Role of peroxiredoxin of the AhpC/TSA family in antioxidant defense mechanisms of 
Francisella tularensis

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Abstract

Francisella tularensis is a Gram-negative, facultative intracellular pathogen and the causative agent of a lethal human disease known as tularemia. Due to its extremely high virulence and potential to be used as a bioterror agent, F. tularensis is classified by the CDC as a Category A Select Agent. As an intracellular pathogen, F. tularensis during its intracellular residence encounters a number of oxidative and nitrosative stresses. The roles of the primary antioxidant enzymes SodB, SodC and KatG in oxidative stress resistance and virulence of F. tularensis live vaccine strain (LVS) have been characterized in previous studies. However, very fragmentary information is available regarding the role of peroxiredoxin of the AhpC/TSA family (annotated as AhpC) of F. tularensis SchuS4; whereas the role of AhpC of F. tularensis LVS in tularemia pathogenesis is not known. This study was undertaken to exhaustively investigate the role of AhpC in oxidative stress resistance of F. tularensis LVS and SchuS4. We report that AhpC of F. tularensis LVS confers resistance against a wide range of reactive oxygen and nitrogen species, and serves as a virulence factor. In highly virulent F. tularensis SchuS4 strain, AhpC serves as a key antioxidant enzyme and contributes to its robust oxidative and nitrosative stress resistance, and intramacrophage survival. We also demonstrate that there is functional redundancy among primary antioxidant enzymes AhpC, SodC, and KatG of F. tularensis SchuS4. Collectively, this study highlights the differences in antioxidant defense mechanisms of F. tularensis LVS and SchuS4.

Introduction

Francisella tularensis is a Gram-negative, facultative intracellular pathogen and the causative agent of a lethal human disease known as tularemia. F. tularensis has a very broad host range and can infect a wide range of ticks, arthropods, and mammals [1]. F. tularensis subsp. tularensis (Type A) cause lethal tularemia in North America. The strains belonging to F. tularensis subsp. holarctica (Type B) are less infectious than the Type A strains and are prevalent...
exist regarding the contents of the manuscript and its authors.

**Competing interests:** The authors have declared that no competing interests exist.

As an intracellular pathogen, *F. tularensis* infects a wide variety of phagocytic cells such as macrophages, neutrophils, dendritic cells and non-phagocytic cells, such as hepatocytes, erythrocytes, and epithelial cells. Macrophages serve as the major reservoir for *F. tularensis* [3,4]. Phagocytic cells including macrophages produce reactive oxygen and nitrogen species (ROS/RNS) in response to *Francisella* infection. To counter these, *F. tularensis* genome encodes a full repertoire of primary antioxidant enzymes. *Francisella* encodes two superoxide dismutases (Sods); an iron-containing SodB (FeSod) and a copper-zinc containing SodC (CuZnSod) for the dismutation of superoxide radicals into hydrogen peroxide (H$_2$O$_2$). SodB is secreted via a major facilitator superfamily (MFS) type Emr multidrug efflux pump extracellularly [5] or in the cytosol of the infected macrophages [6]. A point mutant of the sodB gene is hypersensitive to oxidative stress and attenuated for virulence in mice [7]. SodC of *Francisella* is located in the periplasm of the bacterial cell and is required for resistance from extracellularly generated oxidative stress, and virulence in mice [8]. A catalase encoded by the katG gene converts H$_2$O$_2$ in water and oxygen and therefore prevents generation of other microbicidal ROS such as hydroxyl (HO) radicals or hypochlorous acids (HOCl), and thus play an important role in resistance of *Francisella* against oxidative stress. KatG similar to SodB is secreted in the extracellular environment or the macrophage cytosol [5,9]. The katG gene deletion mutants (ΔkatG) of both *F. tularensis* subspecies holarctica Live Vaccine Strain (LVS) and the highly virulent *F. tularensis* subspecies *tularensis* SchuS4 strain are sensitive to H$_2$O$_2$, but not to RNS, peroxynitrite (ONOO$^-$). The ΔkatG mutant of both the LVS and SchuS4 replicate similar to their respective wild type strains in unstimulated macrophages. However, only the ΔkatG mutant of *F. tularensis* LVS is attenuated for virulence; while the SchuS4 ΔkatG mutant remains virulent in mice [10,11]. In addition to these primary antioxidant enzymes, *Francisella* also encodes glutathione peroxidase (Gpx), MoxR ATPases, Dyp-type Peroxidase, glutaredoxin A (GrxA), and methionine sulfoxide reductase A, A1 and B [12]. A MoxR subfamily protein encoded by FTL_0200 gene of *F. tularensis* LVS provides resistance against oxidative and pH stresses [13]. A gene encoding a protein with sequence similarity to organic hydroperoxide resistance protein Ohr found in several bacterial pathogens is also reported in *F. novicida* and *F. tularensis* LVS. This ohr homolog is required for resistance against organic peroxides as well as NADPH-generated ROS both in vitro and in vivo [4]. It has been reported that FTT_0086 of *F. tularensis* SchuS4 is required for resistance against oxidative stress; while a homolog of this gene is not functional in *F. tularensis* LVS [10]. Collectively, these studies demonstrate that differences do exist between the antioxidant defenses of *F. tularensis* LVS and *F. tularensis* SchuS4.

A highly conserved LysR family of regulators known as OxyR is also present in *F. tularensis*. H$_2$O$_2$ activates OxyR via the modification of an oxidant-sensitive cysteine residue which then binds to the promoter region of the target genes and upregulates their expression. In our previous study, we have demonstrated that OxyR regulates the expression of antioxidant enzyme genes alkyl hydroperoxide reductase (ahpC) and katG [14]. The AhpC belongs to a family of thiol peroxidases (peroxiredoxins) that can scavenge micromolar concentrations of H$_2$O$_2$. The catalases are activated only after AhpC is saturated with millimolar concentrations of H$_2$O$_2$. AhpC uses cysteine thiols to reduce peroxides and acts in conjunction with AhpC reductants; AhpF or AhpD, that recycle AhpC during catalysis [15]. Both these reductants are absent in *F. tularensis*. The open reading frames FTL_1015 in *F. tularensis* LVS and FTT_0557 in *F. tularensis* SchuS4 do not code for AhpC proteins, instead they are more structurally similar to...
peroxidase/peroxiredutase proteins and has been annotated as peroxiredoxin of the AhpC/ 
TSA family. It has been reported that AhpC in *F. tularensis* SchuS4 is required for resistance 
against endogenous H$_2$O$_2$ and ONOO$^-$ [10]. However, very fragmentary information is avail-
able regarding the role of AhpC of *F. tularensis* SchuS4 in the pathogenesis of tularemia 
[10,16]; whereas the role of AhpC of *F. tularensis* LVS is not known. This study was under-
taken to exhaustively investigate the role of AhpC in oxidative stress resistance of *F. tularensis* 
LVS and SchuS4. We report that AhpC of *F. tularensis* LVS confers resistance against a wide 
range of ROS and RNS, and serves as a virulence factor. This study also demonstrates that 
there is a functional redundancy among primary antioxidant enzymes AhpC, KatG and SodC 
of *F. tularensis* SchuS4. However, AhpC serves as a key antioxidant enzyme and contributes to 
robust oxidative and nitrosative stress resistance and intramacrophage survival of the highly 
virulent *F. tularensis* SchuS4 strain.

**Materials and methods**

**Ethics statement**

This study was carried out in strict accordance with the recommendations and guidelines of 
the National Council for Research (NCR) for care and use of animals. All the animal experi-
ments were conducted in the centralized Animal Resources Facility of New York Medical Col-
lege licensed by the USDA and the NYS Department of Health, Division of Laboratories and 
Research and accredited by the American Association for the Accreditation of Laboratory 
Care. The use of animals and protocols were approved by the Institutional Animal Care and 
Use Committee (IACUC) of New York Medical College (Protocol Number 69-2-0914H). Mice 
were administered an anesthetic cocktail consisting of ketamine (5 mg/kg) and xylazine (4 mg/ 
k)g and underwent experimental manipulation only after they failed to exhibit a toe pinch 
reflex. Mice exhibiting more than 25% weight loss, anorexia, dehydration and impairment of 
mobility were removed from the study and euthanized by approved means. Humane end-
points were also necessary for mice which survived at the conclusion of the experiments. Mice 
were administered an anesthetic cocktail of ketamine and xylazine intraperitoneally and then 
euthanized via cervical dislocation followed by cardiac puncture, a method that is consistent 
with recommendations of the Panel on Euthanasia of the American Veterinary Medical Asso-
ciation. In all experimental procedures, efforts were made to minimize pain and suffering. All 
the work with Category A select agent *F. tularensis* SchuS4 was performed in CDC Certified 
Biosafety Level 3 (BSL3) laboratory of New York Medical College (Registration No. 
C20160722-1812) in accordance with protocols approved by Institutional Biosafety Committee 
(Protocol No. 01-2015-3).

**Bacterial strains and growth conditions**

*F. tularensis* subspecies holarctica LVS and *F. tularensis* subspecies *tularensis* SchuS4 used in 
this study were obtained from BEI Resources (Manassas, VA). The ahpC (FTL_1015) gene 
deletion (ΔahpC) mutant of *F. tularensis* LVS and a transcomplemented strain (ΔahpC+pahpC) were generated and used in this study. Previously published ΔsodC mutant of *F. tularensis* LVS available in our laboratory was also used in this study (8). The gene deletion mutants of *F. tularensis* SchuS4; ΔahpC (FTT_0557), ΔkatG (FTT_0721c) and the ΔsodC (FTT_0879), 
and *F. tularensis* LVS ΔkatG (11) mutants were kindly provided by Dr. Andres Sjostedt (Umea 
University, Sweden). All the bacterial strains used in this study are shown in Table 1. All the 
experiments involving *F. tularensis* SchuS4 strain were conducted in the CDC certified BSL3 
laboratory of New York Medical College.
All bacterial strains were grown on Mueller-Hinton (MH)-chocolate agar plates (BD Biosciences, San Jose, CA) at 37˚C with 5% CO₂ or Muller-Hinton broth (MHB) (BD Biosciences, San Jose, CA) supplemented with IsoVitaleX and ferric pyrophosphate at 37˚C with constant shaking (175 rpm). Transcomplemented ΔahpC+pahpC strain of *F. tularensis* LVS was grown on MH-chocolate agar plates supplemented with hygromycin (200 μg/mL). Bacterial strains were grown in MHB to mid-log phase, aliquoted and stored at -80˚C until further use.

**Construction of ΔahpC mutant and transcomplementation**

Allelic replacement method was used to construct the ΔahpC mutant of *F. tularensis* LVS [18]. The entire 557-bp coding region of the *ahpC* gene (*FTL_1015*) was deleted employing an approach described previously [19]. Briefly, a 5’ 1218 bp fragment upstream of the start codon and first 5 bp of the *ahpC* gene was amplified with primers MP241 and 243. A 3’ fragment containing last 10 bp and the stop codon of the *ahpC* gene and 1218 bp of the downstream region was amplified with primers MP245 and 246. Both the upstream and downstream fragments were joined by overlapping extension PCR with primers MP241 and MP246 engineered with BamHI and SalI restriction sites at 5’ ends, respectively. The generated single fragment with *ahpC* gene deletion was digested and cloned into the pJC84 vector using the BamHI and SalI sites. The resultant plasmid, pMM06, was electroporated into the wild type *F. tularensis* LVS as described previously [14,20]. After the primary selection of positive colonies using kanamycin and a counter selection with sucrose, the positive colonies were screened by colony PCR with primers MP260 and MP261 to identify the ΔahpC mutant.

For transcomplementation of the ΔahpC mutant of *F. tularensis* LVS, full-length *ahpC* gene sequence was amplified with primers MP274 and MP275 and cloned into a pMP822 vector at BamHI site generating a plasmid, pMM09. The pMM09 plasmid was transformed into chemically competent *E. coli* DH5α cells and selected on LB-hygromycin plates. The pMM09 was purified, and the orientation of the *ahpC* gene in the pMM09 vector was confirmed by PCR.

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**Table 1. List of bacterial strains and plasmids used in this study.**

| Strains                      | Genotype                          | Source          |
|------------------------------|-----------------------------------|-----------------|
| *Francisella tularensis* LVS | Wild type strain                  | BEI Resources   |
| Δ*ahpC* mutant               | Deletion mutant of *F. tularensis* LVS *ahpC* gene | This study      |
| *ahpC* transcomplement (Δ*ahpC* + *pahpC*) | *F. tularensis* LVS, Δ*ahpC*, pMM09 (pMP822-*ahpC*), Hygro⁺ | This study      |
| Δ*sodC* mutant               | Deletion mutant of *F. tularensis* LVS *sodC* gene | [8]             |
| Δ*katG* mutant               | Deletion mutant of *F. tularensis* LVS *katG* gene | [11]            |
| *Francisella tularensis* SchuS4 | Wild type strain                  | BEI Resources   |
| *F. tularensis* SchuS4 Δ*ahpC* mutant | Deletion mutant of *F. tularensis* SchuS4 *ahpC* gene | [10]            |
| *F. tularensis* SchuS4 Δ*sodC* mutant | Deletion mutant of *F. tularensis* SchuS4 *sodC* gene | [16]            |
| *F. tularensis* SchuS4 Δ*katG* mutant | Deletion mutant of *F. tularensis* SchuS4 *katG* gene | [11]            |
| *E. coli* DH5α               | F- Δφ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ– thi-1 gyrA96 relA1 | Invitrogen      |

**Plasmids**

| Plasmids                  | Source          |
|---------------------------|-----------------|
| pMP822                    | *E. coli-Francisella* shuttle vector, Hygro⁺ | [17] |
| pJC84                     | *E. coli-Francisella* suicide vector, Kan⁺ | [12] |
| pMM06                     | pJC84 + fused flanking fragment of *ahpC* gene, Kan⁺ | This study |

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The pMM09 vector cloned in the correct orientation was electroporated in the ΔahpC mutant. The transformants were selected on MH-chocolate agar plates containing hygromycin (200 μg/mL). The resultant transcomplemented strain was termed as ΔahpC+pahpC and confirmed by PCR. The primer sequences and the vectors used for the generation of ΔahpC and ΔahpC+pahpC strains are shown in Table 2.

### Growth curves

Growth curves were generated by resuspending bacterial cultures grown on MH-chocolate agar plates to an Optical Density at 600 nm (OD\textsubscript{600}) of 0.2 (corresponds to 1x10\(^9\) CFU/mL) in MHB. The bacterial suspensions were grown for 28 hours in the absence or presence of 750 μM H\textsubscript{2}O\textsubscript{2}, and the OD\textsubscript{600} was recorded at 4-hour intervals.

### Disc diffusion assays

Disc diffusion assays were used to determine the sensitivity of F. tularensis LVS, and F. tularensis SchuS4 strains towards superoxide-generating compounds, organic peroxides, and H\textsubscript{2}O\textsubscript{2}. Cultures of wild type F. tularensis LVS, the ΔahpC mutant and the ΔahpC+pahpC transcomplemented strain grown on MH-chocolate agar plates were resuspended in 1mL of sterile PBS and adjusted to an OD\textsubscript{600} of 2.0. The suspensions were then spread on MH-chocolate agar plates using sterile cotton swabs to obtain a heavy bacterial growth. Sterile filter paper discs were impregnated with 10 μL of varying concentrations of superoxide-generating compounds; menadione (1.56 μg/disc), pyrogallol (62.5 μg/disc), paraquat (3.75 μg/disc) (Sigma Aldrich, St. Louis, MO) as well as organic peroxides tert-butyl hydroperoxide (TBH) (437 μg/disc), cumene hydroperoxide (CHP) (125 μg/disc), and H\textsubscript{2}O\textsubscript{2} (6.25 mM/disc) (Sigma Aldrich, St. Louis, MO). An identical protocol was used for disc diffusion assays performed with wild type F. tularensis SchuS4 and the ΔahpC, ΔsodC, and ΔkatG mutants. However, higher concentrations of oxidants than those used for F. tularensis LVS were used. Specifically, the concentrations of menadione (6.25 μg/disc), paraquat (15 μg/disc) and pyrogallol (250 and 500 μg/disc).

### Table 2. List of primer sequences used in this study.

| Primer | Sequence | Purpose |
|--------|----------|---------|
| ahpC gene deletion construct: | | |
| \textit{F. tularensis} LVS *ahpC* upstream fragment | MP241’ | 5’-CAAgatccTCCATTTGCAAGGCTTTTG-3’ | Forward primer with a \textit{BamHI} site |
| | MP243 | 5’-CCTTTTCATAATTACCTAGACTGTACATGCTACACTCTAATG-3’ | Reverse-primer |
| \textit{F. tularensis} LVS *ahpC* downstream fragment | MP245 | 5’-CAAAACAAAGGAGATGACATGACAGAGTCTAAGTTATAGAAAGG-3’ | Forward primer |
| | MP246’ | 5’-tgaatcgtgacGACTAGCTGCCCCTACACTGTTTTA-3’ | Reverse-primer with a \textit{SalI} site |
| \textit{F. tularensis} ΔahpC mutant screening | MP260 | 5’-AATGCAGGTTGGGCTGACAA-3’ | Forward primer for *ahpC* |
| | MP261 | 5’-CCGCCAGAAAAACTTACAGTACTACAA-3’ | Reverse primer for *ahpC* |
| Transcomplementation construct | For transcomplementation of \textit{F. tularensis} LVS ΔahpC mutant | MP274’ | 5’-CAAgatccATGACTAAAAGTTACCTAATG-3’ | Forward primer for *ahpC* with a \textit{BamHI} site |
| | MP275’ | 5’-TGATctcgagTTACTAGACTGTACACCTAATAATCTCAA-3’ | Reverse primer for *ahpC* with an \textit{Xhol} site |

*Underlined lowercase letters denote the restriction enzyme site.

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disc); organic peroxides TBH (3.5mg/disc); CHP (500μg/disc); and H₂O₂ (50 mM/disc) (Sigma Aldrich, St. Louis, MO) were used. The plates were incubated at 37˚C in the presence of 5% CO₂ for 48 hours. The zone of inhibition around the discs was measured in millimeters (mm).

**Spot assays**

Spot assays were performed to determine the sensitivities of *F. tularensis* LVS, the ΔahpC mutant and the ΔahpC+pahpC toward superoxide-generating compounds, peroxides, and RNS generating compounds. Serial two-fold dilutions of superoxide-generating compound menadione (starting concentration 62.5 μg), pyrogallol (155 μg), paraquat (155 μg), TBH (34.7 μg), CHP (27.4 μg), H₂O₂ (4.4mM), sodium nitroprusside (SNP) (375 μg) (Ricca Chemical Company, Arlington, TX) and Sin-1 (0.5 μg) (EMD Millipore corporation, Temecula, CA) were made in a sterile flat bottom 96-well plate in 100 μL volume of MHB. The ΔsodC and ΔkatG mutants of *F. tularensis* LVS were also tested using similar concentrations of menadione, TBH and CHP. The bacterial suspensions of *F. tularensis* LVS, ΔahpC mutant, and the ΔahpC+pahpC cultures grown on MH chocolate agar plate were resuspended in MHB and adjusted to an OD₆₀₀ of 0.2. 100μL of bacterial suspensions were added to each well and mixed. The *F. tularensis* SchuS4 and SchuS4 ΔahpC, ΔsodC and ΔkatG mutants were exposed to 2-fold diluted menadione (Starting concentration 12.5 μg), TBH (875 μg), CHP (62.5 μg), SNP (15.7 μg) and Sin-1 (12.5μg) to test their sensitivities towards these compounds. The plates were incubated at 37˚C in the presence of 5% CO₂ for 1 and 3 hours post-exposure, and 3μL bacterial cultures from each dilution were spotted on MH-chocolate agar plates using a multi-channel pipette. The sensitivity to the compounds tested was determined on the basis of observable growth pattern on the plates after 48 hours of incubation.

**Cell culture assays**

A murine macrophage cell line Raw264.7 was used in cell culture-based assays. The macrophages were infected with the wild-type *F. tularensis* LVS, the ΔahpC mutant, and the ΔahpC+pahpC transcomplemented strain at a multiplicity of infection (MOI) of 10 and 100 in a volume of 1 mL bacterial suspension. In separate experiments, Raw264.7 macrophages were infected with the wild-type *F. tularensis* SchuS4, the ΔahpC, ΔsodC, and ΔkatG mutants at an MOI of 100 as described previously [5,14]. The infected cells were lysed after 4 and 24 hours of infection with 0.1% sodium deoxycholate, diluted 10-fold in sterile PBS and plated on MH-chocolate agar plates. The plates were incubated at 37˚C in the presence of 5% CO₂ for 1 and 3 hours post-exposure, and the colonies were counted. Results were expressed as Mean ± SD of three biological replicates and presented as Log₁₀ colony forming units (CFU)/mL.

**Mouse challenge studies**

All mice studies followed the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of New York Medical College. Six to eight-week-old wild type C57BL/6 and gp91phox⁻/⁻ mice were obtained from Jackson Laboratories. Mice were maintained in a pathogen-free environment in the Animal Facility of New York Medical College (Valhalla, NY) Mice (n = 4 mice/group) were deeply anesthetized by intraperitoneal injection of Ketamine/Xylazine cocktail. The loss of reflexes in anesthetized mice was determined by the loss of toe-pincher reflex. The deeply anesthetized mice were inoculated intranasally with 1x10⁴ CFU of the wild-type *F. tularensis* LVS strain or the ΔahpC mutant resuspended in 20μL PBS (10μL/nare). The infected mice were observed for morbidity and mortality for 21 days. The survival
results were plotted as Kaplan-Meier survival curves, and the data were analyzed statistically by the Log-rank test.

**Statistical analysis**

Statistical analysis was performed by using GraphPad Prism and InStat software. The results were expressed as Mean ± S.E.M. or S.D., and statistical significance between groups was determined by one-way ANOVA followed by Bonferroni’s corrections or student t-test. As detailed earlier, the survival results were expressed as Kaplan-Meier survival curves, and P values were determined by the Log-rank test.

**Results**

The peroxiredoxin of the AhpC/TSA family (ahpC) gene in *F. tularensis* LVS and SchuS4 is transcribed divergently from the LysR family oxidative stress transcriptional regulator gene, oxyR. A similar genomic organization of ahpC gene is also present in *Mycobacterium tuberculosis*. However, in other bacterial pathogens including *Yersinia pestis*, the ahpC gene is not transcribed divergently from the oxyR gene (Fig 1A). To characterize the functional role of the peroxiredoxin AhpC of *F. tularensis* LVS, we generated a gene deletion mutant of ahpC (ΔahpC). The deletion of the ahpC gene was confirmed by PCR followed by DNA sequencing to determine that ahpC gene deletion did not alter reading frames of the downstream genes. A transcomplement of the ΔahpC mutant was generated by providing a copy of ahpC gene intras. Transcomplementation was confirmed by PCR using ahpC gene-specific primers. The ΔahpC mutant was tested for any growth defect under aerobic growth conditions. It was observed that growth pattern of the ΔahpC mutant was identical to that of the wild type *F. tularensis* LVS or the transcomplemented strain when grown aerobically indicating that the loss of ahpC is not associated with any growth defect in the ΔahpC mutant (Fig 1B).

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**Fig 1.** Genomic organization and growth characteristics of the ΔahpC mutant of *F. tularensis* LVS. (A) Genomic organization of the ahpC gene. (B) Growth curves of *F. tularensis* LVS, the ΔahpC mutant and the transcomplemented strains (ΔahpC + pahpC). Equal numbers of bacteria were suspended in Mueller-Hinton broth, and the optical densities (OD₆₀₀) were recorded every 4 hours.

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The ΔahpC mutant of F. tularensis LVS exhibits enhanced sensitivities towards superoxide-generating compounds

The contribution of AhpC of F. tularensis LVS in conferring resistance to superoxide-generating compounds menadione, pyrogallol and paraquat were determined by disc diffusion and spot assays. The ΔahpC mutant of F. tularensis LVS revealed enhanced sensitivities towards superoxide-generating compounds as indicated by significantly larger zones of inhibition around the discs impregnated with menadione (21.6±1.4 mm), pyrogallol (11.6±0.6 mm) and paraquat (26.0±1.0 mm) as compared to those observed for wild type F. tularensis LVS (18.0±0.2, 10.3±0.6, 23.0±1.0 mm, respectively) and the transcomplemented strain (17.1±0.6, 10.0±0.0, 25.6±1.5 mm, respectively) (Fig 2A, 2B and 2C). Similar to the ΔahpC mutant, the ΔsodC and ΔkatG mutants of F. tularensis LVS also exhibited increased susceptibility towards menadione (S1A and S1B Fig).

We next confirmed the results obtained with the disc diffusion assays by performing spot assays that determine the bacterial viability. Wild type F. tularensis LVS, the ΔahpC mutant or...
the transcomplemented strains were exposed to varying concentrations of two-fold serial dilutions of menadione, pyrogallol and paraquat for 1 and 3 hours, and plated to determine the bacterial viability. Reduced viability of the ΔahpC mutant was observed after 1 and 3 hours of exposure to menadione (31.25 and 15.62 μg, respectively), pyrogallol (38.8 and 19.4 μg, respectively) and paraquat (0.30 and 0.15 μg, respectively) as compared to the wild type F. tularensis LVS corroborating the results observed with the disc diffusion assays. The transcomplementation either restored the wild type phenotype or exhibited an intermediate phenotype (Fig 2D, 2E and 2F). Collectively, these results demonstrate that loss of AhpC in F. tularensis LVS is associated with enhanced sensitivities towards the superoxide-generating compounds.

The ΔahpC mutant of F. tularensis LVS exhibits enhanced sensitivity towards organic peroxides and H$_2$O$_2$

The contribution of AhpC of F. tularensis LVS in conferring resistance to organic peroxides TBH and CHP, and H$_2$O$_2$ was determined by disc diffusion assay. The ΔahpC mutant of F. tularensis LVS revealed enhanced sensitivities towards organic peroxides TBH (19.6±1.5 mm), CHP (20.0±1.0 mm) and H$_2$O$_2$ (12.0±0.0 mm) as observed by significantly larger zones of inhibition around the discs impregnated with these compounds as compared to those observed for the wild type F. tularensis LVS (14.3±2.0, 15.3±0.6, 10.0±0.0 mm, respectively) or the transcomplemented strain (15.0±1.0, 17.0±1.0, 10.6±0.5 mm, respectively) (Fig 3A, 3B, and 3C). Similar to the ΔahpC mutant, the ΔsodC and ΔkatG mutants of F. tularensis LVS also exhibited increased susceptibilities towards TBH and CHP (S1C, S1D, S1E and S1F Fig).

We confirmed the results obtained with the disc diffusion assays by performing spot assays and by generating growth curves in the presence of H$_2$O$_2$. The wild type F. tularensis LVS, the ΔahpC mutant or the transcomplemented strains were exposed to varying concentrations of serially diluted TBH, CHP, and H$_2$O$_2$ for 1 and 3 hrs and plated to determine the bacterial viability. The viability of the ΔahpC mutant of F. tularensis LVS was markedly reduced after 1 and 3 hours of exposure to TBH (1.1 μg), CHP (13.7 and 0.9 μg, respectively) and H$_2$O$_2$ (0.2 and 0.05 mM, respectively) as compared to the wild-type F. tularensis LVS. Transcomplementation of the ΔahpC mutant restored the wild type phenotype (Fig 3D, 3E and 3F). The ΔahpC mutant grew very slowly as compared to the wild type or the transcomplemented counterparts when grown in the presence of 750 μM of H$_2$O$_2$ (Fig 3G). Collectively, these results demonstrate that AhpC of F. tularensis LVS plays an important role in providing resistance against organic peroxides and H$_2$O$_2$.

The ΔahpC mutant of F. tularensis LVS exhibits enhanced sensitivity towards RNS

Our preceding results demonstrated that AhpC of F. tularensis LVS provides resistance against superoxide-generating compounds and peroxides. We further tested the role of AhpC in providing resistance against RNS by using nitric oxide (NO) donor sodium nitroprusside (SNP) and Sin-1. Wild type F. tularensis LVS, the ΔahpC mutant or the transcomplemented strains were exposed to varying concentrations of serially diluted SNP and Sin-1 for 1 and 3 hours and plated to determine the bacterial viability. The ΔahpC mutant of F. tularensis LVS was found to be highly sensitive to both SNP (93.8 μg) and Sin-1 (0.1 and 0.05 μg, respectively) as evidenced by marked reduction in viability after 1 and 3 hours of exposure to these compounds as compared to the wild type or the transcomplemented strain (Fig 4A and 4B). These results demonstrate that AhpC of F. tularensis LVS also plays an important role in providing resistance against RNS.
Fig 3. The ΔahpC mutant of *F. tularensis* LVS exhibits enhanced sensitivity towards organic peroxides and H₂O₂. The sensitivities of the wild type *F. tularensis* (Ft) LVS, the ΔahpC mutant, and the transcomplemented strain ΔahpC+pahpC as determined by disc diffusion and spot assays against organic peroxides tert-butyl hydroperoxide (TBH) (A and D), cumene hydroperoxide (CHP) (B and E) and H₂O₂ (C and F). For disc diffusion assays, the results are expressed as zone of inhibition in millimeters obtained using the indicated concentrations of the compounds and are expressed as Mean ± S.D. of triplicate samples. In spot assays, *Francisella* strains were exposed...
The ΔahpC mutant of *F. tularensis* LVS does not exhibit intramacrophage growth defect but is attenuated for virulence in mice

The contribution of AhpC of *F. tularensis* LVS in intramacrophage survival was determined by macrophage gentamicin protection assay. Almost equal numbers of wild type *F. tularensis* LVS, the ΔahpC mutant, and the transcomplemented bacteria invaded the cells at 4 hours post-infection. Nearly 2-fold fewer ΔahpC mutant bacteria as compared to the wild type *F. tularensis* LVS were recovered from macrophages infected with 10 MOI. Similarly, after 24 hours of infection, 2-fold fewer ΔahpC mutant bacteria (8.7±0.1 Log_{10} CFU/mL) as compared to the wild type *F. tularensis* LVS (8.9±0.0 Log_{10} CFU/mL) were recovered from the macrophages infected with 100 MOI. However, the fold-increase at 24 hours for both the wild type *F. tularensis* LVS and the ΔahpC mutant remained similar. These results indicate that *ahpC* is not required for intramacrophage survival of *F. tularensis* LVS (Fig 5A).

We next examined the contribution of AhpC of *F. tularensis* LVS in virulence in mice. Since our preceding results indicated that the ΔahpC mutant is highly sensitive to ROS, we also determined the contribution of NADPH oxidase-dependent ROS in clearance of ΔahpC mutant of *F. tularensis* LVS by infecting *Phox^-/-* mice. These mice are defective in ROS generation. Wild-type C57BL/6 and *Phox^-/-* mice were infected intranasally with 1×10^4 CFUs of...
either the wild-type *F. tularensis* LVS or the ΔahpC mutant and observed for mortality for 21 days. 100% of wild type C57BL/6 mice infected with the ΔahpC mutant survived the infection; while mice infected with similar doses of the wild type *F. tularensis* LVS succumbed to infection by day 8 post-infection, indicating that AhpC is required for virulence. On the other hand, 100% of *Phox*−/− mice infected either with *F. tularensis* LVS, or the ΔahpC mutant succumbed to infection indicating that NADPH-oxidase induced ROS is required for clearance of the ΔahpC mutant (Fig 5B).

**AhpC of *F. tularensis* SchuS4 is a major antioxidant enzyme that protects against oxidative stress induced by superoxide-generating compounds**

Previous studies conducted with mutants of *F. tularensis* LVS deficient in SodB, SodC, or KatG have reported that loss of only one antioxidant enzyme results in an enhanced sensitivity
of *F. tularensis* LVS to oxidative stress, attenuated intramacrophage growth and virulence in mice [7,8,11]. The results obtained in this study with the ΔahpC mutant of *F. tularensis* LVS also support this notion. On the contrary, the reported phenotype of the SchuS4 ΔkatG mutant is quite different from that reported for the corresponding mutant of *F. tularensis* LVS [11]. Moreover, unlike *F. tularensis* LVS mutants, the ΔkatG, ΔsodC and ΔahpC mutants of *F. tularensis* SchuS4 retain their virulence in mice [10,11,16]. We next investigated to establish if AhpC is one of the major antioxidant enzymes of *F. tularensis* SchuS4 by determining the sensitivities of the ΔahpC, ΔsodC and ΔkatG mutants of *F. tularensis* SchuS4 to oxidants and RNS.

Exposure of *F. tularensis* SchuS4, the ΔahpC, ΔsodC and the ΔkatG mutants to the superoxide-generating compound menadione revealed that the ΔahpC mutant was extremely sensitive to menadione as evident by significantly enlarged zone of inhibition (25.3±1.1 mm) as compared to the wild type *F. tularensis* SchuS4, ΔsodC and ΔkatG mutants (6.0±0.0 mm for all the three strains, respectively). No differences in sensitivity towards menadione were observed between the wild type *F. tularensis* SchuS4 or the ΔsodC and the ΔkatG mutants (Fig 6A). We further confirmed these findings by performing spot- and bacterial killing assays. Results from the spot assays (Fig 6B) demonstrated that exposure to increasing concentrations of menadione resulted in reduced viability of the ΔahpC mutant as compared to wild type *F. tularensis* SchuS4, or the ΔkatG mutant. In another approach, equal numbers of wild type *F. tularensis* SchuS4 and the ΔahpC mutant were exposed to menadione (6.25μg/mL) for 1 and 4 hours, diluted 10-fold, and the bacterial killing was determined. The results demonstrated that after 1-hour post-treatment with menadione, significantly lower numbers of the ΔahpC mutant bacteria (4.7±0.1 Log10 CFU/mL) survived as compared to the wild type *F. tularensis* SchuS4 strain (6.7±0.3 Log10 CFU/mL). After 4 hours of treatment, no colonies of the ΔahpC mutant were recovered, while the viability of the wild type *F. tularensis* SchuS4 was only reduced by 10-fold (5.8±0.1 Log10 CFU/mL). The viability of both *F. tularensis* SchuS4 and the ΔahpC mutant were not affected in the PBS control or exposure to the volume of ethanol that was used to resuspend menadione (Fig 6C).

Exposure to paraquat resulted in a significantly larger zone of inhibition for the ΔahpC mutant (31.67 ± 1.53 mm) as compared to the wild type *F. tularensis* SchuS4 strain (26.3±0.5 mm). However, treatment of ΔsodC (26.0±1.0 mm) and ΔkatG (28.0±1.0 mm) mutant strains with paraquat did not show any enhanced sensitivity as compared with the wild type *F. tularensis* SchuS4 (Fig 6D). Disc diffusion assays using pyrogallol (250 and 500μg/disc) displayed similar results, with ΔahpC mutant strain showing a significantly enlarged zone of inhibition (21.6 ±1.5 and 27.3±1.5 mm, respectively) as compared to the wild type *F. tularensis* SchuS4 strain (17.6±1.1 and 21.3±0.5 mm, respectively). Further, similar to paraquat, the ΔsodC (18.6±0.5 and 23.0±2.0 mm, respectively) and ΔkatG (16.3±1.5 and 21.3±0.5 mm, respectively) mutant strains did not show any increased sensitivity to pyrogallol when compared with the wild type *F. tularensis* SchuS4 (Fig 6E). Collectively, these results indicate that AhpC of *F. tularensis* SchuS4 is primarily responsible for providing resistance against oxidative stress induced by superoxide radicals. These results also demonstrate that both the SodC and KatG are dispensable, as the loss of these antioxidant enzymes do not alter the sensitivities of the ΔsodC and ΔkatG mutants to superoxide-generating compounds and remain similar to the wild type *F. tularensis* SchuS4 strain.

AhpC of *F. tularensis* SchuS4 protects against oxidative stress induced by peroxides

Disc diffusion assays using peroxides TBH, CHP and H2O2 exhibited results similar to those observed following treatment with superoxide-generating compounds. Exposure of ΔahpC
mutant to 3.5mg/disc of TBH demonstrated a significantly larger zone of inhibition (28.00 ± 2.0 mm) as compared to the wild type F. tularensis SchuS4 strain (9.3±1.1mm) (Fig 7A). However, the ΔsodC mutant strain (6.0±0.0mm) was observed to be more resistant to TBH than the wild type SchuS4 strain. The susceptibility of the ΔkatG mutant (10.0±0.0 mm) to TBH treatment remained similar to that observed for the wild type F. tularensis SchuS4. The spot assay demonstrated similar results as observed for the disc diffusion assays; the ΔahpC mutant was more sensitive to increasing concentrations of TBH than the wild type F. tularensis SchuS4 strain. However, the sensitivity of the ΔkatG mutant remained similar to that observed for the wild type F. tularensis SchuS4 strain (Fig 7B).

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Fig 6. AhpC of F. tularensis SchuS4 is a major antioxidant enzyme that protects against oxidative stress induced by superoxide generating compounds. The sensitivities of the wild type F. tularensis (Ft) SchuS4, the ΔahpC, ΔsodC and the ΔkatG mutants of SchuS4 as determined by disc diffusion (A), spot assay (B) and bacterial killing assay (C) against superoxide-generating compound, menadione. The sensitivity of the indicated strains against paraquat (D) and pyrogallol (E) was determined using the indicated concentration of the compounds by disc diffusion assay. For the disc diffusion assays, the results are expressed as a zone of inhibition in millimeters and are expressed as Mean ± S.D. The red arrows in (B) indicate enhanced killing of the SchuS4 ΔahpC mutant at the indicated concentrations of menadione. For bacterial killing assay (C) indicated bacterial strains were exposed to menadione (6.25μg/mL) and the bacterial numbers were enumerated after 1 and 4 hours of exposure. PBS, and ethanol required for suspension of menadione were used as controls. The data shown are representative of 2 independent experiments each conducted with 3 biological replicates and were analyzed by one-way ANOVA. **P<0.01; ***P<0.001.
Exposure of wild type \textit{F. tularensis} SchuS4 and the \textit{ΔahpC}, \textit{ΔsodC} and the \textit{ΔkatG} mutants to 500μg/disc of CHP demonstrated that the \textit{ΔahpC} mutant was significantly more sensitive to the compound (29.0 ± 1.0 mm) as compared to the wild type \textit{F. tularensis} SchuS4 (19.3 ± 1.1 mm). The sensitivity of the \textit{ΔkatG} mutant to CHP (16.6 ± 1.5 mm) remained similar to that observed for the wild type \textit{F. tularensis} SchuS4 strain (Fig 7C) as determined by the disc diffusion assays as well as by spot assay (Fig 7D). The \textit{ΔahpC} mutant demonstrated higher sensitivity to 50mM/disc of \textit{H}_{2}\text{O}_{2} as indicated by a greater zone of inhibition (28.00±1.4 mm) compared to the wild type \textit{F. tularensis} SchuS4 strain (Fig 7E), whereas, the sensitivities of the \textit{ΔsodC} and \textit{ΔkatG} mutants remained similar to those observed for the wild type SchuS4 strain (20.5±0.7 and 21.0 ±1.1 mm, respectively). Collectively, these results indicate that the requirement of AhpC for resistance against oxidative stress induced by superoxide radicals and peroxides.

Fig 7. AhpC of \textit{F. tularensis} SchuS4 protects against oxidative stress induced by peroxides. The sensitivities of the wild type \textit{F. tularensis} (Ft) SchuS4, the \textit{ΔahpC}, \textit{ΔsodC} and the \textit{ΔkatG} mutants of SchuS4 as determined by disc diffusion assays against tert-butyl hydroperoxide (TBH) (A), cumene hydroperoxide (CHP) (C) and \textit{H}_{2}\text{O}_{2} (E), and by spot assays against TBH (B) and CHP (D). For the disc diffusion assays, the results are expressed as zone of inhibition in millimeters and are expressed as Mean ± S.D. The red arrows in (B and D) indicate enhanced killing of the \textit{ΔahpC} mutant at the indicated concentrations of the compounds. The data shown are representative of two independent experiments each conducted with 3 biological replicates and were analyzed by one-way ANOVA, and \( p \) values were recorded. * \( P < 0.05; ** P < 0.001.

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Exposure to NO-generating compounds results in the enhanced killing of ΔahpC mutant of *F. tularensis* SchuS4

We next investigated the role of *F. tularensis* SchuS4 antioxidants in providing resistance to RNS. Results of this assay demonstrated that the ΔahpC mutant was highly sensitive to increasing concentrations of SNP and SIN-1 (Fig 8A and 8B) as compared to the wild type *F. tularensis* SchuS4 or the ΔkatG mutant. However, the ΔsodC mutant showed enhanced resistance to SNP as compared to the wild type *F. tularensis* SchuS4 strain (Fig 8A). These results demonstrate that AhpC in addition to ROS also protects *F. tularensis* SchuS4 against RNS.

The ΔahpC mutant of *F. tularensis* SchuS4 is attenuated for intramacrophage growth

To determine the role of *F. tularensis* SchuS4 antioxidants in intramacrophage survival, we infected Raw264.7 macrophages with the wild type *F. tularensis* SchuS4 and the ΔahpC, ΔsodC and the ΔkatG mutants at an MOI of 100 and lysed the cells 4 and 24 hours post-infection. It was observed that significantly lower numbers of the ΔahpC mutant bacteria (6.2±0.1 Log_{10}...
CFU/mL) were recovered from Raw264.7 cells at 24 hours post-infection as compared to the wild type *F. tularensis* (Ft) SchuS4 strain (6.9±0.1 Log10 CFU/mL). Higher numbers of ΔkatG mutant bacteria were taken up by the macrophages as compared to the wild type *F. tularensis* SchuS4, the ΔahpC, ΔsodC, and the ΔkatG mutants at 4 hours post-infection. However, both ΔkatG and ΔsodC mutants survived and replicated similarly to the wild type *F. tularensis* SchuS4 strain and equal numbers of bacteria (7.0±0.1 and 7.0±0.2 Log10 CFU/mL, respectively) were recovered at 24 hours post-infection. The wild type *F. tularensis* SchuS4 and the ΔsodC bacteria showed a 25-fold increase at 24 hours post-infection than those recovered from macrophages after 4 hours of infection. The ΔkatG mutants exhibited 20-fold increase; while the ΔahpC mutants increased by 17-fold at 24 hours post-infection. These results demonstrate that AhpC contributes to intramacrophage growth of *F. tularensis* SchuS4 (Fig 9).

**Discussion**

*Francisella tularensis* during its intracellular residence encounters a number of oxidative and nitrosative stresses. To overcome these, *F. tularensis* has evolved a multitude of mechanisms. *Francisella* counts the phagocyte induced oxidative stress by relying on two divergent approaches; neutralize the ROS/RNS produced by the phagocytic cells and inhibit the assembly of NADPH oxidase [21]. The roles of the primary antioxidant enzymes SodB, SodC and KatG of *F. tularensis* LVS have been characterized in previous studies [7,11,22]. It has been reported that these antioxidant enzymes are required for resistance of *F. tularensis* LVS against oxidative...
Oxidative stress resistance mechanisms of *Francisella tularensis*

Majority of Gram-negative bacteria encode AhpC belonging to 2-Cys peroxiredoxins to protect bacteria from ROS and RNS-induced cell damage [29]. A conserved peroxidatic cysteine in AhpC reacts with H$_2$O$_2$ or organic peroxides to form sulfenic acid and then subsequently releases water or the corresponding alcohols. The oxidized AhpC is reduced and regenerated by an NADH-dependent oxidoreductase AhpF [29]. The AhpC of *F. tularensis* differs from other members of the peroxiredoxin family of proteins. *F. tularensis* AhpC is a 1-Cys peroxiredoxin containing a conserved peroxidatic cysteine; however, it lacks the resolving cysteine as well as the reducing partner AhpF. Similar to *F. tularensis*, AhpC in mycobacteria...
protects against RNS and hydroperoxides [30]. Mycobacterial AhpC catalyzes the conversion of ONOO\(^{-}\) to nitrite very rapidly and prevents its spontaneous decomposition into highly microbicidal nitrogen dioxide and hydroxyl radicals [31]. However, unlike \textit{F. tularensis}, the \textit{M. tuberculosis} AhpC is a 3-Cys peroxiredoxin containing the peroxidatic cysteine, the putative resolving cysteine and the third cysteine with unknown catalytic role [32]. The peroxidatic cysteine of the mycobacterial AhpC attacks ONOO\(^{-}\) and gets oxidized to cysteine sulfenic acid residues; while the resolving cysteine completes the catalytic cycle. A thioredoxin-like protein known as AhpD reduces the oxidized AhpC in mycobacteria [31]. The mechanisms through which the AhpC of \textit{F. tularensis} neutralizes ONOO\(^{-}\) in the absence of a resolving cysteine and how AhpC is regenerated in \textit{F. tularensis} in the absence of AhpD/AhpF homologs is yet to be elucidated.

Collectively, this study highlights differences in antioxidant defense mechanisms of \textit{F. tularensis} LVS and SchuS4 and their abilities to counter oxidative and nitrosative stresses. Nearly 4–5 times the concentration of oxidants and RNS generating compounds used for \textit{F. tularensis} LVS were required to get tangible results with \textit{F. tularensis} SchuS4 mutants. One hundred percent of the wild type \textit{F. tularensis} LVS bacteria were killed when the concentrations of the compounds used in assays with \textit{F. tularensis} SchuS4 were applied. However, these concentrations either did not affect or only moderately affected the viability of \textit{F. tularensis} SchuS4. To conclude, our results demonstrate that AhpC of \textit{F. tularensis} LVS confers resistance against a wide range of ROS and RNS, and serves as a virulence factor. In highly virulent \textit{F. tularensis} SchuS4 strain, AhpC serves as a key antioxidant enzyme and contributes to its robust oxidative and nitrosative stress resistance, and intramacrophage survival. It also becomes evident from these results that \textit{F. tularensis} SchuS4 can compensate for the loss of KatG and SodC with other antioxidant enzymes, but may not do so when AhpC is absent. The results from this study further indicate that differences in virulence attributes of \textit{F. tularensis} LVS and SchuS4 may be due to the inherent differences in their antioxidant defense mechanisms.

**Supporting information**

\textbf{S1 Fig.} The sensitivities of the wild type \textit{F. tularensis} (Ft) LVS, the \textit{ΔsodC} mutant, and the \textit{ΔkatG} mutants. The sensitivities of \textit{Ft} LVS, \textit{ΔsodC} mutant, and the \textit{ΔkatG} mutants were determined by disc diffusion and spot assays against superoxide-generating compounds menadione (A and B), TBH (C and D), and CHP (E and F). For disc diffusion assays, the results are expressed as a zone of inhibition in millimeters obtained using the indicated concentrations of the compounds and are expressed as Mean ± S.D. of triplicate samples. All the results shown are representative of 3 independent experiments conducted. The \(p\) values were determined by one-way ANOVA and a \(p\)-value of <0.05 is considered statistically significant. \(^*p<0.05; \quad **p<0.01; \quad ***p<0.001.\)

\(\text{TIF}\)

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