# DNA Repair Is More Important than Catalase for *Salmonella* Virulence in Mice

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## Abstract

Pathogenic microorganisms possess antioxidant defense mechanisms for protection from reactive oxygen metabolites such as hydrogen peroxide ($H_2O_2$), which are generated during the respiratory burst of phagocytic cells. These defense mechanisms include enzymes such as catalase, which detoxify reactive oxygen species, and DNA repair systems which repair damage resulting from oxidative stress. To determine the relative importance of these two potentially protective defense mechanisms against oxidative stress encountered by *Salmonella* during infection of the host, a *Salmonella typhimurium* double mutant unable to produce either the HPI or HPII catalase was constructed, and compared with an isogenic *recA* mutant deficient in DNA repair. The *recA* mutant was hypersusceptible to $H_2O_2$ at low cell densities in vitro, while the catalase mutant was more susceptible to high $H_2O_2$ concentrations at high cell densities. The catalase mutant was found to be resistant to macrophages and retained full murine virulence, in contrast to the *recA* mutant which previously was shown to be macrophage-sensitive and attenuated in mice. These observations suggest that *Salmonella* is subjected to low concentrations of $H_2O_2$ while at relatively low cell density during infection, conditions requiring an intact DNA repair system but not functional catalase activity. (*J. Clin. Invest.* 1995. 95:1047–1053.) Key words: oxidative stress • pathogenesis • macrophage • hydrogen peroxide • *recA*

## Introduction

Phagocytic cells are able to generate superoxide, hydrogen peroxide ($H_2O_2$), and other reactive oxygen metabolites which are capable of damaging microbial DNA, proteins, and membranes (1). Microorganisms possess multiple defenses to oxidative stress which detoxify oxygen species, repair damage, and compete with phagocytes for oxygen (1–3). The enzyme catalase plays a central role in these defenses, catalyzing the disproportionation of toxic $H_2O_2$ to water and oxygen.

A correlation between catalase activity and virulence has been noted for several pathogenic bacterial species, including *Staphylococcus aureus* (4), *Neisseria meningitidis* (5), *Legionella pneumophila* (6), *Nocardia asteroides* (7), and *Mycobacterium tuberculosis* (8, 9). However, these studies were conducted using naturally occurring strains, raising the possibility that additional virulence determinants other than catalase might have differed between isolates. Two *Listeria monocytogenes* mutants lacking catalase activity were found to be fully virulent in mice (10), although these mutants interestingly were not susceptible to $H_2O_2$ in vitro. Catalase-deficient *Shigella flexneri* was found to be only modestly attenuated for virulence (11).

The ability to survive within phagocytic cells has been demonstrated to be an essential aspect of *Salmonella* virulence (12), suggesting that resistance to the phagocyte respiratory burst might be important to *Salmonella*. Moreover, *Salmonella* is one of the most common opportunistic pathogens in patients with chronic granulomatous disease, a genetic disorder characterized by a deficient phagocyte respiratory burst (13). Experimental data regarding the importance of oxidative killing mechanisms in *Salmonella* infection have been inconclusive (14–18). Attempts to define the role of catalase in *Salmonella* virulence have been hampered by the lack of defined mutants in isogenic strains (17).

Like *Escherichia coli* (19), *Salmonella* possesses two catalase proteins, hydroperoxidase I (HPI)[1] and hydroperoxidase II (HPII). HPI is encoded by the *katG* gene (20) and is regulated by *oxyR* in response to oxidative stress (21). HPII is encoded by *katE* (22) is and regulated by an alternative sigma factor encoded by *katF* (*rpoS*), in response to starvation (23, 24). *Salmonella* mutants lacking either of the catalase regulatory loci, *oxyR* (12, 21) or *katF* (24), have been shown to be attenuated for virulence in mouse models, but these effects cannot be attributed to catalase alone since both *oxyR* and *katF* regulate multiple genes.

A *katE/katG S. typhimurium* mutant unable to produce either the HPI or HPII catalases was constructed and evaluated with respect to susceptibility to $H_2O_2$ and phagocytic cells in vitro, and virulence in BALB/c mice. The double catalase mutant was compared with isogenic *S. typhimurium* mutants deficient in either catalase alone, with *recA* mutant *S. typhimurium* which is deficient in the ability to repair DNA damage, and with the virulent wild-type parent strain *S. typhimurium* 14028s.

## Methods

*Media.* Luria-Bertani (LB) broth (tryptone, 10 mg/ml; yeast extract, 5 mg/ml; NaCl, 10 mg/ml) at 37°C was used for all bacterial cultivation. Agar (1.5%) was added to solid medium. Media were supplemented with 250 μg/ml penicillin or 15 μg/ml tetracycline (Sigma Chemical

1. *Abbreviations used in this paper:* HP, hydroperoxidase; LB, Luria-Bertani; NBT, nitroblue tetrazolium.

## Table I. Bacterial Strains

| Strain | Relevant characteristics | Reference |
|---|---|---|
| 14028s | Wild-type | 12 |
| CL1000 | recA | 15 |
| MS7953 | phoP::Tn10 Tet$^r$ | 12 |
| SF1005 | katF::pRR10ΔtrfA Pen$^r$ | 24 |
| MS4999 | katE::Tn10 | 12 |
| XF1000 | katG::pRR10ΔtrfA Pen$^r$ | This work |
| XF1001 | katE/katG | This work |
| TH2423 | Contains P22 on a mini-F plasmid | 27 |
| E. coli S17-1 | Tra$^+$ recA pro thi hsdR chr::RP4-2 | 28 |
| pRR10 | RK2 mini-replicon | 29 |



*Figure 1.* Catalase gel of *Salmonella* catalase mutants. Gels were prepared by the method of Gregory and Fridovich (32). Cell extracts were examined from wild-type (lane *1*), *katE* mutant (lane *2*), *katG* mutant (lane *3*), and *katE/katG* mutant (lane *4*) *S. typhimurium.* The two bands corresponding to the HPI catalase (KatG) and the single band corresponding to the HPII catalase (KatE) are designated. The dark band corresponds to peroxidase activity, which is unaffected by the catalase mutations.

Co., St. Louis, MO) as indicated. Green plates (25) were used to identify cells not harboring bacteriophage P22 as a pseudo-lysogen. Macrophages were cultured in endotoxin-free RPMI plus 10% fetal calf serum (26).

*Bacterial strains and plasmids.* Strains used in this study are listed in Table I. All studies were performed using wild-type *S. typhimurium* ATCC 14028s or isogenic derivatives. The construction of catalase-deficient *S. typhimurium* MS4999s (*katE*), *S. typhimurium* XF1000 (*katG*), and *S. typhimurium* XF1001 (*katE/katG*) is described in Results. *S. typhimurium* TH2423 contains bacteriophage P22 on a mini-F plasmid (27) and was used for preparation of transducing lysates from rough *Salmonella* strains. *E. coli* S17-1 (28) is a donor strain used to mobilize the pRR10-based suicide vector for the construction of *S. typhimurium* XF1000. pRR10 is a mobilizable RK2 mini-replicon encoding β-lactam resistance (29).

*PCR.* PCR was used to amplify an internal 796-bp sequence of *katG* from *S. typhimurium* 14028s genomic DNA by a published method (30). The primers 5'-TGGTGGCCGAACAAGCTTCGCGTGG-3' and 5'-TGCTGGCCCAACTTAAGCCCTGCGC-3' were derived from the published *katG* sequence (20).
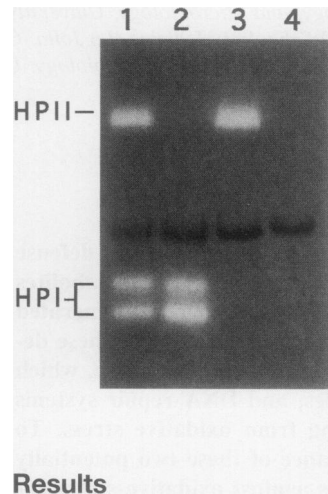
*Catalase assay.* Quantitative catalase assays were performed with an oxygraph (31), with activity expressed as units per milligram of dry weight. Catalase activity was visualized on 9.5% nondenaturing polyacrylamide gels (32).

*Hydrogen peroxide susceptibility assay.* The susceptibility of stationary phase bacterial cells to $H_2O_2$ was initially determined by adding $H_2O_2$ to bacteria in LB broth to a final concentration of 15 mM. Stationary phase bacteria were chosen for study since many critical antioxidant defenses are preferentially expressed during stationary phase (23, 33). Aliquots of bacteria were removed at timed intervals, diluted, and plated onto LB agar for quantitation of viable cells (24). Alternatively, 400 μM or 4 mM $H_2O_2$ was added to stationary phase bacteria diluted to either 5 × $10^5$ or $10^8$ cells/ml in M9 broth as indicated, before quantitation by plating at timed intervals.

*Macrophage survival assay.* The 24-h survival of *Salmonella* mutants in macrophages was assayed using proteose peptone–elicited macrophages as previously described (26). Short-term survival assays were performed in a similar manner but without the addition of gentamicin to the cultures. Production of a respiratory burst by macrophages was detected by the reduction of nitroblue tetrazolium (NBT) in a microplate assay (34). The production of $H_2O_2$ was measured using a microassay based on horseradish peroxidase–dependent oxidation of phenol red (34).

*Mouse virulence assay.* The virulence of the *Salmonella* catalase mutants was determined in 7-wk-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) after intragastric or intraperitoneal adminstration (26).

## Results

*Construction of catalase-deficient Salmonella mutants.* A 796-bp PCR-amplified internal *katG* fragment (20) was cloned into a suicide vector based upon the RK2-derivative pRR10 (29) and conjugally transferred into *S. typhimurium* 14028s. Insertional inactivation of *katG* resulted from homologous recombination of the suicide vector into the bacterial chromosome. This *katG* mutant strain was designated XF1000. The rough *katE* Tn10 mutant MS4999r was obtained (12) and the *katE* mutation was transduced into a smooth wild-type (14028s) background using TH2423 (27), resulting in strain MS4999s. The same P22 lysate was used to introduce the *katE* mutation into XF1000, resulting in the *katE/katG* mutant *S. typhimurium* strain XF1001.
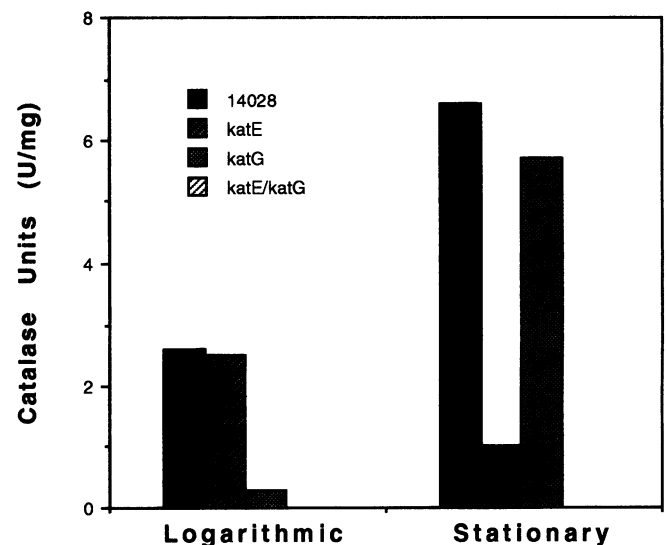


*Figure 2.* Catalase activity expressed by *Salmonella* catalase mutants. Catalase activity was assayed using an oxygraph (31). In wild-type *S. typhimurium* 14028s, the *katE*-encoded HPII accounts for 11 and 83% of the total catalase activity during logarithmic growth and stationary phase, respectively. The measured catalase activity from *katE/katG* mutant *S. typhimurium* during both logarithmic growth and stationary phase was < 0.01 U/ml.
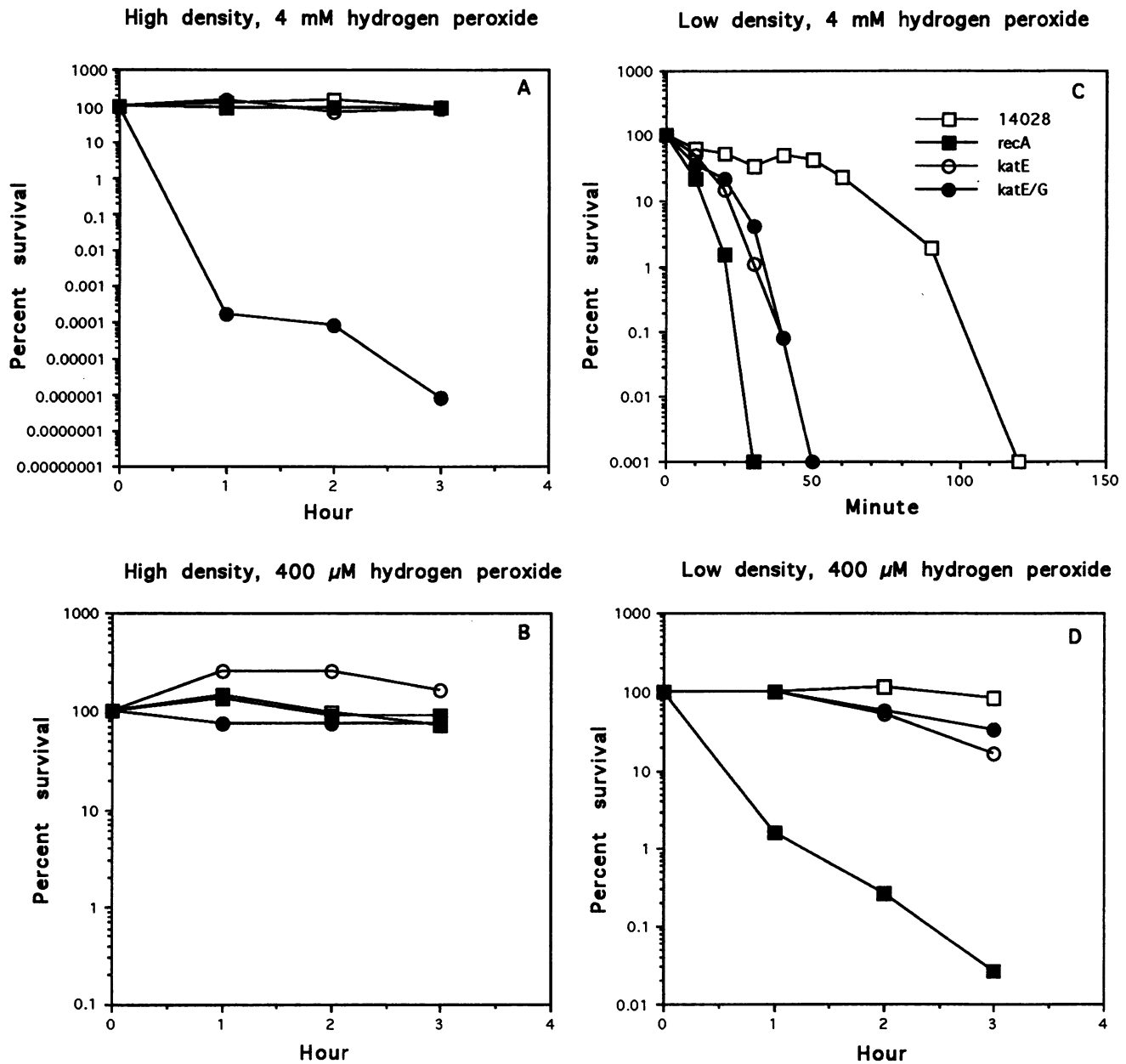
**High density, 4 mM hydrogen peroxide**

**Low density, 4 mM hydrogen peroxide**

**High density, 400 μM hydrogen peroxide**

**Low density, 400 μM hydrogen peroxide**



*Figure 3.* Sensitivity of *Salmonella* at high and low density to hydrogen peroxide. Stationary phase wild-type (*open squares*), *rec*A mutant (*closed squares*), *kat*E mutant (*open circles*), and *kat*E/*kat*G mutant (*closed circles*) *S. typhimurium* were resuspended in M9 broth to either $10^8$/ml (*A* and *B*) or $5 \times 10^5$/ml (*C* and *D*) and added to hydrogen peroxide to give a final concentration of 4 mM (*A* and *C*) or 400 μM (*B* and *D*). Samples were incubated at 37°C and aliquots were removed at timed intervals and plated for viable counts. Percent survival was calculated using the mean of duplicate samples at timed intervals compared to 0 h. Each sensitivity assay was performed three times.

The catalase-deficient phenotypes of the mutant *S. typhimurium* strains were confirmed by analyzing bacterial lysates on catalase activity gels (32). As shown in Fig. 1, XF1000 expresses only the *kat*E-encoded HPII catalase, MS4999s expresses only the *kat*G-encoded HPI catalase which is visualized as a doublet (35), and XF1001 expresses neither catalase. Catalase activity expressed by the mutant strains as measured by oxygraph is shown in Fig. 2. The *kat*E-encoded HPII catalase accounted for 11% of all catalase activity during logarithmic growth and 83% of total catalase activity during stationary phase.

*Susceptibility of catalase-deficient Salmonella to hydrogen*

*peroxide.* Susceptibility of the catalase-deficient *S. typhimurium* strains to $H_2O_2$ was initially assessed using 1.0 ml of an overnight culture ($\sim 10^9$ bacteria/ml) and 15 mM $H_2O_2$, a concentration well tolerated by wild-type *S. typhimurium* (24). Both the *kat*E and *kat*G single mutants were resistant to 15 mM $H_2O_2$, but the double *kat*E/*kat*G mutant XF1001 was highly susceptible to 15 mM $H_2O_2$ with greater than 3 logs killing in 30 min and no viable cells detected by 60 min.

Although the *kat*E/*kat*G mutant was extremely sensitive to $H_2O_2$ as originally tested at a density of $10^9$ cells/ml, the protective effect of microbial catalase is known to be cell density–dependent (36). Therefore, $H_2O_2$ assays were repeated using
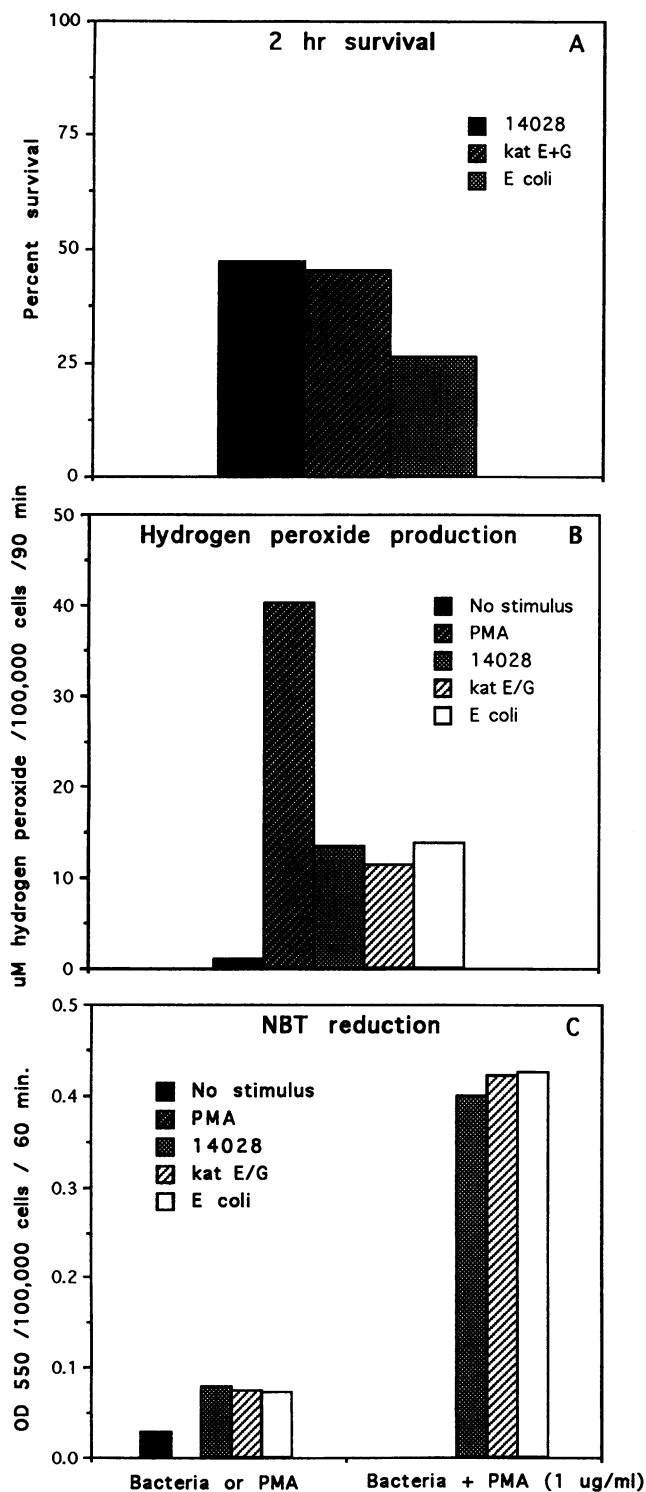
*Figure 4.* Survival and stimulation of oxidative burst by *Salmonella* in peritoneal exudate macrophages. (*A*) Macrophages were infected with 20 bacteria/cell for 15 min, washed four times and fresh medium containing no antibiotics was added. Triplicate wells were lysed and plated at 0 and 2 h (26). Percent survival is 2 h viable count/0 h viable count. (*B*) $10^5$ macrophages were infected with 20 bacteria/cell or stimulated with 1 $\mu$g/ml PMA and incubated for 90 min in media containing phenol red and horseradish peroxidase as described for the microassay (34). Hydrogen peroxide levels were determined at $OD_{650}$ and compared with a standard curve. (*C*) $10^5$ macrophages were infected with 20 bacteria/ cell or stimulated with 1 $\mu$g/ml PMA in media containing 1 mg/ml

bacterial densities of $10^8$ cells/ml and $5 \times 10^5$ cells/ml, which may be more similar to bacterial densities during infection studies. In these experiments, the catalase mutants were compared with the isogenic *rec*A mutant *S. typhimurium* CL1000, which is sensitive to DNA-damaging compounds and attenuated for virulence in both macrophages and mice (15). At low cell densities, the *rec*A mutant was the most sensitive strain tested to $H_2O_2$. Low concentration (400 $\mu$M) $H_2O_2$ killed only the *rec*A strain in a significant manner, while the catalase mutants of *S. typhimurium* were virtually resistant to 400 $\mu$M $H_2O_2$ (Fig. 3). When the concentration of $H_2O_2$ was increased 10-fold to 4 mM, the *rec*A mutant was inactivated most rapidly, followed by the catalase mutants and then wild-type organisms. In contrast, at high cell density the double catalase mutant of *S. typhimurium* displayed the greatest susceptibility to high concentration (4 mM) $H_2O_2$.

*Susceptibility of catalase-deficient Salmonella to macrophages.* Survival of the double *kat*E/*kat*G mutant *S. typhimurium* (XF1001) was compared with that of wild-type 14028s in peptone-elicited macrophages. There was no difference in survival in a 24-h assay (data not shown). Bacterial killing during the first 2 h of infection when the peak oxidative burst is produced was then examined. Peritoneal exudate macrophages were infected with 20 bacteria/cell for 15 min, washed extensively before fresh media without antibiotics were added, and incubated for another 2 h. There was no difference in the susceptibility of the double *kat*E/*kat*G mutant to killing by macrophages compared to wild-type *S. typhimurium* (Fig. 4 *A*).

The respiratory burst of the peritoneal exudate macrophages was measured in parallel to verify that the macrophages were producing oxidative products, to determine whether the bacterial inoculum used was sufficient to trigger a burst, and to determine whether infection with *S. typhimurium* inhibited the respiratory burst when triggered by a second stimulus. After stimulation with 1 $\mu$g/ml PMA, the peritoneal macrophages produced $H_2O_2$ (Fig. 4 *B*) and reduced NBT (Fig. 4 *C*). Infection with bacteria stimulated less oxidative burst than the PMA, however both *S. typhimurium* 14028s and the *kat*E/*kat*G mutant stimulated the production of similar amounts of oxidative products as *E. coli* (Fig. 4 *C*), which was used as a control organism carrying LPS but lacking the ability to survive in macrophages. The detectable concentration of $H_2O_2$ in the cultures was $\sim$ 10 $\mu$M after stimulation with bacteria. Infecting macrophages with bacteria at the same time that PMA was added did not reduce the oxidative burst, but enhanced it twofold over PMA alone as detected by NBT reduction (Fig. 4 *C*).

*Virulence of catalase-deficient Salmonella in BALB/c mice.* Virulence of the catalase mutants was assayed in BALB/c mice. The double catalase *Salmonella* mutant XF1001 was not attenuated as measured by either lethal dose or time until death. All mice given $10^3$ CFU of the *kat*E/*kat*G mutant intraperitoneally were dead by day 5, which was identical to mice infected with wild-type 14028s (Fig. 5 *A*). Mice infected with the *kat*F mutant SF1005 displayed no signs of infection, providing evidence of

NBT and incubated for 60 min. Reduction of NBT was measured as OD at 550 nm (34). For double stimulation, cells were infected with 20 bacteria/cell using media containing 1 $\mu$g/ml PMA and incubated for 60 min. Each survival or stimulation assay was performed three times.
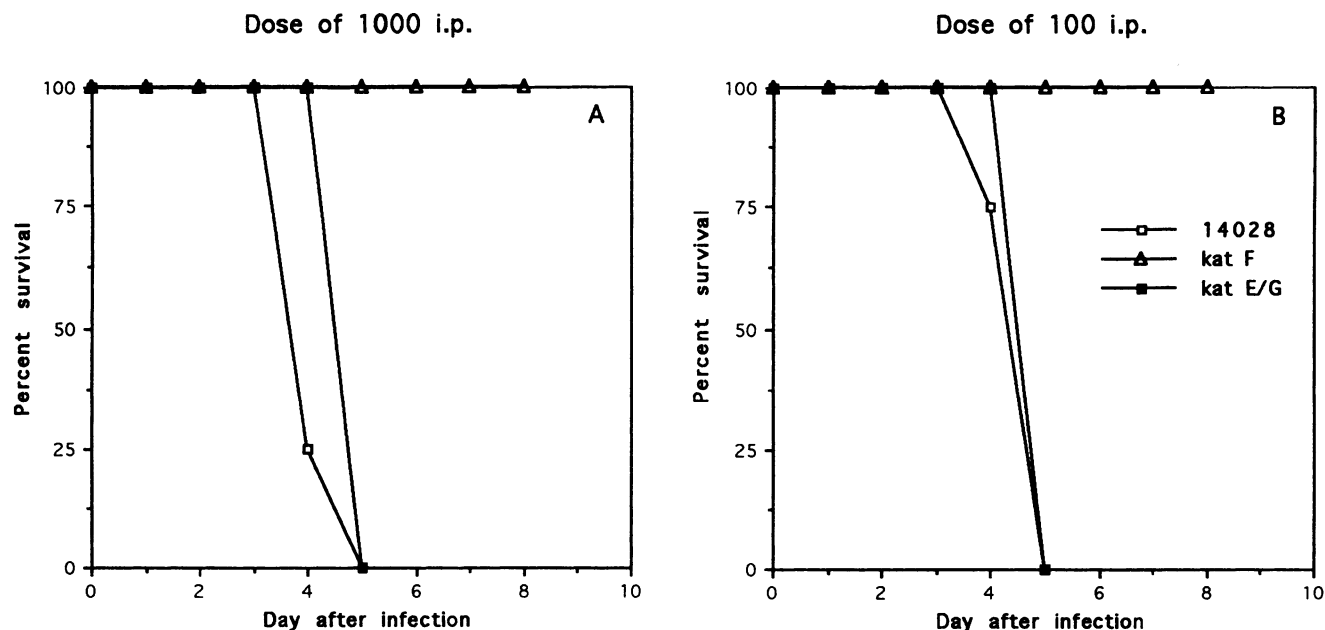
**Dose of 1000 i.p.**

**Dose of 100 i.p.**

Legend:
- 14028 (open squares)
- kat F (open triangles)
- kat E/G (closed squares)

Y-axis: Percent survival (0, 25, 50, 75, 100)
X-axis: Day after infection (0, 2, 4, 6, 8, 10)

A / B

*Figure 5.* Virulence of catalase mutants in mice. Groups of female BALB/c mice were infected intraperitoneally with either $10^3$ CFU (*A*) or $10^2$ CFU (*B*) of bacteria. No significant difference in the virulence of wild-type (*open squares*) and *katE/katG* mutant (*closed squares*) *S. typhimurium* was seen. *katF* mutant *S. typhimurium* (*open triangles*) was included as an attenuated control strain (24). Each intraperitoneal virulence determination was performed twice, using four mice per group.

its attenuation as previously described (24). The *recA* mutant CL1000 recently has been demonstrated to have attenuated virulence in mice (15), hence virulence testing with this strain was not repeated. Reducing the infecting dose of the *katE/katG* mutant to $10^2$ bacteria intraperitoneally still failed to reveal any evidence of attenuation (Fig. 5 *B*). Infection by the oral route similarly showed no reduction in virulence of the *katE/katG* mutant (data not shown).

## Discussion

Hydrogen peroxide ($H_2O_2$) is an important component of the respiratory burst. Although $H_2O_2$ itself is considered to be only weakly microbicidal (37), it is an important component of the phagocyte respiratory burst because of its ability to diffuse rapidly across membranes. Once within the bacterial cell, $H_2O_2$ may react with iron or copper ions to generate highly cytotoxic hydroxyl radicals ($OH \cdot$) via the Fenton reaction (2). The enzyme catalase catalyzes the disproportionation of $H_2O_2$ into water and oxygen. This reaction might be anticipated to be particularly important in the virulence of intracellular pathogens such as *Salmonella*, which reside within the phagosomes of host phagocytic cells (38).

However, this study demonstrates that catalase does not play a necessary role in *S. typhimurium* virulence in mice. Mutations in both the *katE*- and *katG*-encoded catalases of *S. typhimurium* were constructed to eliminate ambiguities arising from the use of nonisogenic strains and to avoid multigenic effects resulting from disruption of pleiotropic regulatory genes. *katE/katG* mutant *S. typhimurium* lacks detectable catalase activity and demonstrates increased susceptibility to $H_2O_2$ in vitro. Nonetheless, this mutant shows no significant attenuation for virulence by either oral or intraperitoneal infection in BALB/c mice. This

is in contrast to *katF* (*rpoS*) mutant *S. typhimurium*, which is attenuated for virulence; this finding confirms that the avirulence of the *katF* mutant results from disrupted expression of *katF*-regulated genes other than *katE*, such as the *spv* plasmid virulence genes (24). Moreover, although *katE/katG* mutants are hypersusceptible to $H_2O_2$ killing in vitro, single *katE* or *katG* mutants did not demonstrate increased $H_2O_2$ susceptibility in a conventional $H_2O_2$-killing assay. Since *katF* mutant *S. typhimurium* is $H_2O_2$ sensitive despite intact *katG* expression (24), this implicates *katF*-regulated functions other than catalase in the mechanism of *Salmonella* resistance to $H_2O_2$, perhaps involving the *dps* (33) or *xthA* loci (23).

The *Salmonella* catalase proteins were also found to be unnecessary for either long-term (24 h) or short-term (2 h) survival in macrophages in vitro. This finding complements earlier observations that induction of catalase expression does not appear to enhance survival of *E. coli* in polymorphonuclear neutrophils (39). Other investigators have recently shown that mutations in *oxyR* or the *oxyR*-regulated *katG* gene encoding the HPI catalase also fail to significantly alter *S. typhimurium* LT2 survival after exposure to neutrophils (40). The peritoneal exudate macrophages used in this study were demonstrated to be capable of producing a respiratory burst in response to phagocytosis of bacteria or PMA stimulation. Only low levels of $H_2O_2$ (10 $\mu$M) were detected in the tissue culture medium of stimulated macrophages, but this undoubtedly underestimates $H_2O_2$ production since rapid catalatic or peroxidatic breakdown of $H_2O_2$ occurs under these assay conditions (41). No inhibition of the respiratory burst by *Salmonella* was observed. Macrophages infected with *Salmonella* produced similar levels of reactive oxygen products compared to *E. coli*–infected macrophages. Simultaneously stimulating with PMA and infecting with *Salmonella* produced an amplified oxidative burst. Simi-

larly, other investigators using *Salmonella typhi* or *S. typhimurium* LT2 have shown that *Salmonella* triggers the production of hydrogen peroxide in murine resident peritoneal macrophages (14). Francis and Gallagher (42) have recently provided evidence that intracellular *Salmonella* comes into contact with $H_2O_2$. Using *Salmonella* containing an $H_2O_2$-responsive Mu*d-lux* gene fusion, they demonstrated maximal expression of the reporter gene 80–90 min after *Salmonella* infection of J774 macrophages (42).

One of the most striking findings of this study is the variation in relative susceptibility of catalase-deficient (*katE/katG*) and DNA repair-deficient (*recA*) *Salmonella* depending upon the in vitro conditions of $H_2O_2$ concentration and cell density. Catalase-deficient mutants are more susceptible to high $H_2O_2$ concentrations at high cell density than the repair-deficient mutant, while the repair-deficient mutant is more susceptible to $H_2O_2$ at low cell density. These observations help to extend and reconcile prior conflicting reports regarding the relative importance of catalase and DNA repair mechanisms in bacterial susceptibility to $H_2O_2$ (22, 43, 44). Imlay and Linn (44) have previously suggested that DNA damage is the predominant lethal action caused by low concentrations of $H_2O_2$ ($\leq 1$ mM). Therefore, *recA* mutant *S. typhimurium* is highly susceptible to $H_2O_2$ killing occurring at low peroxide concentrations and low cell density. At higher $H_2O_2$ concentrations, cell death results from a mixture of DNA damage and injury to other undefined cell targets (1). Hence, *recA* and DNA repair play only a partial role in the cellular defenses against this mode of damage (44). At high cell densities in vitro, catalase-containing bacteria are able to lower ambient $H_2O_2$ concentrations by mass effect. However, the protection afforded by catalase decreases proportionally at low cell densities (36), because the rate of $H_2O_2$ diffusion into single cells appears to exceed the rate of degradation by cellular catalase. As predicted by this model, the protective effect of high cell density is abrogated in mutant *S. typhimurium* unable to produce catalase (Fig. 3).

The identification of mutants with differential susceptibility to killing by $H_2O_2$ under various in vitro conditions provides biological sensors to determine the oxidative stress experienced by *Salmonella* within host phagocytes. While *katE/katG* mutant *S. typhimurium* retains full virulence in vivo and the ability to survive in macrophages in vitro, *recA* mutant *S. typhimurium* demonstrates attenuated virulence in mice and reduced survival in macrophages (15), which appears to be dependent upon the respiratory burst capability of the phagocytic cells. The in vitro conditions of low $H_2O_2$ concentration (400 $\mu$M) and low cell density (5 × $10^5$ cells/ml), to which the *recA* strain is most susceptible of the mutants tested, appear to best reflect the conditions encountered by *Salmonella* during in vivo infection (15). Under these conditions, the ability to repair DNA damage is more important to microbial survival than the ability to directly inactive $H_2O_2$.

## Acknowledgments

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