 (17). Therefore, virulent strains of *F. tularensis* possess KatG-independent mechanisms contributing to oxidative stress resistance that are lacking in LVS. *FTT0086* is an example of a putative KatG-independent, ROS-protective mechanism, since the encoding

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TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid ^a	Origin, genetic feature, or reference(s)
Strains	
<i>F. tularensis</i>	
SCHU S4	Human, USA, 1941
ΔFTT0086 mutant	This study
ΔkatG mutant	17
ΔkatG ΔFTT0086 mutant	This study
ΔahpC mutant	29
Δgpx mutant	29
FSC200	Human ulcer
LVS	ATCC 29684
FSC741	18, 19
<i>E. coli</i>	
S17-1	38
BL21	23
Plasmids	
pDMK2	20
pBAD24	23
pBAD24::yfeX	This study
pBAD24::FTT0086	This study
pBAD24::FTL1773	This study

^a The mutant strains listed were derived from the SCHU S4 strain of *F. tularensis* subsp. *tularensis*.

gene is predicted to encode a DyP-type peroxidase, but the gene is truncated in the LVS strain and, therefore, it has been postulated to lack peroxidase activity (18, 19).

In the present study, the aim was to obtain an improved understanding of ROS-detoxifying enzymes for the ability of *F. tularensis* to counteract the toxic effects of various types of ROS by investigating the phenotypes of relevant mutants under defined conditions *in vitro*. Moreover, we also wanted to compare how the *in vitro* phenotypes correlated to the ability of intramacrophage replication and virulence. To this end, we investigated the roles of the SCHU S4 mutants lacking expression of the FTT0086, KatG, Gpx, or AhpC enzyme for ROS detoxification and for the intracellular survival and virulence of the pathogen. We also investigated the effects of combining the gene deletions to better understand if the detoxifying functions are overlapping or complementary. In addition, it was assessed if the FTT0086 peroxidase plays a role in the differential detoxifying capabilities of SCHU S4 and LVS since it has been postulated to be enzymatically inactive in the latter strain.

MATERIALS AND METHODS

Plasmids and bacterial strains. All strains and plasmids used in this study are described in Table 1. *F. tularensis* LVS was originally obtained from the American Type Culture Collection (ATCC 29684). *F. tularensis* strains SCHU S4 (*F. tularensis* subsp. *tularensis*) and FSC200 (*F. tularensis* subsp. *holarctica*) were obtained from the *Francisella* Strain Collection of the Swedish Defense Research Agency, Umeå, Sweden. In-frame deletion of the FTT0086 gene was constructed by allelic exchange using a previously described technique based on integration and excision of a suicide plasmid carrying upstream and downstream sequences of the target gene (17). Briefly, 1,500 bp of the upstream and downstream regions of each of the genes was amplified by PCR. The PCR fragments for each gene contained complementary sequences in the 3' end of the upstream fragment and the

5' end of the downstream fragment, which were annealed during a second round of PCR. After restriction enzyme digestion and purification, the PCR fragments were cloned into the suicide vector pDMK2, which was later transformed into *Escherichia coli* S17-1. Conjugation to *F. tularensis* SCHU S4 was carried out as described previously (20). Conjugants were selected on media containing 10 μg/ml kanamycin and 50 μg/ml of polymyxin B and confirmed by PCR. To select for a second recombination event, conjugants were plated on medium containing 5% sucrose, and the deletion of the genes was verified by sequencing and PCR (see Fig. S1 in the supplemental material).

Plasmids used for *trans*-complementation studies were constructed by PCR amplification of the *ahpC* or *katG* genes and introduction of them into plasmid pKK289Km, to allow constitutive expression from the *groEL* promoter. Plasmids were transferred into the SCHU S4 strain by electroporation. The FTT0086 gene was used for *cis*-complementation of LVS as previously described (19). FTT0086 was introduced into LVS by conjugation and localized in the chromosome under the control of its native promoter.

Escherichia coli strain BL21 and plasmids pBAD24 and pBAD24::yfeX were gifts from Cécile Wandersman (Institut Pasteur, Paris, France). The genes FTL1773 and FTT0086 were amplified by PCR from genomic DNA of LVS and SCHU S4, respectively. These fragments were cloned into pBAD24 using XbaI and SalI restriction sites to give pBAD24::FTL1773 and pBAD24::FTT0086.

Mice. For virulence testing, specific-pathogen-free female BALB/c mice were purchased from Charles River Laboratories, St. Constant, Quebec, Canada. Mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care *Guide to the Care and Use of Experimental Animals* in federally licensed, select agent-approved, small-animal containment level 3 facilities at the National Research Council, Ottawa, Ontario, Canada.

Bone marrow-derived macrophages (BMDM) were derived from C57BL/6 mice, which were purchased from Charles River Laboratories, Germany. Mice were maintained and used in accordance with the recommendations of the Swedish Guidelines for Animal Care.

Preparation of growth media. Chamberlain's defined medium (CDM) with iron depleted by chelation (C-CDM) was produced as described previously (10, 21, 22). Briefly, 1% (wt/vol) Chelex-100 (Bio-Rad, Hercules, CA) was added to CDM without FeSO₄, and the mixture was kept in rotation for 24 h at 4°C. The Chelex-100 was removed by filtering the medium through a 0.45-μm-pore-size Millipore filter (Biosciences, Stockholm, Sweden), and the chelation step was repeated once. The medium was thereafter supplemented with essential cations (MgSO₄, 0.55 mM; ZnCl₂, 1.4 μM; CuSO₄, 0.2 μM; MnCl₂, 1.0 μM; CaCl₂, 5 μM). Sterility was ensured by a second filtration through a 0.22-μm-pore-size Millipore filter (Biosciences, Stockholm, Sweden).

Deferoxamine (DFO) is an iron chelator and was used to prepare agar plates lacking free iron in the medium (DFO plates). These plates were composed of 1 part 4% agar GC medium base (BD Diagnostic Systems, Sparks, MD), 1 part C-CDM without FeSO₄, and 25 μg/ml of DFO (Sigma-Aldrich, St. Louis, MO). Modified Mueller-Hinton (MC plates) contained 1% (wt/vol) hemoglobin (Oxoid, Ltd., Hampshire, United Kingdom), 3.6% (wt/vol) GC agar base (BD Diagnostic Systems), and 1% (vol/vol) IsoVitaleX (BD Diagnostic Systems), and plates containing this medium were used to propagate strains.

Heme-dependent growth. SCHU S4 and LVS and their respective isogenic ΔFTT0086 and ΔFTL1773 mutants were grown on C-CDM plates overnight. These iron-depleted strains were inoculated into C-CDM supplemented with heme (200 μg/ml) and the iron chelator deferoxamine (10 μg/ml). The growth of the respective strain was followed by measuring the optical density at 600 nm.

H₂O₂ susceptibility assay. Bacteria cultivated overnight on MC plates were resuspended and diluted in phosphate-buffered saline (PBS) to an estimated density of 3 × 10⁶ bacteria/ml. The bacterial suspension of each *F. tularensis* strain was divided into test tubes with H₂O₂ or control tubes

that were untreated. The tubes were incubated for 2 h at room temperature (RT) without shaking. Thereafter, the samples were serially diluted and spread on MC plates that were incubated at 37°C in 5% CO₂ for 3 days before enumeration of the CFU. The concentrations of H₂O₂ used were chosen to best reflect the differences among the strains and were not intended to mirror any physiological concentrations.

Paraquat susceptibility assay. Bacteria cultivated overnight on MC plates were resuspended and diluted in PBS to an estimated density of 3×10^7 bacteria/ml. One hundred microliters of bacterial suspension was spread on an MC plate. Once dried, a Whatman paper disc containing 10 μ l of a paraquat (Sigma) solution (H₂O) was deposited on each plate and incubated overnight at 37°C in 5% CO₂ before determination of the inhibition zone. The concentrations of paraquat used were chosen to best reflect the differences among the strains and were not intended to mirror any physiological concentrations.

SIN-1 susceptibility assay. Bacteria cultivated overnight on MC plates were resuspended and diluted in PBS to an estimated density of 3.0×10^6 bacteria/ml. The bacterial suspension of each *F. tularensis* strain was divided into test tubes with SIN-1 or control tubes that were untreated. The tubes were incubated for 4 h at RT without shaking. Thereafter, the samples were serially diluted and spread on MC plates that were incubated at 37°C in 5% CO₂ for 3 days before enumeration of the CFU. The concentrations of SIN-1 used were chosen to best reflect the differences among the strains and were not intended to mirror any physiological concentrations.

Deferrochelate assay. pBAD24::FTL1773 and pBAD24::FTT0086 were introduced into the *E. coli* C600 strain by electrotransformation. C600 strains containing the different vectors were streaked on LB agar plate supplemented with 0.2% L-arabinose. After an overnight incubation at 37°C, fluorescence was investigated by near-UV light irradiation (405 nm) (23).

Infection of macrophages. J774A.1 macrophages or bone marrow-derived macrophages (BMDM) from C57BL/6 mice were used in the cell infection assays. J774A.1 macrophages were cultured and maintained in DMEM (GIBCO BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO). BMDM were obtained by flushing bone marrow cells from the femurs and tibias of C57BL/6 mice. These cells were cultured for 7 days in DMEM containing 10% FBS, 5 μ g/ml gentamicin, and 20% conditioned medium (CM) from L929 cells (ATCC no. CCL-1), after which they were grown in medium lacking gentamicin. CM was replaced every 2 to 3 days. The day before infection, macrophages were seeded at a density of 4×10^5 in a 24-well tissue culture plates in DMEM with 10% FBS. Following incubation overnight, cells were washed, reconstituted with fresh culture medium, and allowed to recover for at least 30 min prior to infection. A multiplicity of infection (MOI) of 30 was used for nonstimulated cells and an MOI of 200 was used for IFN- γ -stimulated cells, and phagocytosis of the bacteria was allowed to proceed for 90 min. Thereafter, the monolayer was washed twice and incubated with gentamicin (5 μ g/ml) to eradicate any remaining extracellular bacteria. The time point after 30 min of gentamicin treatment was defined as 0 h. For the remaining time of the experiment, the medium was supplied with 2 μ g/ml gentamicin. To some wells, 1,000 U/ml of IFN- γ (PeproTech, London, United Kingdom) was supplied 12 h before bacteria were added and thereafter. This treatment elicits effective control of intracellular *F. tularensis* (8) and primes the cells for ROS production (24).

At 0 and 18 h, the macrophage monolayers were lysed with 200 μ l of 0.1% deoxycholate, and the number of intracellular bacteria was determined by plating 10-fold serial dilutions of the lysates in PBS. The assay was performed in triplicate and repeated three times.

Mouse infections. For virulence testing, specific-pathogen-free female BALB/c mice were injected intradermally with *F. tularensis* strains in a volume of 50 μ l. Actual concentrations of inocula were determined by plating 10-fold serial dilutions. Mice were examined daily for signs of infection and were euthanized by CO₂ asphyxiation as soon as they displayed signs of irreversible morbidity.

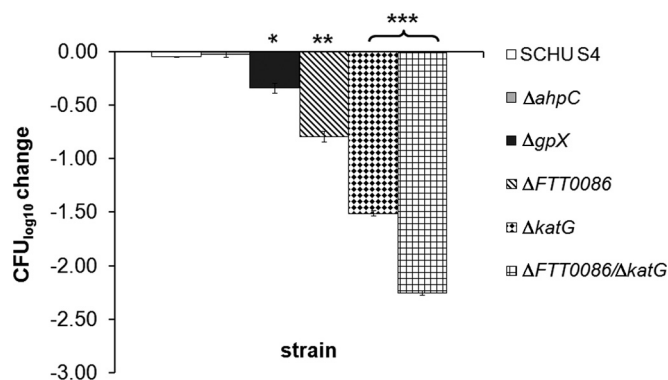


FIG 1 Changes in bacterial numbers of indicated *F. tularensis* strains after exposure to 4 mM H₂O₂ for 2 h. The bars represent the average decrease in bacterial viability from three replicates, and the error bars represent the standard error of the mean (SEM) (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Similar results were observed in two additional experiments. The bacterial number at the initiation of the experiment for each strain was approximately $6.0 \log_{10}$ CFU.

To determine the proliferative capacity of the strains, the bacterial numbers in liver, spleen, lungs, and blood from infected BALB/c mice were analyzed. The mice were given approximately 20 CFU intradermally of the indicated *F. tularensis* strain. At day 4 of infection, the mice were euthanized by CO₂, and organs and blood were withdrawn. The number of bacteria was determined by plating 10-fold serial dilutions of homogenized organs in PBS.

Statistical analysis. Independent-sample *t* tests (two-tailed) were used to identify differences between groups. Statistical analyses were carried out using SPSS software, version 21.0.

RESULTS

Identification of *F. tularensis* enzymes for resistance to H₂O₂-mediated killing. The roles of the potential ROS-detoxifying enzymes AhpC, KatG, Gpx, and FTT0086 of SCHU S4 were assessed by analyzing the survival of the corresponding deletion mutants after exposure to various concentrations of H₂O₂. FTT0086 belongs to the superfamily of heme-containing peroxidases with structures distinct from other peroxidases that show wide substrate specificities, but their roles to protect against ROS have been little studied (25).

After 2 h of exposure to 4 mM H₂O₂, the viability of SCHU S4 or the Δ ahpC mutant was not affected, whereas the viabilities of the Δ gpx, Δ FTT0086, Δ katG, and Δ FTT0086 Δ katG mutants decreased significantly (Fig. 1). Of the single mutants, the viability of the Δ gpx strain decreased the least ($P < 0.05$ versus SCHU S4), and the Δ katG strain was the most susceptible, whereas the Δ FTT0086 strain showed an intermediate phenotype ($P < 0.001$ and $P < 0.01$, respectively). The Δ FTT0086 Δ katG mutant was more susceptible than any of the single mutants ($P < 0.001$), showing a $>2\text{-log}_{10}$ decrease (Fig. 1). The Δ katG strain complemented with *katG* in *trans* exhibited essentially the same susceptibility to 4 mM H₂O₂ as SCHU S4 (see Fig. S2 in the supplemental material). When the Δ katG and Δ FTT0086 strains were exposed to 8 mM H₂O₂, the Δ katG mutant showed a complete loss of survival (a decrease of $>6 \log_{10}$ CFU), whereas the viability of the Δ FTT0086 mutant decreased 1.5 \log_{10} CFU and that of SCHU S4 decreased 0.5 \log_{10} CFU.

In summary, AhpC does not contribute to the protection against H₂O₂, whereas FTT0086 as well as Gpx and KatG protect

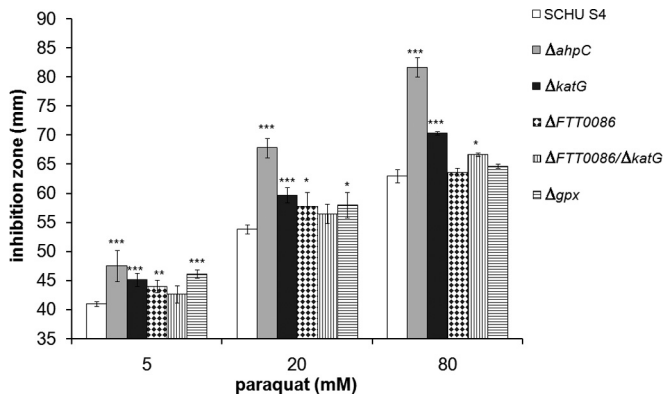


FIG 2 Inhibition of the growth of *F. tularensis* strains after exposure to paraquat. A disk containing 10 μ l of the indicated concentration of paraquat was placed on an agar plate. The bars represent the average of the growth inhibition zone (diameter) from three separate plates. The error bars represent the SEM (*, $P < 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

F. tularensis against relatively low or intermediate concentrations of H_2O_2 . Among the mutants tested, KatG is the most important enzyme to protect SCHU S4 against high concentrations of H_2O_2 .

Identification of *F. tularensis* enzymes for resistance to paraquat-mediated killing. The SCHU S4, Δ FTT0086, Δ katG, Δ FTT0086 Δ katG, and Δ gpx mutant strains were exposed to paraquat using a disc diffusion assay. Paraquat reacts with components of the respiratory chain in bacteria, leading to the reduction of O_2 and the generation of superoxide anion O_2^- (26). By the action of cytoplasmic superoxide dismutases, or spontaneously, this leads to generation of H_2O_2 . Thus, addition of paraquat leads to increased intracellular levels of H_2O_2 . The most marked effect of paraquat was observed for Δ ahpC, which was highly susceptible ($P < 0.001$) compared to SCHU S4 (Fig. 2). The other mutants tested, genotypes Δ katG, Δ FTT0086, Δ gpx, and Δ FTT0086 Δ katG, were less susceptible ($P < 0.01$) than the Δ ahpC mutant but more susceptible ($P < 0.01$) than SCHU S4 to the relatively low concentrations of 5 or 20 mM (see Fig. S3 in the supplemental material). At the intermediate concentration of 80 mM, the Δ ahpC and Δ katG strains were still more susceptible than SCHU S4, whereas the other mutants were not (see Fig. S3). This increased susceptibility was sustained for Δ ahpC alone at concentrations up to 500 mM (see Table S1 in the supplemental material). The Δ ahpC strain complemented with *ahpC* in *trans* exhibited essentially the same susceptibility to paraquat as SCHU S4 (see Fig. S3).

In summary, AhpC appears to constitute the primary detoxifying mechanism against the paraquat-induced toxicity among the mutants tested, whereas the other enzymes had less important roles, and differences were observed only at relatively low concentrations of paraquat.

Identification of *F. tularensis* enzymes for resistance to SIN-mediated killing. A variant of highly reactive and bactericidal species is represented by ONOO $^-$, resulting from the reaction between O_2^- and nitric oxide. SIN-1 generates O_2^- and nitric oxide, which spontaneously form ONOO $^-$. The effect of this agent on the survival of SCHU S4 or the Δ ahpC, Δ FTT0086, Δ katG, Δ FTT0086 Δ katG, or Δ gpx mutant was investigated. No killing was observed when the strains were exposed to a concentration of 4.8 mM for 5 h (data not shown), whereas 8.0 mM resulted in significant killing of the Δ ahpC mutant ($P < 0.01$ versus SCHU

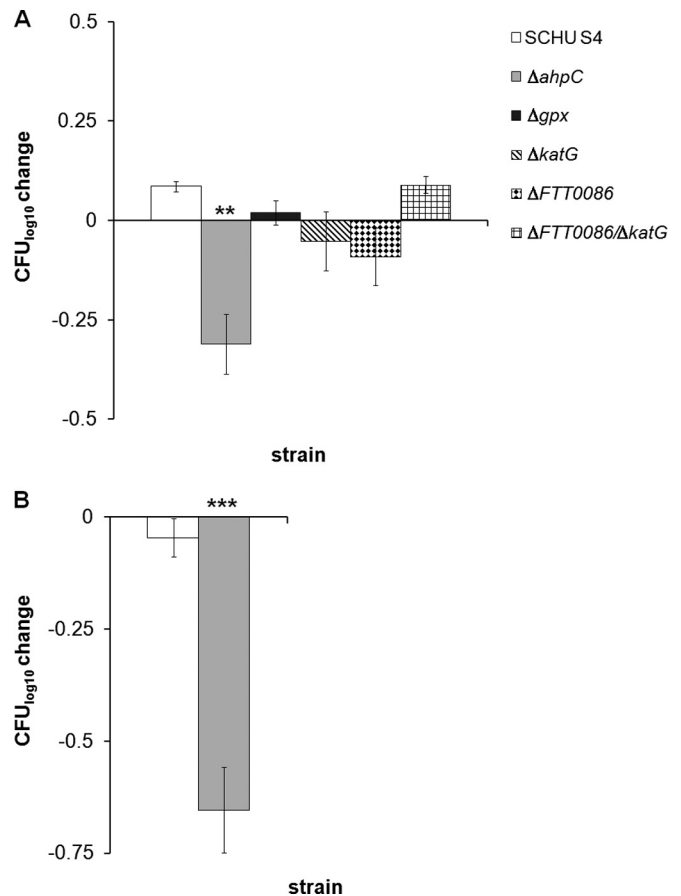


FIG 3 Changes in bacterial numbers of indicated *F. tularensis* strains after exposure to SIN-1. The bars represent the average change in bacterial viability from three replicates, and the error bars represent the SEM (**, $P \leq 0.01$; ***, $P \leq 0.001$). Similar results were observed in two additional experiments. (A) Exposure to 8.0 mM SIN-1 for 2 h. (B) Exposure to 10 mM SIN-1 for 2 h. The bacterial number at the initiation of the experiment for each strain was approximately $6.0 \log_{10}$ CFU.

S4) but not of the other mutants or SCHU S4 (Fig. 3A). When the concentration was increased to 10 mM, the effect on the Δ ahpC mutant was even more marked ($P < 0.001$), whereas SCHU S4 was not affected (Fig. 3B).

Intracellular replication and virulence of mutants defective for ROS-detoxifying enzymes. The Δ gpx, Δ ahpC, Δ katG, Δ FTT0086, and Δ FTT0086 Δ katG mutants were tested for intracellular growth in J774 macrophage-like cells, and all of them showed the same degree of replication as SCHU S4. In BMDM, which demonstrate a higher state of activation than J774 cells (27), Δ ahpC, but not the other mutants, showed compromised replication versus SCHU S4 ($P < 0.01$) (Fig. 4). Importantly, the Δ ahpC mutant grew as well as SCHU S4 in CDM (data not shown). We hypothesized that the role of each of the enzymes would become more critical if the macrophages were endowed with enhanced bactericidal capabilities. The essential role of IFN- γ in this respect is very well documented (5, 6, 8, 28). In IFN- γ stimulated BMDM, the net growth of SCHU S4 was marginal, and there was no difference in this regard compared to the mutant strains ($P > 0.05$) (Fig. 4).

The virulence of the Δ FTT0086 and Δ FTT0086 Δ katG mutants was tested after intradermal injection of 10 CFU into mice. The

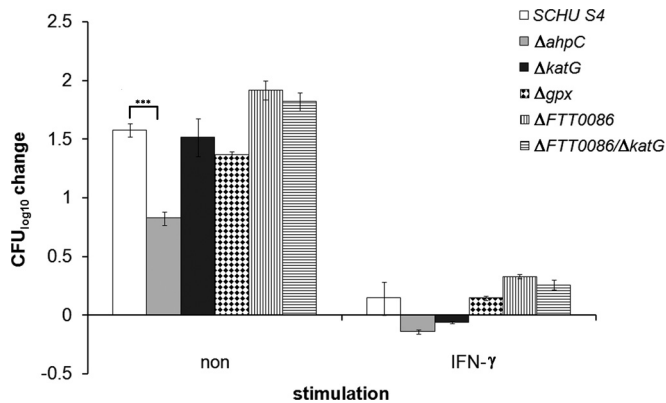


FIG 4 Intracellular replication of the indicated *F. tularensis* strains in BMDM with or without IFN- γ . Cells were infected at an MOI of 30 (without IFN- γ) or 200 (with IFN- γ), and bacterial numbers were determined after 24 h. ***, $P < 0.001$.

$\Delta FTT0086$ mutant killed mice as quickly as SCHU S4, whereas the average time to death of mice infected with the $\Delta FTT0086$ $\Delta katG$ mutant was 6.8 ± 0.26 days, significantly delayed ($P < 0.01$) compared to mice infected with SCHU S4 (5.4 ± 0.32 days) (Fig. 5). Previously, we observed that the $\Delta ahpC$ mutant displayed significantly delayed time to death (9.4 days versus 6.0 days for SCHU S4 [29]), and we now investigated if the replication of the $\Delta FTT0086$ $\Delta katG$ and $\Delta ahpC$ mutants was affected in internal organs after intradermal infection. This was indeed the case, since both mutants showed lower bacterial numbers than SCHU S4; however, although there was an approximately 1- \log_{10} difference for the $\Delta FTT0086$ $\Delta katG$ mutant, this was not significant, whereas the $\Delta ahpC$ mutant displayed significantly compromised replication, almost 2 \log_{10} lower CFU, in liver, spleen, and lung ($P < 0.01$ for spleen and liver and $P < 0.001$ for lung) (Table 2). No $\Delta ahpC$ bacteria were recovered from blood and significantly, lower numbers were recovered from blood of $\Delta FTT0086$ $\Delta katG$ strain-infected mice versus SCHU S4-infected mice ($P < 0.05$) (Table 2).

Previously, we observed that the Δgpx and $\Delta katG$ mutants showed no attenuation with regard to time to death (17, 29). Nevertheless, significantly fewer $\Delta katG$ bacteria were recovered from skin, spleen, and liver of infected mice (17).

Thus, the data demonstrate that neither FTT0086, KatG, nor Gpx is crucial for intramacrophage growth or *in vivo* infection.

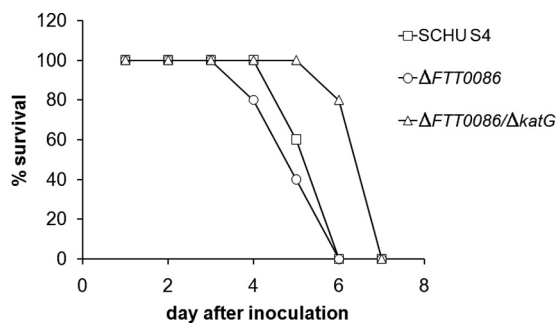


FIG 5 Survival of C57BL/6 mice after infection with SCHU S4 (squares), the $\Delta FTT0086$ mutant (circles), and the $\Delta FTT0086$ $\Delta katG$ mutant (triangles). Mice were injected intradermally with 10 CFU of the indicated strain. Each group contained 5 mice.

TABLE 2 Bacterial burdens in organs and blood of BALB/c mice infected with *F. tularensis*

Strain	Bacterial burden in ^a :			
	Organ (\log_{10} CFU/organ) ^b			Blood (\log_{10} CFU/ml) ^b
	Liver	Spleen	Lung	
SCHU S4	6.81 ± 0.25	7.7 ± 0.38	3.84 ± 0.49	3.38 ± 0.44
$\Delta katG$ $\Delta FTT0086$ mutant	6.08 ± 0.33	6.95 ± 0.36	2.90 ± 0.55	$1.27 \pm 0.78^*$
$\Delta ahpC$ mutant	$5.36 \pm 0.34^{**}$	$6.00 \pm 0.30^{**}$	$0.26 \pm 0.26^{***}$	$0 \pm 0^{***}$

^a Organs were harvested from mice at day 4 after intradermal infection with approximately 20 CFU of the indicated *F. tularensis* strain per mouse. Asterisks indicate significant difference versus SCHU S4-infected mice for the indicated organ: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

^b Values are means \pm SEM from 5 mice.

Nevertheless, infection with the strain with combined $\Delta FTT0086$ and $\Delta katG$ mutations resulted in significantly delayed time to death of mice. AhpC was required for effective replication in BMDM and also demonstrated an important role *in vivo*, since replication of the $\Delta ahpC$ mutant in internal organs was very significantly impaired.

Presence of an altered Dyp-type peroxidase FTT0086 homolog in *F. tularensis* LVS. Most of the genes encoding the ROS-detoxifying enzymes of *F. tularensis* are highly conserved among subsp. *tularensis* and *holarctica*, with the exception of FTT0086. A BLAST analysis of the protein sequence using the PeroxiBase database (<https://peroxibase.toulouse.inra.fr>) confirmed that FTT0086 belongs to the DyP-type peroxidase B class and demonstrates 38% protein identity to CtesDyPrx01 of *Comamonas testosteroni* and 25% identity to YfeX from *E. coli*. FTT0086 is predicted to reside in the cytosol but lacks signal peptide and the TAT motif. The predicted primary structure reveals the presence of a heme-binding site in the C-terminal part of the protein.

The encoding gene is highly conserved in the published *F. tularensis* genomes, with the exception of the LVS homolog FTL1773, which contains a deletion of 93 nucleotides in the C-terminal part (18, 19). It has been previously postulated that the deletion would inactivate the peroxidase function (18, 19); however, our bioinformatic analysis demonstrated that it contains all the conserved residues required for heme-binding and peroxidase activities, as illustrated in the protein alignment (Fig. 6). The motif GXXDG corresponds to the catalytic domain of the peroxidase and is strictly conserved. Other conserved residues important for heme binding like D134, H204, and R220 are present in both proteins. The C-terminal truncation of 31 amino acids in FTL1773 may, however, affect the tertiary structure and thereby the function of the protein.

In view of the uncertain function of the peroxidase in the LVS strain, we investigated the roles of the two *Francisella* homologues for resistance to ROS. LVS and the $\Delta FTL1773$ mutant were exposed to a concentration of 1 mM H_2O_2 . At this concentration, a decrease of slightly more than 1.0 \log_{10} was observed for both strains (Fig. 7). Thus, the lack of FTL1773 did not affect the susceptibility of LVS to H_2O_2 . In contrast, FSC741—LVS complemented with the SCHU S4 FTT0086 gene (18, 19)—showed less than a 0.5- \log_{10} decrease in bacterial numbers when exposed to 1 mM H_2O_2 (Fig. 7), which was significantly less than LVS ($P < 0.001$). Thus, our findings suggest that FTT0086 but not FTL1773 has the capacity to degrade H_2O_2 .

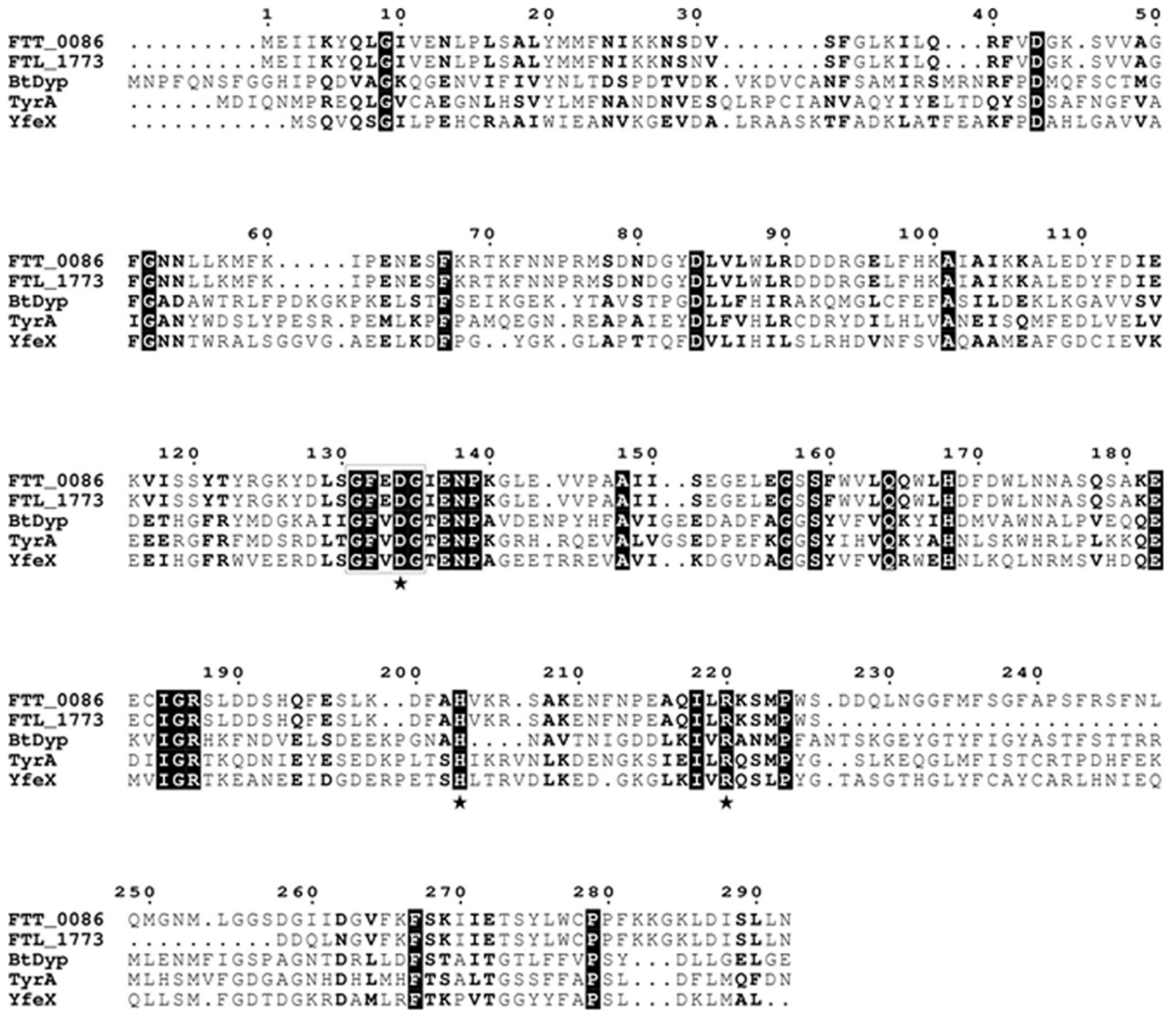


FIG 6 Multiple sequence alignments of FTT0086, FTL1773, and homologous DyP-type peroxidases. The sequence alignments were performed using ClustalW, and ESPript was used to display the protein alignment (36, 37). Conserved residues are indicated in white on black. Similar amino acid residues are in bold. The D134, H203, and R220 residues of DyP are indicated with black stars. The GXXDG motif is boxed. The sequences of TyrA and BtDyP were retrieved from PDB under accession no. 2hag and 2gvk, respectively. BtDyp is the Dyp homologue from *Bacteroides thetaiotaomicron* and TyrA the homologue from *Shewanella oneidensis*.

We also investigated if FTT0086 possessed deferrohchelata se activity like its homolog YfeX in *E. coli* (23). To this end, YfeX and the two *F. tularensis* homologs were expressed in a recombinant system by *E. coli* C600, and it was found that YfeX led to the release of iron from the heme, and this resulted in the accumulation of proporphyrin, which was detected as red fluorescence (see Fig. S4 in the supplemental material). When FTT0086 or FTL1773 was expressed under the same conditions as yfeX, there was no manifestation of red fluorescence (see Fig. S4). The expression in *E. coli* of the proteins encoded by FTT0086 and FTL1773, respectively, was confirmed by SDS-PAGE (see Fig. S5 in the supplemental material). Therefore, we conclude that FTT0086 is not involved in iron acquisition from heme through deferrohchelation, like its ho-

molog YfeX. In line with this result, SCHU S4 and LVS and their respective ΔFTT0086 and ΔFTL1773 isogenic mutants grew with heme as the exclusive iron source (data not shown).

DISCUSSION

F. tularensis is highly adapted to the intracellular habitat and capable of multiplying in many cell types, most notably within macrophages, a normally highly bactericidal cell type (18, 19). The intracellular growth of *Francisella*, which occurs in the cytosol, is considered a key factor for the virulence of the bacterium since it allows for escape from many immune mechanisms and access to nutrients and thereby enables its multiplication in the host. The prerequisite of intracellular growth for the bacterial virulence is

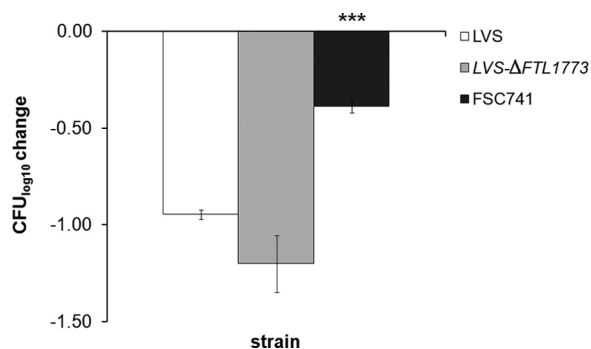


FIG 7 Changes in bacterial numbers of the indicated *F. tularensis* strains after exposure to 1 mM H₂O₂ for 2 h. The bars represent the average decrease in bacterial viability from three separate tubes, and the error bars represent the SEM (***, $P \leq 0.001$). Similar results were observed in two additional experiments. The bacterial number at the initiation of the experiment for each strain was approximately 6.0 log₁₀ CFU.

demonstrated by the close correlation between the capability of *F. tularensis* mutants to replicate in macrophages and their virulence in the mouse model. To survive within the macrophage, the bacterium must first counteract the normally bactericidal environment of the phagosome, which is replete with antibacterial agents, such as ROS and RNS, which indicates that potent defenses against such reactive species are critical to successful intracellular bacteria, such as *F. tularensis* (30, 31). The bactericidal effects of various types of macrophages vary considerably; however, activation almost invariably endows them with highly effective capabilities to control infection and IFN- γ (8, 24).

In the present study, the aim was to investigate the roles of ROS-detoxifying enzymes for the survival of *F. tularensis* *in vitro* in the presence of specific bactericidal agents and to determine how this susceptibility is correlated to intramacrophage growth and virulence. Thereby, we hypothesized that we would be able to understand indirectly how infection is controlled. Previously, we demonstrated that KatG plays an essential role for the resistance of SCHU S4 and LVS to ROS-mediated killing *in vitro* and that the enzyme is essential for the virulence of LVS but not that of SCHU S4 (17). Here, we demonstrated that not only KatG but also Gpx and FTT0086 of SCHU S4 contributed significantly to the resistance to H₂O₂. At high concentrations, however, resistance relied exclusively on expression of KatG. As the putative Dyp peroxidase of LVS, FTL1773, is truncated in its C terminus, we wanted to know if this truncation altered the peroxidase activity. Although we observed that the known characteristic features of DyP-type peroxidases were preserved in FTL1773, the corresponding mutant, in contrast to the SCHU S4 FTT0086 mutant, showed no enhanced susceptibility to H₂O₂, indicating that, in fact, it is non-functional. Presumably, the lack of the C terminus affects the overall protein conformation, leading to impairment of its peroxidase function, or the protein is inherently unstable. In further support of a functional difference between FTT0086 and FTL1773, we observed that FSC741 (LVS complemented with the SCHU S4 FTT0086 gene) showed increased resistance to H₂O₂. Interestingly, as previously demonstrated, the virulence of the complemented strain is not enhanced (19). Therefore, our findings collectively suggest that the ability of LVS to degrade ROS—for example, via the function of the KatG—is sufficient for its survival *in vivo* and that the enhanced detoxifying capability conferred by a

functional DyP peroxidase does not confer a survival advantage in the mouse.

FTT0086 endows SCHU S4 with an efficient tool to handle oxidative stress that is missing from LVS; however, since the single mutants and the $\Delta katG \Delta FTT0086$ double mutant showed significant survival at fairly high H₂O₂ concentrations, the bacterium must possess additional detoxifying mechanisms besides KatG and FTT0086. The most likely culprits in this regard are the peroxiredoxin, AhpC, and glutathione peroxidase, Gpx, since both enzymes have been shown to be potent detoxifiers of H₂O₂ in other bacterial species (32). Indeed, we observed that the susceptibility to H₂O₂ of the Δgpx mutant was increased to the same extent as for the $\Delta FTT0086$ mutant. AhpC is involved in resistance to endogenous H₂O₂ and has been demonstrated to be essential for the virulence of the facultative intracellular bacterium *Brucella abortus* (33). We observed no enhanced susceptibility of the mutant to exogenous H₂O₂, but in view of its postulated role to protect against endogenous H₂O₂, we tested its sensitivity to paraquat, a molecule that via generation of O₂⁻ leads to generation of intracellular H₂O₂, and SIN-1, which indirectly generates ONOO⁻ (26). In both instances, the $\Delta ahpC$ mutant was found to be highly susceptible, and the *ahpC* deletion resulted in much diminished bacterial load and significantly prolonged time to death for infected mice.

Altogether, the data indicate that virulent *F. tularensis* strains rely on an intricate network of overlapping functions to protect themselves against the effects of ROS. Apparently, each of the FTT0086 and Gpx enzymes possesses a significant ability to degrade H₂O₂; however, these functions are not critical for the bacterium to survive in macrophages or *in vivo*, presumably due to their overlapping activities. As we previously demonstrated, this also holds true for the $\Delta katG$ mutant of SCHU S4 (17). In our previous study, we observed, however, that the $\Delta katG$ mutant showed some degree of attenuation since the bacterial numbers in liver and spleen were decreased, although this did not affect the survival of the mice. Here, we observed that SCHU S4 was dependent on KatG to degrade high concentrations of H₂O₂, and in fact, the mutant was eradicated by a concentration of 8 mM, but presumably, such high concentrations are not encountered *in vivo*, and therefore not even KatG is critically required. In contrast, the findings on the $\Delta ahpC$ mutant show that the function of AhpC is distinct from the other enzymes studied, since it was not required for resistance to H₂O₂ but was the only enzyme that played a critical role in protection against the paraquat- and ONOO⁻-mediated effects. Thus, the findings imply that AhpC performs a very important role for detoxification in view of the enhanced susceptibility to the two compounds. In view of these findings, it was somewhat surprising that the mutant was capable of nearly normal intramacrophage replication; however, it showed marked attenuation *in vivo* as evidenced by very impaired growth in liver, spleen, and lung. Together, the findings indicate that the critical role of AhpC for virulence may not be related to the macrophage infection. Also, the $\Delta katG \Delta FTT0086$ double mutant showed attenuation *in vivo* as evidenced by significantly longer time to death than SCHU S4.

Collectively, our data suggest that *F. tularensis* possesses complementary detoxifying systems, meaning that the absence of one or the other of these detoxifying enzymes will not be detrimental. In addition to the enzymes studied here, there are also *F. tularensis* enzymes that may contribute to the overall capability of virulent *F.*

tularensis strains to withstand oxidative stress. One such example is the FTN1133 homolog that originally was identified in *Francisella novicida* and also in LVS was found to contribute to resistance to organic hydroperoxides but not to H₂O₂ (9), and also the MoxR ATPase that was important for resistance of the LVS strain to H₂O₂ (13). In addition, each of the LVS SodB and SodC mutants has been demonstrated to display an attenuated phenotype (9); however, we observed no attenuation of the SCHU S4 *sodC* mutant (29), presumably again emphasizing the more elaborate network of resistance to ROS in the highly virulent *F. tularensis* strains than in the LVS strain. The critical role of the antioxidative defense of *F. tularensis* is further emphasized by the recent demonstration that an *emrA1* mutant is highly susceptible to hydrogen peroxide and attenuated for virulence by affecting secretion of SodB and KatG (34).

The correlation we found between the lack of detoxifying ability and attenuation demonstrated the relevance of the *in vitro* assays for the situation *in vivo*; however, exactly when during the life cycle the critical roles of the ROS-detoxifying enzymes are executed are unclear. Since *F. tularensis* replicates in the cytosol and appears to be capable of effective phagosomal escape even in activated macrophages, control of infection must be mediated in the macrophage cytosol. However, no cytosolic effector mechanisms have been directly identified, although inhibition of RNS partially reverses the effects (17). These facts, together with the present findings that the Δ *ahpC* mutant was capable of normal intramacrophage replication, indicate that the relevance of ROS detoxification may be most important either during the extracellular phase or in cell types other than macrophages.

It may be difficult to pinpoint a definite role for many enzymes *in vivo* since their activities may be overlapping, as is the case for FTT0086. It has been found that *F. tularensis* gene expression inside macrophage was characterized by the upregulation of many genes involved in resistance to oxidative stress during the early phagosomal phase of infection (11). These findings together with our present and previous findings indicate that the redundancy and overlapping enzymatic functions that we observe with regard to detoxification of ROS reflect the very important overall roles of these functions for the survival of intracellular pathogens, such as *F. tularensis*. It would be possible to further delineate these overlapping and redundant functions by combining deletions of the detoxifying genes, as exemplified by the Δ *katG* Δ *FTT0086* double mutant. However, we have repeatedly attempted to generate other combinations, such as Δ *ahpC* plus Δ *katG*, but these attempts have been futile. One possible explanation is that such combinations severely compromise the ability of the bacterium to cope with the oxidative levels normally encountered, and, therefore, the combinations are not possible to select for during the genetic recombination.

Future studies will be aimed at identifying the regulations and roles of the enzymes implicated in oxidative stress resistance at different stages of infection with *F. tularensis*. Their regulation should also be analyzed in relation to iron metabolism, since we have demonstrated that the high degree of oxidative stress resistance of *F. tularensis* subsp. *tularensis* is related to low levels of intracellular free iron (35).

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