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Magali Hébrard, Julie Viala, Stéphane Méresse, Frédéric Barras, Laurent Aussel. Redundant Hydrogen Peroxide Scavengers Contribute to Salmonella Virulence and Oxidative Stress Resistance. Journal of Bacteriology, 2009, 191, pp.4605 - 4614. 10.1128/jb.00144-09 . hal-03269585

HAL Id: hal-03269585
https://amu.hal.science/hal-03269585

Submitted on 24 Jun 2021

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Redundant Hydrogen Peroxide Scavengers Contribute to *Salmonella* Virulence and Oxidative Stress Resistance▽†

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***Salmonella enterica* serovar Typhimurium is an intracellular pathogen that can survive and replicate within macrophages. One of the host defense mechanisms that *Salmonella* encounters during infection is the production of reactive oxygen species by the phagocyte NADPH oxidase. Among them, hydrogen peroxide (H_2O_2) can diffuse across bacterial membranes and damage biomolecules. Genome analysis allowed us to identify five genes encoding H_2O_2 degrading enzymes: three catalases (KatE, KatG, and KatN) and two alkyl hydroperoxide reductases (AhpC and TsaA). Inactivation of the five cognate structural genes yielded the HpxF$^-$ mutant, which exhibited a high sensitivity to exogenous H_2O_2 and a severe survival defect within macrophages. When the phagocyte NADPH oxidase was inhibited, its proliferation index increased 3.7-fold. Moreover, the overexpression of *katG* or *tsaA* in the HpxF$^-$ background was sufficient to confer a proliferation index similar to that of the wild type in macrophages and a resistance to millimolar H_2O_2 in rich medium. The HpxF$^-$ mutant also showed an attenuated virulence in a mouse model. These data indicate that *Salmonella* catalases and alkyl hydroperoxide reductases are required to degrade H_2O_2 and contribute to the virulence. This enzymatic redundancy highlights the evolutionary strategies developed by bacterial pathogens to survive within hostile environments.**

Salmonella is a facultative intracellular pathogen that is associated with gastroenteritis, septicemia, and typhoid fever. This gram-negative bacterium survives and replicates in macrophages during the course of infection and can be exposed to a number of stressful environments during its life cycle (16). One of the host defense mechanisms that *Salmonella* encounters upon infection is the production of superoxide anion $O_2^{\bullet -}$ by the phagocyte NADPH oxidase (1, 25). This radical can pass the outer membrane of the bacteria and represents one of the major weapons used by the macrophage to kill engulfed pathogens (18). Evidence that phagocyte-produced superoxide is a key mechanism for avoiding *Salmonella* infection is clear: mice and humans who are genetically defective in superoxide production are significantly more susceptible to infection (36, 38). Superoxide dismutases, located in the bacterial periplasm and in the cytoplasm, dismutate superoxide $O_2^{\bullet -}$ to hydrogen peroxide H_2O_2 and molecular oxygen. Unlike superoxide, hydrogen peroxide can diffuse readily across bacterial membranes and form $HO^{\bullet}$ hydroxyl radicals in the presence of Fe(II) (18). These reactive oxygen species (ROS) can oxidize and damage proteins, nucleic acids, and cell membranes.

To scavenge and degrade H_2O_2 molecules generated either as a by-product of aerobic metabolism or by the phagocyte NADPH oxidase, *Salmonella* has evolved numerous defense mechanisms. The KatE and KatG catalases are involved in H_2O_2 degradation, with *katE* being described as a member of the RpoS regulon (17, 22) and *katG* being OxyR dependent (26, 39). Both enzymes share the ability to reduce hydrogen peroxide to water and molecular oxygen, and their role was shown to be predominant at millimolar concentrations of H_2O_2 since they do not require any reductant (32). This observation is of particular importance, since these enzymes are not limited by the availability of a reductant, such as NADH, which cannot be generated fast enough to face a burst of H_2O_2. However, the *katG* and *katE* simple mutants, as well as the *katE katG* double mutant, did not show any increased susceptibility in macrophage or virulence attenuation in mice (5, 27). A possible reason could be the presence of a third nonheme and manganese-dependent catalase called KatN (30). This enzyme may contribute to hydrogen peroxide resistance under certain environmental conditions, but its involvement in virulence remains unknown. Moreover, *katE*, *katG*, and *katN* single mutants did not show any susceptibility to exogenous millimolar H_2O_2, essentially due to the compensatory function of the remaining catalases (5, 30).

Another family of enzymes was shown to play an alternative role in H_2O_2 scavenging: the alkyl hydroperoxide reductases. These proteins directly convert organic hydroperoxides to alcohols, e.g., hydrogen peroxide to water. The alkyl hydroperoxide reductase AhpC belongs to the two-cysteine peroxiredoxin family, and the gene encoding this enzyme was identified as a member of the OxyR regulon (26, 39). The redox system consists of two proteins, AhpC and AhpF, with the latter being a thioredoxin reductase-like protein that contains two disulfide centers and transfers electrons from NADH to AhpC (13).

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† Supplemental material for this article may be found at http://jb.asm.org/.

AhpC was shown to be a predominant scavenger at low concentrations of H_2O_2, mainly because its catalytic efficiency was better than those of catalases (32). Recently the alkyl hydroperoxide reductase from *Helicobacter hepaticus*, TsaA (*T*hiol-*S*pecific *A*ntioxidant), was characterized (24). The *tsaA* mutant was found to be more sensitive to oxidizing agents like superoxide anion or *t*-butyl hydroperoxide. Surprisingly, this mutant was more resistant than the wild-type to H_2O_2, essentially because the level of catalase was increased in this background (24). In gastric pathogens, TsaA plays a critical role in the defense against oxygen toxicity that is essential for survival and growth (2). Interestingly, *Salmonella* contains two genes encoding alkyl hydroperoxide reductases, *ahpC* and *tsaA*, whereas a single copy was found in *Escherichia coli* (*ahpC*) or in *Helicobacter pylori* (*tsaA*).

The redundancy of these antioxidant proteins could explain the extremely high resistance of *Salmonella* to hydrogen peroxide. It has been shown by Imlay and coworkers that in *E. coli*, three genes were involved in H_2O_2 scavenging: two catalase genes (*katE* and *katG*) and an alkyl hydroperoxide reductase gene (*ahpC*) (32). Simultaneous inactivation of the *katE*, *katG*, and *ahpCF* genes negated H_2O_2 degradation. As a consequence, this triple mutant, called the Hpx$^-$ mutant, accumulates intracellular H_2O_2 (32). Moreover, H_2O_2 generated by aerobic metabolism was found to be sufficient to create toxic levels of DNA damage in such a background (28). In the present study, we deleted the *Salmonella katE*, *katG*, and *ahpCF* genes and two more genes absent in *E. coli*, *katN* and *tsaA*, to obtain the HpxF$^-$ mutant, which lacks three catalases and two alkyl hydroperoxide reductases. HpxF$^-$ cells exhibited the incapacity to degrade micromolar concentrations of H_2O_2, whereas this phenotype was not observed for the Kat$^-$ (*katE katG katN*) and Ahp$^-$ (*ahpCF tsaA*) mutants. Therefore, the HpxF$^-$ mutant exhibited a high sensitivity to this compound. Moreover, this mutant did not show any proliferation within macrophages and presented reduced virulence in mice, suggesting that *Salmonella* catalases and alkyl hydroperoxide reductases form a redundant antioxidant arsenal essential for survival and replication within host cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Strains were routinely grown in Luria-Bertani (LB) medium at 37°C. Minimal medium (M9) contained $MgSO_4$ (1 mM), $CaCl_2$ (200 μM), and thiamine (1 μg/ml). As a carbon source, glucose was added at a final concentration of 0.2%. Ampicillin (50 μg/ml), kanamycin (25 μg/ml), and chloramphenicol (25 μg/ml) were added when necessary. Deletions of various genes and concomitant insertion of an antibiotic resistance cassette were carried out using lambda-Red-mediated recombination (11). Mutations were moved to the wild-type strain 12023 by P22 transductions. To avoid the outgrowth of suppressed strains, *katE*, *katG*, *katN*, *ahpCF* and *tsaA* mutations were selected anaerobically on LB agar supplemented with bovine liver catalase (2,000 U/plate; Sigma-Aldrich). In some cases, antibiotic resistance cassettes were removed by using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (11).

In silico genome analysis. The genome analyzed was that of *Salmonella enterica* serovar Typhimurium LT2 (23) (www.genome.jp/dbget-bin/www_bfind?S.typhimurium). STM4106, STM1318, and STM0608 correspond to the catalases KatG and KatE and the alkyl hydroperoxide reductase AhpC, respectively. STM1731 was characterized as a third catalase, KatN (30). STM0402 was annotated as a putative thiol-alkyl hydroperoxide reductase, and we renamed it TsaA (thiol-specific antioxidant) for homology with *Helicobacter hepaticus* TsaA (58.5% identity) (24).

TABLE 1. Bacteria and plasmids used in this study

Bacterium/plasmid	Relevant characteristic(s)	Source or reference
S. Typhimurium strains		
12023	Wild type	Laboratory stock
Ahp$^-$ mutant	Δ*ahpCF*::kan Δ*tsaA*::cat	This study
Kat$^-$ mutant	Δ*katE* Δ*katG* Δ*katN*::kan	This study
HpxT$^-$ mutant	Δ*katE* Δ*katG* Δ*ahpCF*::kan	This study
HpxF$^-$ mutant	Δ*katE* Δ*katG* Δ*katN* Δ*ahpCF* Δ*tsaA*	This study
E. coli strains		
MG1655	F$^-$; wild type	Laboratory stock
Hpx$^-$ mutant	Δ*katE*::tet Δ*katG*::cat Δ*ahpCF*::kan	This study
Plasmids		
pACYC184	cat (Cm^r) tet (Tc^r), p15A, *ori*	7
p*katG*	pACYC184 derivative carrying *katG* and its promoter (Cm^r)	This study
p*tsaA*	pACYC184 derivative carrying *tsaA* and its promoter (Cm^r)	This study
pKD3	Template plasmid; contains an FLP recombination target-flanked chloramphenicol resistance gene (Cm^r)	11
pKD4	Template plasmid; contains an FRT-flanked kanamycin resistance gene (Km^r)	11
pCP20	Thermal induction of FLP synthesis	11
pKD46	Red recombinase expression plasmid	11

Measurement of hydrogen peroxide accumulation in cell culture. Cells were grown overnight in M9 minimal medium containing 0.2% glucose and supplemented with Casamino Acids (1 mg/ml), diluted to an optical density at 600 nm (OD_{600}) of 0.05, and grown for four generations. These cells were then washed in fresh minimal medium containing 0.05% glucose and incubated with shaking at 37°C for 25 min. Aliquots were removed every 5 min, and hydrogen peroxide levels were determined using the Amplex red/horseradish peroxidase method (Invitrogen). The low glucose concentration was used in order to minimize H_2O_2 production by catalyzed glucose autooxidation.

Hydrogen peroxide scavenging by whole cells. Bacterial cultures were grown overnight aerobically in M9 minimal medium supplemented with Casamino Acids, diluted to an OD_{600} of 0.01, and grown to an OD_{600} of 0.3. Cells were diluted in fresh prepared minimal medium at an OD_{600} of 0.1. H_2O_2 was added at a final concentration of 10 μM. Each 90 s, aliquots were removed and assayed immediately for H_2O_2 content by the Amplex red/horseradish peroxidase method (Invitrogen).

Hydrogen peroxide sensitivity assay. Bacterial cultures were grown overnight in LB, diluted to an OD_{600} of 0.05, grown to an OD_{600} of 0.3 in LB, and treated with 1 mM hydrogen peroxide. To determine viability, aliquots were taken immediately before treatment and 1 h and 2 h after treatment, serially diluted, and plated onto LB supplemented with catalase.

Bacterial infection of macrophages and survival assays. RAW 264.7 macrophages were seeded at a density of 4×10^5 cells per well in six-well tissue culture plates containing Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) (HyClone) and supplemented with gamma interferon (IFN-γ) (10 U/ml; ImmunoTools) 24 h before use. Bone marrow cells were isolated from femurs of 8- to 10-week-old C57BL/6 female mice and differentiated into macrophages as described previously (12). Bacteria were cultured overnight at 37°C with shaking and opsonized in DMEM containing FCS and normal mouse serum (10%; Perbio) for 30 min. The macrophages were activated with 0.2 μM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) before infection. Where indicated, diphenyleneiodonium (DPI) (Sigma Aldrich) was added at various concentrations to inhibit NADPH oxidase. Bacteria were added to the monolayers at a MOI of ≈70:1, centrifuged at $400 \times g$ for 5 min at 4°C, and incubated for 30 min at 37°C in 5% CO_2. The macrophages were washed three times and

incubated with DMEM containing FCS and 100 μg/ml gentamicin for 90 min, after which the gentamicin concentration was decreased to 10 μg/ml for the remainder of the experiment. For enumeration of intracellular bacteria, macrophages were washed two times with phosphate-buffered saline and lysed with 0.1% Triton X-100, and a dilution series was plated on LB agar supplemented with catalase.

Competition assays. Eight- to 10-week-old C57/B6 mice were inoculated intraperitoneally with equal amounts of two bacterial strains for a total of 10^5 bacteria per mouse. The spleens were harvested 48 h after inoculation and homogenized. Bacteria were recovered and enumerated after plating a dilution series onto LB agar and LB agar with the appropriate antibiotics, both supplemented with catalase. Competitive indexes (CIs) were determined for each mouse (3). The CI is defined as the ratio between the mutant and wild-type strains within the output (bacteria recovered from the mouse after infection) divided by their ratios within the input (initial inoculum). A one-sample *t* test was used to determine whether the CI was significantly different from 1. All statistical analyses were performed by using the Prism software program (GraphPad, San Diego, CA). The two-tailed *P* value was calculated.

Competition assays in LB medium were performed as described previously (10). Briefly, bacterial mixtures were incubated with equal amounts at 37°C for 24 h under shaking. Cells were diluted and plated on LB agar supplemented with catalase, and the resulting colonies were plated on the selective medium to determine the relative percentage of each strain recovered. The CI was calculated as described above.

RNA extraction and quantitative PCR. RNA extraction from intracellular bacteria was adapted from a previously described method (14). In brief, activated RAW 264.7 macrophages were seeded in 10 tissue-culture-treated plates (128 by 86 mm). The infection was carried out as described above with few modifications: bacteria were added to the monolayers at a MOI of 50:1, and supplemented DMEM containing 66 μg/ml gentamicin was added for 45 min following the three washes. Six hours postinfection, cells were washed twice with phosphate-buffered saline and lysed, and RNA was stabilized with a solution of 0.3% sodium dodecyl sulfate, 1% acidic phenol, and 19% ethanol for 30 min on ice. RNA extraction was performed with the SV Total RNA isolation system (Promega). cDNAs were synthesized from 1 μg RNA with the Superscript II reverse transcriptase and random primers (Invitrogen). Real-time PCR was performed on a Mastercycler ep realplex instrument (Eppendorf) by using the SYBR Premix Ex *Taq* (Perfect Real Time) PCR kit (Takara Bio Group, Japan). Specific primers are described in Table S1 in the supplemental material. Melting curves were analyzed to control for the specificities of the PCRs. Copy numbers were calculated from a standard curve plotting four different dilutions of genomic DNA against the PCR cycle number at which the measured fluorescence intensity reached the threshold, specified so that it was significantly above the noise band of the baseline (10 times the standard deviation).

Plasmid construction. The cloning vector used was pACYC184. The insert carrying the 200-bp-upstream *katG* start codon and the *katG* gene was PCR amplified from *S.* Typhimurium 12023 by using the forward primer 5′-CCCAAGCTTCCGGGAGCTTTATTACAACTC-3′ and the reverse primer 5′-GGGGGATCCCTGGTTGTGCATAACATAGGC-3′. PCR products were digested using BamHI and HindIII and cloned into the pACYC184 vector to generate p*katG*, and the insert was verified by sequencing. The insert carrying the 200-bp-upstream *tsaA* start codon, and the *tsaA* gene was PCR amplified by using the forward primer 5′-CCCTCTAGAAGCGAGGGCGTGCGTCAGATC-3′ and the reverse primer 5′-GGGGGATCCGAAGTGGCGCTGGAAGCGTTG-3′. PCR products were digested using BamHI and XbaI and cloned into the pACYC184 vector to generate p*tsaA*, and the insert was verified by sequencing.

RESULTS

Three catalases and two alkyl hydroperoxide reductases allow *Salmonella* to tolerate an anaerobic/aerobic shift. To assay the importance of *Salmonella* catalases and alkyl hydroperoxide reductases during aerobic metabolism, two mutants were built: a *katE katG ahpCF* mutant (HpxT$^-$, for Hpx$_{three}^-$) and a *katE katG katN ahpCF tsaA* mutant (HpxF$^-$, for Hpx$_{five}^-$). These strains were precultured in LB anaerobically and then diluted in aerobic medium, and their phenotypes were compared to that of the *E. coli katE katG ahpCF* mutant (Hpx$^-$). *Salmonella* HpxT$^-$ cells did not present any growth defect, whereas the HpxF$^-$ mutant initially grew and then stalled for 2 to 3 h, finally catching up at the end of the stationary phase (Fig. 1A). At OD_{600}s of 0.1, 0.5, and 1, aliquots of each culture were removed and observed under a microscope. Whereas *Salmonella* wild-type and HpxT$^-$ cells appeared rod shaped, HpxF$^-$ cells formed long filaments (Fig. 1C). This trend was emphasized at an OD_{600} of 0.5 and correlated with the lag observed during growth but decreased at an OD_{600} of 1 when the cells escaped from the lag and resumed growing. Similar observations were made with the *E. coli* Hpx$^-$ mutant, which presented a growth defect and filamentation when the cells were submitted to an anaerobic/aerobic shift (Fig. 1B and C). Presumably, upon the switch to aerobiosis, cells experience a sudden burst in ROS that catalases and alkyl hydroperoxide reductases are able to scavenge. These results are consistent with the characterization of the *E. coli* Hpx$^-$ strain already reported (28). Taken together, these data indicated that inactivation of three catalases and two alkyl hydroperoxide reductases led to a growth lag and filamentation of *Salmonella* under an aerobic shift.

Viability of the HpxF$^-$ mutant is not affected by endogenous hydrogen peroxide. To establish the impact of endogenous H_2O_2 on *Salmonella* fitness, we built two supplementary mutants, which we referred to as the Kat$^-$ mutant, which lacks three catalases (*katE*, *katG*, and *katN*), and the Ahp$^-$ mutant, which lacks two alkyl hydroperoxide reductases (*ahpCF* and *tsaA*). Then, we evaluated their ability to eliminate endogenously produced H_2O_2. In order to monitor H_2O_2 accumulation generated during aerobic growth, the strains were grown in minimal medium supplemented with Casamino Acids for at least four generations and immediately resuspended in fresh medium. H_2O_2 was then quantified every 5 min over a period of 25 min. In wild-type and Kat$^-$ strains, the H_2O_2 levels remained constant, accumulating at a rate of 1.2 nM/min (Fig. 2A). The Ahp$^-$ mutant also showed only a minor accumulation of H_2O_2 (2 nM/min), reaching a final concentration of 0.14 μM. Within HpxF$^-$ cells, the H_2O_2 concentration increased 2.6-fold in 25 min to reach 0.36 μM, with a rate of 8.8 nM/min (Fig. 2A). Thus, a HpxF$^-$ mutant accumulated much more endogenous H_2O_2 than Kat$^-$ or Ahp$^-$ mutants, underlying the synergistic action of both type of enzymes on H_2O_2 degradation. Then, to estimate the impact of endogenous H_2O_2 accumulation on their fitness, the four bacterial strains were grown aerobically in LB and viability was assayed at various times. Growth was monitored at 600 nm, and the growth curves of the four strains were found to be identical (data not shown). Then, aliquots from each culture were removed at OD_{600}s of 0.5 and 2 and plated on LB agar medium supplemented with catalase. The results showed that the wild type and the three mutants shared identical plating recovery, indicating that none of the mutants presented any viability defect in comparison to its wild-type parent (Fig. 2B). Thus, the HpxF$^-$ mutant clearly accumulated H_2O_2 during growth, but the concentrations reached in LB medium were not high enough to compromise viability. Finally, the wild-type strain and the three mutants (the Ahp$^-$, Kat$^-$, and HpxF$^-$ mutants) were grown in minimal medium under aerated conditions. The growth of all the mutants was slowed down, with HpxF$^-$ being slightly more affected (Fig. 2C). Thereby, the nutrient limitation led to a growth rate reduction for the Ahp$^-$, Kat$^-$, and HpxF$^-$ mutants. Surprisingly, addition of Casamino Acids did not signif-

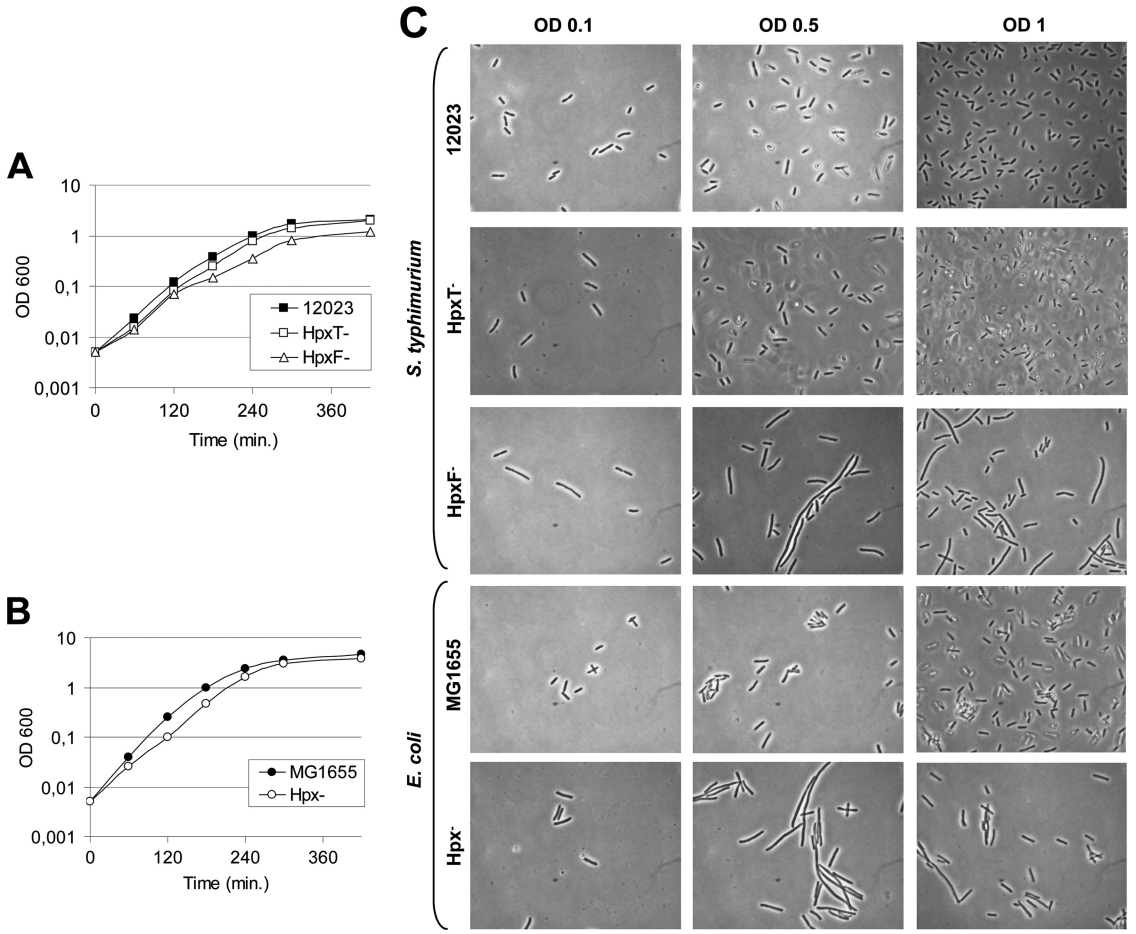


FIG. 1. *E. coli* Hpx$^-$ and *Salmonella* HpxF$^-$ strains share the same growth defect linked to filamentation. *S.* Typhimurium 12023 (wild type; filled squares), *E. coli* MG1655 (wild type; filled circles), *S.* Typhimurium HpxF$^-$ (*katE katG katN aphCF tsaA*; open triangles), *S.* Typhimurium HpxT$^-$ (*katE katG aphCF*; open squares), and *E. coli* Hpx$^-$ (*katE katG aphCF*; open circles) cells were grown in LB medium anaerobically until early log phase. The cells were then diluted in aerobic LB medium to an OD_{600} of 0.05, and aerobic growth was monitored at 600 nm (A and B). At OD_{600}s of 0.1, 0.5, and 1, cells from the five strains were removed for microscopic observations (C) (magnification, ×100).

icantly change the growth rate of the HpxF$^-$ mutant (data not shown). This last observation ruled out the possibility that this mutation led to amino acid auxotrophy as described for the *E. coli sodA sodB* mutant (6).

The HpxF$^-$ mutant is highly sensitive to exogenous hydrogen peroxide. To evaluate the role of catalases and alkyl hydroperoxide reductases when *Salmonella* is submitted to exogenous stress, the wild-type strain and the three mutants were exposed to various exogenous H_2O_2 levels. The bacterial strains were first assayed for their ability to scavenge micromolar concentrations of H_2O_2. Bacterial cultures were grown to an OD_{600} of 0.3 and diluted in minimal medium, and H_2O_2 was added at the final concentration of 10 μM. The wild-type, Ahp$^-$, and Kat$^-$ strains scavenged H_2O_2 in less than 7 min, whereas the HpxF$^-$ mutant failed to do so (Fig. 3A). A wild-type strain heat killed at 80°C displayed a degradation profile similar to that of the HpxF$^-$ mutant (Fig. 3A). These data demonstrated that the combination of mutations in catalase- and alkyl hydroperoxide reductase-encoding genes prevented scavenging of exogenous H_2O_2 at micromolar concentrations. They also highlighted the catalytic efficiency of catalases and alkyl hydroperoxide reductases, since the Ahp$^-$ and Kat$^-$ mutants could completely degrade 10 μM H_2O_2 in a few minutes.

Next, to evaluate the impact of millimolar concentrations of H_2O_2 on *Salmonella* fitness, strains were grown to an OD_{600} of 0.3 in LB medium and treated with 1 mM H_2O_2, and growth was monitored at 600 nm over time. The Kat$^-$ and HpxF$^-$ mutant growth was abolished following the addition of exogenous H_2O_2 (Fig. 3B). In contrast, growth of the wild type and the Ahp$^-$ strain was not affected, reflecting the poor efficiency of AhpC and TsaA in scavenging millimolar concentrations of H_2O_2. Finally, we examined cell viability after exposure to 1 mM H_2O_2. Bacterial cells were collected just before and 1 and 2 h after H_2O_2 treatment. We observed that 2 h after stress, plating recovery of the HpxF$^-$ mutant exhibited a 25-fold drop, from $\approx 10^8$ to 4×10^6 CFU/ml, whereas plating recovery of the wild-type strain showed a 10-fold increase (Fig. 3C). Viability of the Kat$^-$ strain was also affected, since its plating recovery showed a threefold decrease 2 h after stress, whereas the Ahp$^-$ mutant survived as well as its wild-type parent. Differences in plating recovery between the Kat$^-$ and HpxF$^-$ mutants also assigned a scavenging effect to the Ahp enzymes, observed only

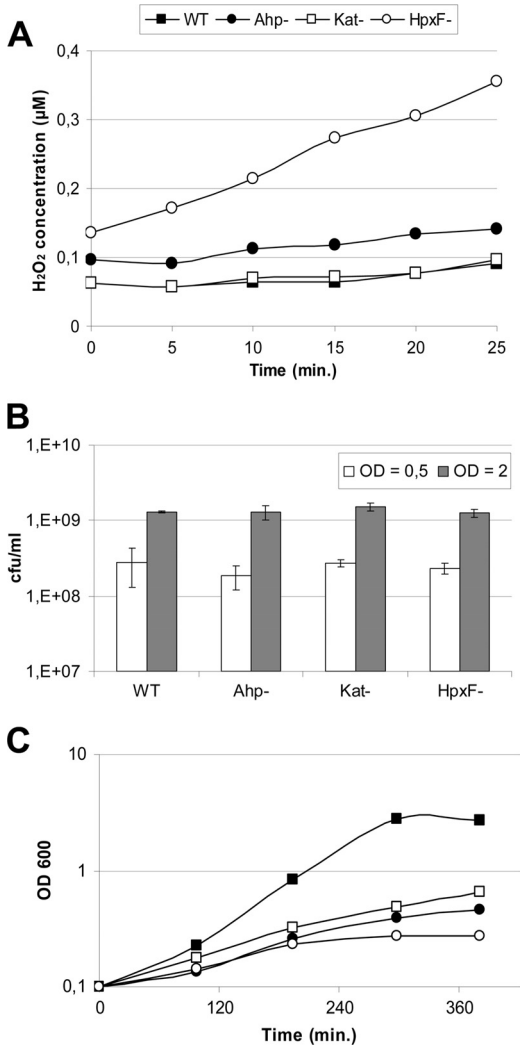


FIG. 2. Endogenous H_2O_2 does not affect HpxF$^-$ mutant viability. (A) Cultures of *Salmonella* Typhimurium 12023 (wild type; filled squares), the Ahp$^-$ mutant (*aphCF tsaA*; filled circles), Kat$^-$ (*katE katG katN*, open squares), and the HpxF$^-$ mutant (*katE katG katN aphCF tsaA*; open circles) were grown overnight aerobically in minimal medium supplemented with Casamino Acids, diluted to an OD of 0.05, and grown for four generations. The cells were then washed in fresh medium and incubated, and aliquots were removed every 5 min to measure H_2O_2 concentrations as described in Materials and Methods. (B) Wild-type, Kat$^-$, Ahp$^-$, and HpxF$^-$ strains were grown in LB medium. At OD_{600}s of 0.5 (white bars) and 2 (gray bars), aliquots were removed from each culture and plated on LB agar supplemented with catalase to determine cell viability. Results are the means ± standard deviations for three independent experiments, each in triplicate. (C) Wild-type, Kat$^-$, Ahp$^-$, and HpxF$^-$ cells were grown in minimal medium overnight. The cells were then diluted to an OD_{600} of 0.1, and aerobic growth was monitored at 600 nm.

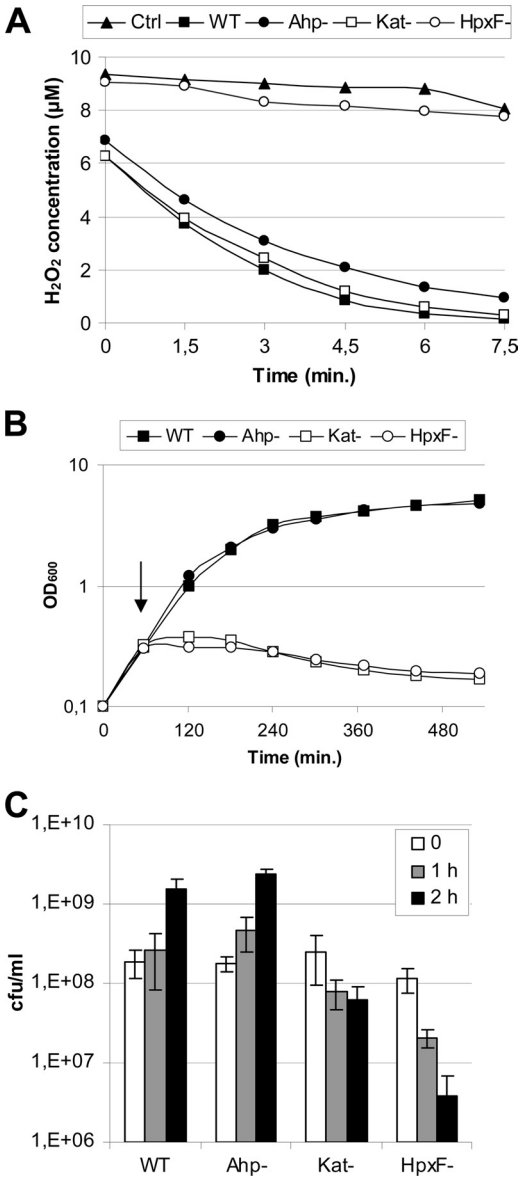


FIG. 3. The HpxF$^-$ mutant is highly sensitive to exogenous hydrogen peroxide. (A) Wild-type (filled squares), Ahp$^-$ (filled circles), Kat$^-$ (open squares), HpxF$^-$ (open circles), and heat-killed wild-type (filled triangles) cells were grown in minimal medium to an OD_{600} of 0.3 and then diluted at an OD_{600} of 0.1. H_2O_2 was added at a final concentration of 10 μM. Aliquots were removed every 90 s to assay H_2O_2 levels as described in Materials and Methods. (B) Bacterial cultures (wild type, Ahp$^-$, Kat$^-$, and HpxF$^-$) grown to an OD_{600} of 0.3 in LB medium were treated with 1 mM hydrogen peroxide (arrow). The growth was then monitored at 600 nm. (C) Viability was assayed, respectively, before (white bars) and 1 h (gray bars) and 2 h (black bars) after H_2O_2 treatment by plating the bacterial cells on LB agar supplemented with catalase. Results are the means ± standard deviations for three independent experiments, each in triplicate.

when the catalases were inactivated (Fig. 3C). All together, these experiments highlighted the sensitivity of the HpxF$^-$ mutant when subjected to exogenous stress and the importance of the catalases in degrading millimolar concentrations of exogenous hydrogen peroxide.

The HpxF$^-$ mutant is defective for proliferation within macrophages. To investigate the involvement of catalases and alkyl hydroperoxide reductases in intracellular proliferation, we infected two kind of cells: macrophages freshly prepared from murine bone marrow and considered highly oxidant and RAW 264.7 mouse macrophages. Both kind of cells were activated with IFN-γ and PMA and infected with the wild-type, Ahp$^-$, Kat$^-$, and HpxF$^-$ *Salmonella* strains. Bacterial proliferation was assayed by calculating the proliferation index as a ratio of

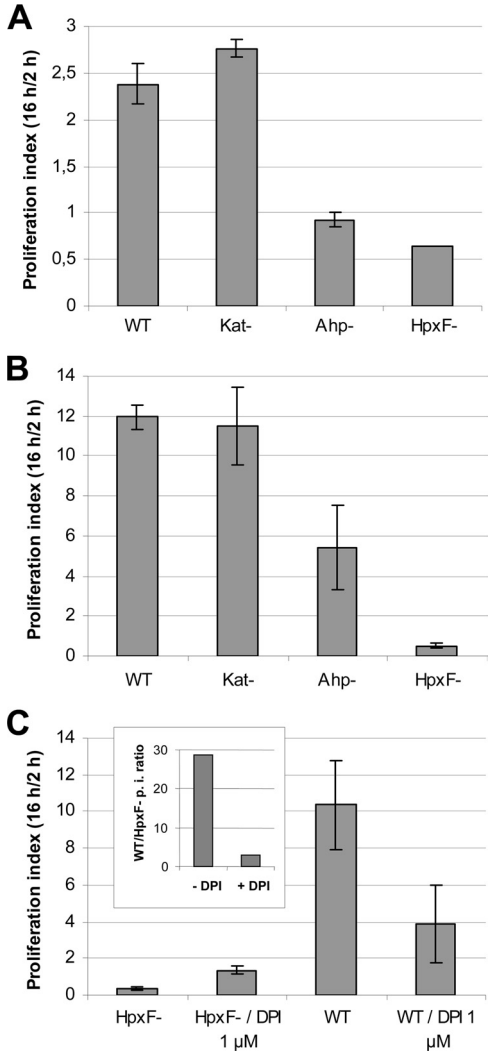


FIG. 4. HpxF$^-$ mutations led to a drastic attenuation of intracellular proliferation within bone marrow-derived and RAW 264.7 macrophages. Opsonized bacteria (Wild type or Kat$^-$, Ahp$^-$, or HpxF$^-$ mutant) were phagocytosed by freshly prepared bone marrow-derived macrophages (A) or RAW 264.7 cells (B), both activated with IFN-γ and PMA. Two and sixteen hours postinfection, mouse macrophages were lysed for enumeration of intracellular bacteria (gentamicin protected), determined by CFU counts. The values shown represent the proliferation index, calculated as a ratio of the intracellular bacteria between 16 and 2 h postinfection. (C) The HpxF$^-$ mutant and the wild-type strain were tested for infection of activated RAW 267.4 cells without or with 1 μM DPI, which inhibits the phagocyte NADPH oxidase. The proliferation index was calculated as described above. The inset represents the proliferation index ratio (wild type/HpxF$^-$) without or with 1 μM DPI. Results presented in the three panels are the means ± standard deviations for at least three independent experiments, each in triplicate.

the intracellular bacteria between 16 and 2 h postinfection. Within murine bone marrow, the proliferation indexes of the wild-type and Kat$^-$ cells were found to be nearly similar (2.38 ± 0.21 and 2.76 ± 0.10, respectively), whereas the Ahp$^-$ and HpxF$^-$ mutants were defective for proliferation (0.92 ± 0.07 and 0.64 ± 0.01, respectively) (Fig. 4A). Within RAW cells, the proliferation index was also very near for the wild-type and Kat$^-$ cells (11.9 ± 0.6 and 11.5 ± 1.9, respectively) whereas the Ahp$^-$ and HpxF$^-$ mutants exhibited proliferation indexes of 5.5 ± 2.1 and 0.5 ± 0.1, respectively (Fig. 4B). These results indicated that the HpxF$^-$ mutant was unable to proliferate within bone marrow or RAW 264.7 macrophages. Moreover, they highlighted the contribution of Ahp enzymes to intracellular replication within macrophages. Next, to test if the lack of proliferation of the HpxF$^-$ mutant was due to exogenous oxidative stress generated by the phagocyte NADPH oxidase, we infected macrophages in the presence of various concentrations of DPI, an inhibitor of this enzymatic complex. The proliferation index of the HpxF$^-$ strain increased 3.7-fold in the presence of 1 μM DPI (Fig. 4C). Conversely, the proliferation index of the wild-type strain decreased from 10.4 ± 2.4 to 3.9 ± 2.1 when DPI was added, indicating that this molecule had an undesirable effect on the bacterial proliferation (Fig. 4C). Thus, we calculated the comparative wild type/HpxF$^-$ proliferation index ratio, which dropped from 28.8 to 2.9 in the presence of the NADPH oxidase inhibitor (Fig. 4C). These results showed that despite the nonspecific effect of DPI, the oxidative stress generated by the NADPH oxidase was partially responsible for the proliferation defect of the HpxF$^-$ mutant.

The HpxF$^-$ mutant exhibited attenuated virulence in mice. Finally, we tested whether mutation of catalase- and alkyl hydroperoxide reductase-encoding genes contributed to virulence in the animal. To address this issue, we used mixed infections of wild-type and mutant strains in mice to determine the CI, a very sensitive technique to compare the virulence levels of different strains (3). The lack of three catalases and two alkyl hydroperoxide reductases significantly decreased *Salmonella* virulence, since the CI of the HpxF$^-$ mutant was found to be 0.07 (Table 2). In contrast, the CIs of the Kat$^-$ and Ahp$^-$ mutants did not show any significant alteration in virulence (Table 2). These results indicate that deletion of all catalase- and Ahp-encoding genes strongly affects *Salmonella* virulence in the mouse model. As a control, a competition assay was performed in LB, in which the mutant and wild-type strains were mixed one to one and grown under aerated conditions. The CIs of the Kat$^-$, Ahp$^-$, and HpxF$^-$ mutants were found to be 1.10, 0.86, and 0.69, respectively (Table 3). Thus, the three mutants tested could compete with the wild-type strain in rich culture medium. All in all, these data provided evidence for a contribution of catalases and alkyl hydroperoxide reductases in allowing resistance against oxidative stress generated during the infection process.

TABLE 2. Competition assays with mice[a]

Strain description	Median CI	No. of mice	*P* value[b]	Fold attenuation
Kat$^-$	1.11	4	NS[c]	NS
Ahp$^-$	1.20	4	NS	NS
HpxF$^-$	0.07	5	<0.0001	14

[a] Mice were inoculated intraperitoneally with a mixture of two strains. At day 2 after injection, spleens were harvested for bacterial counts. CIs of wild-type versus Kat$^-$, wild-type versus Ahp$^-$, and wild-type versus HpxF$^-$ strains were determined.

[b] A one-sample *t* test was used to determine whether the CI was significantly different from 1.

[c] NS, not significant.

TABLE 3. Competition assays with LB aerated cultures[a]

Strain description	Median CI	No. of samples	*P* value[b]	Fold attenuation
Kat$^-$	1.10	3	NS[c]	NS
Ahp$^-$	0.86	3	NS	NS
HpxF$^-$	0.69	3	NS	NS

[a] Bacterial mixtures were incubated with equal amounts for 24 h with shaking. Cells were then diluted and plated to determine the relative percentage of each strain recovered. CI of wild-type versus Kat$^-$, wild-type versus Ahp$^-$, and wild-type versus HpxF$^-$ strains were determined.

[b] A one-sample *t* test was used to determine whether the CI was significantly different from 1.

[c] NS, not significant.

Gene expression pattern in LB and macrophages. To monitor the expression of *Salmonella* catalase- and Ahp-encoding genes, we performed quantitative reverse transcription-PCR using a wild-type strain grown either within RAW 264.7 macrophages for 6 h or in LB to an OD_{600} of 2. Within macrophages, *ahpC* and *tsaA* were strongly transcribed (3×10^{10} and 4.7×10^{10} copies/μg RNA, respectively) (Fig. 5). In comparison, the three catalase-encoding genes presented much lower expression levels (4.3×10^9, 4.7×10^8, and 4.5×10^8 copies/μg RNA for *katG*, *katE*, and *katN*, respectively) (Fig. 5). These data highlight the efficient transcription of *ahpC* and *tsaA*, both being strongly expressed in LB and within macrophages.

The *katG* and *tsaA* genes can complement the HpxF$^-$ mutant. To evaluate the contributions of individual genes in H_2O_2 scavenging, we cloned *katG* and *tsaA* into the low-copy-number plasmid pACYC184. Wild-type and HpxF$^-$ strains were transformed with the p*katG* and p*tsaA* plasmids, and these strains were grown in LB. When submitted to 1 mM H_2O_2, the HpxF$^-$ strain carrying p*katG* grew as well as its wild-type parent (Fig. 6A). In contrast, the HpxF$^-$ strain carrying p*tsaA* showed a growth defect similar to that of the HpxF$^-$ strain carrying pACYC184 (Fig. 6A). Thus, the presence of *katG* carried on a low-copy-number plasmid (10 to 15 copies/cell)

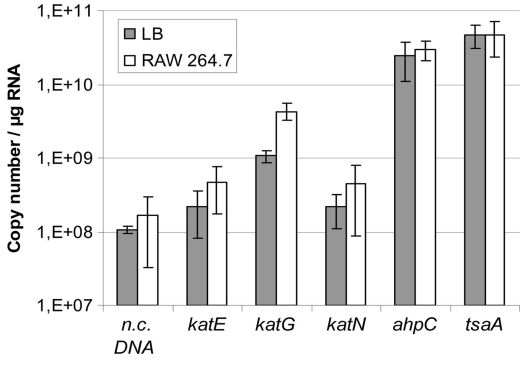


FIG. 5. Expression pattern of catalase- and alkyl hydroperoxide reductase-encoding genes in LB and RAW 264.7 macrophages. RNA was extracted from the *Salmonella* wild-type strain grown either in LB medium to an OD_{600} of 2 or within activated RAW 264.7 cells during 6 h. cDNA were synthesized from 1 μg RNA, and real-time PCR was performed to amplify the *katE*, *katG*, *katN*, *ahpC*, *tsaA* genes. A noncoding (n.c.) DNA domain located between STM0413 and STM0414 was used as a control to monitor the basal level of expression due to residual genomic DNA. The results are the means ± standard deviations for three independent experiments.

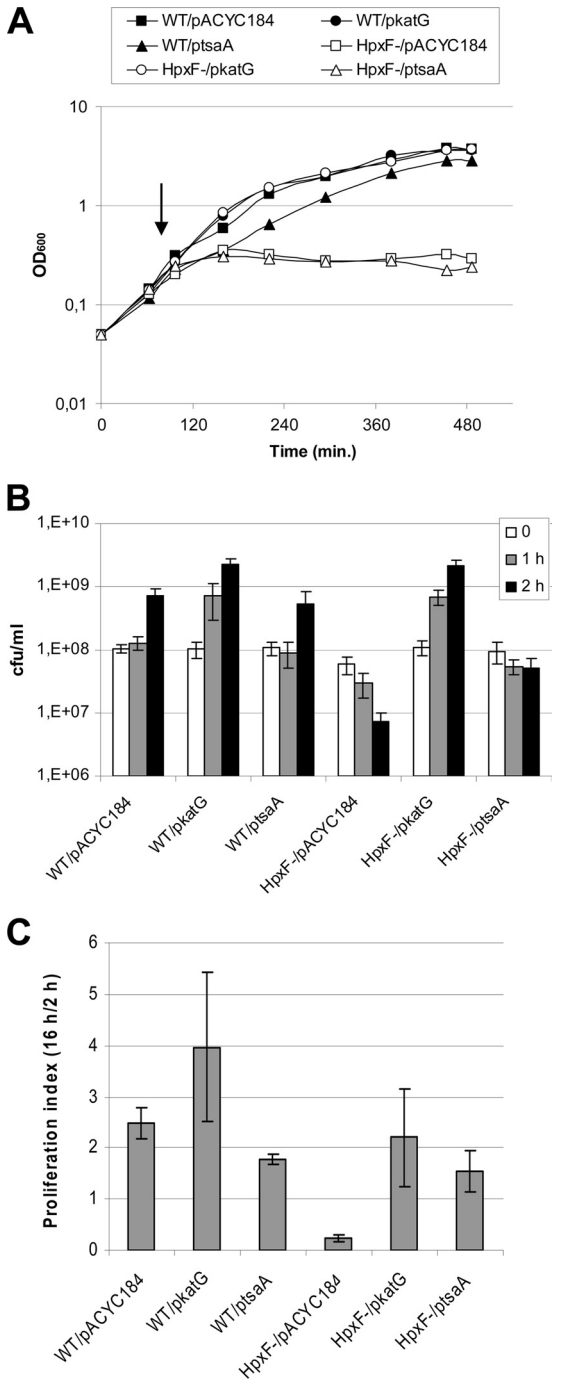


FIG. 6. p*katG* and p*tsaA* complement the HpxF$^-$ mutant with different efficiencies. Bacterial cultures (wild type, filled symbols; HpxF$^-$, open symbols) were transformed with empty vector (squares), the p*katG* plasmid (circles), or the p*tsaA* plasmid (triangles). The cells grown to an OD_{600} of 0.3 and were treated with 1 mM H_2O_2 (arrow). Growth was monitored at an OD of 600 nm (A), and the viability was assayed before (white bars) and 1 h (gray bars) and 2 h (black bars) after H_2O_2 treatment (B). Wild-type and HpxF$^-$ strains were transformed with empty vector, p*katG*, and p*tsaA* and were tested for infection within activated RAW 264.7 cells. Bacterial cells were plated and counted on LB agar supplemented with chloramphenicol and catalase. The values shown represent the proliferation index, calculated as a ratio of the intracellular bacteria between 16 and 2 h postinfection (C). Results presented in panels B and C are the means ± standard deviations for at least three independent experiments, each in triplicate.

was sufficient to allow wild-type resistance to exogenously added H_2O_2. On the other hand, the *tsaA*-encoded enzyme did not support a catalytic efficiency strong enough to allow the HpxF$^-$ mutant to be resistant to 1 mM H_2O_2.

We also investigated cell viability after exposure to 1 mM H_2O_2. HpxF$^-$ cells carrying p*katG* exhibited a wild-type plating recovery ($\approx 10^8$ to $\approx 2 \times 10^9$ CFU/ml 2 h after stress), compared to a 10-fold decrease in viability of the HpxF$^-$, pACYC184-carrying control (Fig. 6B). Moreover, in a wild-type background, the presence of p*katG* yielded an increased plating recovery of $\approx 2 \times 10^9$ CFU/ml 2 h after stress, compared to $\approx 7 \times 10^8$ CFU/ml obtained with pACYC184. The contribution of *tsaA* was less pronounced but increased 4.5-fold the bacterial cell survival level: 54% of the HpxF$^-$ cells carrying p*tsaA* survived for 2 h after the stress, compared to 12% for the HpxF$^-$ cells carrying pACYC184 (Fig. 6B). Thus, *katG* was found to restore the viability of the HpxF$^-$ cells, whereas a slight contribution was attributed to *tsaA*.

The contribution of *katG* and *tsaA* was also assayed under infection conditions. All proliferation indexes were found to decrease in the presence of the pACYC184 plasmid and its derivatives, as described previously (19). Transformation of the wild-type strain with p*katG* or p*tsaA* did not significantly change the intracellular proliferation compared to results with the empty vector (1.6-fold and 0.73-fold, respectively) (Fig. 6C). In contrast, the presence of *katG* or *tsaA* noticeably increased the proliferation index of the HpxF$^-$ mutant (9.6-fold or 6.7-fold, respectively). Moreover, the proliferation indexes of the HpxF$^-$ strain carrying p*katG* and wild-type strain carrying pACYC184 were found to be quite similar (2.21 ± 0.95 versus 2.47 ± 0.31) (Fig. 6C). This observation suggested that an overexpressed single gene suppressed a phenotype generated by the mutation of five different genes. Thus, these experiments assigned a direct role for both the *tsaA* and *katG* genes in intracellular bacterial growth, showing a link between resistance to oxidative stress and proliferation within macrophages.

DISCUSSION

Resistance to oxidative stress is one of the key processes that allow pathogens to survive within macrophages. Hence, pathogenic bacteria have developed a series of antioxidative defenses which either eliminate ROS or repair their damages (18, 31). In recent years, several attempts have been made to assess the contribution of these defenses to the overall virulence of bacteria. Surprisingly, the outcome was not as straightforward as one would have anticipated. Thus, in several cases, inactivation of genes encoding ROS-eliminating enzymes failed to cause a significant defect in bacterial virulence. For instance, inactivation of catalase-encoding genes had little if any effect on pathogenicity-associated traits of *Salmonella* Typhimurium (5), *Yersinia pestis* (15), *Francisella tularensis* (21), *Staphylococcus aureus* (9), and *Neisseria gonorrhoeae* (34). In the present study, we addressed the question of the involvement of cytoplasmic H_2O_2-degrading enzymes in *Salmonella* virulence. Our study revealed that efficient proliferation of this bacterium in macrophages relies on the activities of three catalases and two alkyl hydroperoxide reductases. It illustrates how *Salmonella* has evolved numerous and redundant defense mechanisms to survive within such hostile and oxidative environments.

Analysis of the *S.* Typhimurium genome allowed us to identify five genes encoding H_2O_2 degrading enzymes: two alkyl hydroperoxide reductases, AhpC and TsaA, and three catalases, KatE, KatG, and KatN. Alkyl hydroperoxide reductase activity was found to contribute to macrophage infection, since the proliferation index of a strain lacking both *ahpC* and *tsaA* was decreased by twofold. This observation could be linked to the oxidative microenvironment present within a *Salmonella*-containing vacuole (SCV), where ROS and reactive nitrogen species are massively synthesized after macrophage phagocytosis (18, 36). Stimulation of NADPH oxidase and NO synthase, both located on the SCV membrane of the macrophages, leads to the production of $O_2^{\bullet -}$ and NO$^\bullet$, respectively. Both molecules (i) are used directly as a reactive species against *Salmonella*, (ii) are enzymatically transformed into another reactive species (H_2O_2 or $NO_2^\bullet$), or (iii) react with each other to generate a peroxynitrite anion, $ONOO^-$. In contrast, a Kat$^-$ mutant strain, lacking all three catalases, exhibited a wild-type-like proliferation index within macrophages and mice. This is consistent with previous results in which a *Salmonella katE katG* mutant retained full murine virulence (5). Alternatively, the wild-type phenotype of the Kat$^-$ mutant could be related to the low expression levels of *katE*, *katG*, and *katN* under infection conditions. However, this possibility can be discarded on the basis that a Kat$^-$ mutant failed to resist to millimolar exogenous H_2O_2. This implies that the level of expression recorded was sufficient for participation in H_2O_2 scavenging. Moreover, one can anticipate that the presence of catalases was the very thing accounting for the resistance of the Ahp$^-$ mutant to millimolar concentrations of H_2O_2. These observations fit with the *E. coli* model, wherein AhpC was proposed to be of primary importance in scavenging micromolar concentrations of H_2O_2 whereas the role of catalases was dominant at millimolar concentrations of H_2O_2 (32).

An HpxF$^-$ mutant lacking the three catalases and the two alkyl hydroperoxide reductases was unable to proliferate or even survive within macrophages. These mutations also contributed to the attenuation of *Salmonella* virulence, since the HpxF$^-$ cells exhibited a reduced proliferation in mice. Exogenous ROS were found to be partially responsible for the proliferation defect of the HpxF$^-$ mutant, since its intracellular replication increased when NADPH oxidase was inhibited. But the DPI did not allow this mutant to reach a wild-type replication efficiency, and its effects appeared quite difficult to sort out since it altered proliferation of the wild-type strain as well. Still, the fact that DPI increased the replication of the HpxF$^-$ mutant is fully consistent with the "exogenous ROS" hypothesis. Other causes could account for the reduced proliferation of the HpxF$^-$ cells. For instance, this mutant was found to accumulate H_2O_2 during aerobic growth much faster than its wild-type parent. Thus, endogenous ROS generated during aerobic metabolism in the bacterial cytoplasm might contribute to cumulative oxidative damages. Moreover, the HpxF$^-$ mutant was found to grow poorly in minimal medium, and nutrient limitation within the SCV could amplify this trend. Finally, the two detrimental effects could act synergistically and explain the drastic attenuation of proliferation of the HpxF$^-$ mutant within macrophages and mice.

The HpxF$^-$ mutant exhibited much stronger deficiencies than the Ahp$^-$ or Kat$^-$ mutant. The additive effect of the *ahpC tsaA* and *katE katG katN* mutations opened the possibility that both types of scavenging systems contribute in a specific way to the replication process. For instance, H_2O_2 concentrations were found to vary during infection, increasing massively just after phagocytosis and decreasing during the next 6 h (36). Then, both type of activity would be exploited at different time points during the whole process. An alternative possibility is that the substrate repertoire of alkyl hydroperoxide reductases is much more expanded than that of catalases: ethyl, *t*-butyl, cumene, and linoleic acid hydroperoxides, among others, were efficiently reduced by *Salmonella* AhpC (29). Also, this enzyme was shown to protect *Salmonella* against reactive nitrogen intermediates and to detoxify peroxynitrite ($ONOO^-$) to nitrite fast enough to forestall DNA oxidation (4, 8). Hence, catalases could act as an H_2O_2 scavenger while alkyl hydroperoxide reductases could intervene in eliminating micromolar concentrations of H_2O_2 and other hydroperoxides.

The redundancy of *Salmonella* enzymes is striking even in a comparison with the closely related *E. coli*, which requires only three genes, *ahpC*, *katE*, and *katG*, to scavenge H_2O_2. It is a classic hypothesis that occurrence of redundancy can be rationalized by genetic regulation. In the present work, the expression levels of *Salmonella tsaA* and *ahpC*, quantified 6 h after macrophage infection, were found to be high. Conversely, the three catalase-encoding genes were poorly transcribed. The importance of fine-tuning gene expression was also illustrated by the fact that a moderate increase in gene dosage (15 copies per cell) allowed either *katG* or *tsaA* to confer a proliferation index similar to that of the wild type. This demonstrated that increased synthesis of only one type of H_2O_2-degrading activity is sufficient to restore a wild-type replication level within macrophages. However, a certain degree of redundancy also arises at the regulatory level, with OxyR being the transcriptional activator of *katG* and *ahpC* whereas *katE* and *katN* transcription is RpoS dependent. Our ongoing work aims at assessing a link between redundancy and genetic regulation by carrying out a thorough gene regulation study with *Salmonella*-infected macrophages.

Previous studies had revealed that an important aspect of *Salmonella* survival within macrophages was the production of two periplasmic Cu/Zn superoxide dismutases, SodCI and SodCII. These enzymes combat phagocytic superoxide $O_2^{\bullet -}$ by dismutation to H_2O_2 and O_2. SodCI but not SodCII was found to play a role during infection of mice by *Salmonella* (10, 20, 35). In this context, SodCI and SodCII were considered the front line for combating NADPH-oxidase-generated superoxide. A remaining question was the management of H_2O_2 produced by dismutation of superoxide. The H_2O_2 flux into the bacterial cytoplasm was shown to be rapid, with a high permeability coefficient of the membrane (33). Here we showed that cytoplasmic catalases and alkyl hydroperoxide reductases constitute a second line for combating H_2O_2 produced by SodCI and SodCII in the periplasm. Hence, together with these previous studies, the present work contributes to a better understanding of the strategies used by *Salmonella* to resist host-produced ROS. It will now be extremely important to bring into the picture the proposal by Fang and coworkers that *Salmonella* pathogenicity island 2 allows bacteria to avoid NADPH oxidase-dependent killing by macrophages (37). Their data suggested that the NADPH phagocyte oxidase could be excluded from SCV in a *Salmonella* pathogenicity island 2-dependent manner. Thus, an emerging comprehensive picture would be that *Salmonella* relies on two alternative ways of coping with oxidative stress: excluding the NADPH phagocyte oxidase and metabolizing ROS produced by the host. Investigation of the interplay between the two strategies represents a major challenge for the future.

ACKNOWLEDGMENTS

Thanks are due to the members of the F.B. group, A. Latifi and P. Moreau (LCB, Marseille, France), for fruitful discussions and to R. Stevens (GAFL, Avignon, France) for critical reading of the manuscript.

This work was funded by the ANR (Programme Blanc "Stress Oxydant" and Programme M.I.E. "Salmo-sensor"), the CNRS, and the Université de la Méditerranée. M.H. was supported by the Ministère de la Recherche and J.P.M.V. by the Fondation pour la Recherche Médicale (FRM).

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