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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 (H₂O₂), 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 two of these 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 H₂O₂ at low cell densities in vitro, while the catalase mutant was more susceptible to high H₂O₂ 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 H₂O₂ 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 (H₂O₂), 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 H₂O₂ 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 asteroids (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 H₂O₂ 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, hydperoxidase I (HPI) and hydperoxidase 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) and is regulated by an alternative sigma factor encoded by katF (ρΩS), 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 H₂O₂ 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

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Abbreviations used in this paper: HP, hydperoxidase; LB, Luria-Bertani; NBT, nitroblue tetrazolium.
Table 1. Bacterial Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14028s</td>
<td>Wild-type</td>
<td>12</td>
</tr>
<tr>
<td>CL1000</td>
<td>recA</td>
<td>15</td>
</tr>
<tr>
<td>MS7953</td>
<td>phoP::Tn10 Tet’</td>
<td>12</td>
</tr>
<tr>
<td>SFI005</td>
<td>katE::pRR10ΔtrfA Pen’</td>
<td>24</td>
</tr>
<tr>
<td>MS4999</td>
<td>katE::Tn10</td>
<td>12</td>
</tr>
<tr>
<td>XFI1000</td>
<td>katG::pRR10ΔtrfA Pen’</td>
<td>This work</td>
</tr>
<tr>
<td>XFI1001</td>
<td>katE/katG</td>
<td>This work</td>
</tr>
<tr>
<td>TH2423</td>
<td>Contains P22 on a mini-F plasmid</td>
<td>27</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>Tra’ recA pro thi hsdR chr::RP4-2</td>
<td>28</td>
</tr>
<tr>
<td>pRR10</td>
<td>RK2 mini-replon</td>
<td>29</td>
</tr>
</tbody>
</table>

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 1. 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-replon 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’TGGTGGCCGAACAAAGCTTGCCTGTTG-3’ and 5’TGGTGGCCGGACACTTTAAGCCTGGCGC-3’ were derived from the published katG sequence (20).

Catalase assay. Quantitative catalase assays were performed with an oxgraph (31), with activity expressed as units per milligram of dry weight. Catalase activity was visualized on 9.5% non-denaturing polyacrylamide gels (32).

Hydrogen peroxide susceptibility assay. The susceptibility of stationary phase bacterial cells to H₂O₂ was initially determined by adding H₂O₂ 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 H₂O₂ was added to stationary phase bacteria diluted to either 5 × 10⁵ or 10⁶ 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 protease 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 H₂O₂ 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 administration (26).

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 conjugaingly 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.

Results

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.

Figure 2. Catalase activity expressed by Salmonella catalase mutants. Catalase activity was assayed using an oxgraph (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.
Figure 3. Sensitivity of Salmonella at high and low density to hydrogen peroxide. Stationary phase wild-type (open squares), recA mutant (closed squares), katE mutant (open circles), and katE/katG mutant (closed circles) S. typhimurium were resuspended in M9 broth to either $10^9$ 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 $400$ $\mu$M (B and D). Samples were incubated at $37^\circ C$ 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 katE-encoded HPII catalase, MS4999s expresses only the katG-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 katE-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 katE and katG single mutants were resistant to 15 mM $H_2O_2$, but the double katE/katG 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 katE/katG 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
peritoneal containing bacteria/cell

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) \(10^3\) 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<sub>500</sub> and compared with a standard curve. (C) \(10^3\) 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 \(5 \times 10^7\) cells/ml, which may be more similar to bacterial densities during infection studies. In these experiments, the catalase mutants were compared with the isogenic recA 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 recA mutant was the most sensitive strain tested to \(H_2O_2\). Low concentration (400 \(\mu\)M) \(H_2O_2\) killed only the recA 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 recA 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 katE/katG 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 katE/katG 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 katE/katG 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. Infected 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 mutant Salmonella XF1001 was not attenuated as measured by either lethal dose or time until death. All mice given \(10^3\) CFU of the katE/katG mutant intraperitoneally were dead by day 5, which was identical to mice infected with wild-type 14028s (Fig. 5 A). Mice infected with the katF 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.
Figure 5. Virulence of catalase mutants in mice. Groups of female BALB/c mice were infected intraperitoneally with either 10^5 CFU (A) or 10^7 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.

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·) 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 μM) 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 H2O2. Using *Salmonella* containing an H2O2-responsive MudA 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 H2O2 concentration and cell density. Catalase-deficient mutants are more susceptible to high H2O2 concentrations at high cell density than the repair-deficient mutant, while the repair-deficient mutant is more susceptible to H2O2 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 H2O2 (22, 43, 44). Imlay and Linn (44) have previously suggested that DNA damage is the predominant lethal action caused by low concentrations of H2O2 (∼1 mM). Therefore, recA mutant *S. typhimurium* is highly susceptible to H2O2 killing occurring at low peroxide concentrations and low cell density. At higher H2O2 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 H2O2 concentrations by mass effect. However, the protection afforded by catalase decreases proportionally at low cell densities (36), because the rate of H2O2 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 H2O2 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. In the in vitro conditions of low H2O2 concentration (400 μM) and low cell density (5 × 10³ cells/ml), to which the recA strain is most susceptible of the mutants tested, appeared 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 H2O2.

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**References**


