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# Loss of Catalase Activity in Tn*1545*-Induced Mutants Does Not Reduce Growth of *Listeria monocytogenes* In Vivo

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**Two catalase-negative mutants of *Listeria monocytogenes* were obtained by chromosomal insertions of the conjugative transposon Tn*1545*. The loss of catalase activity did not reduce the level of virulence of these mutants in mice. Indeed, both mutants were capable of growing in host tissues at the same rate as the parental catalase-positive strain. These results favor the view that catalase does not play a critical role in the resistance of *L. monocytogenes* to macrophage killing.**

There is evidence that the facultative intracellular pathogen *Listeria monocytogenes* is able to resist killing by host macrophages (15). Since *L. monocytogenes* is readily ingested by phagocytes (5, 15, 17, 18) and induces a significant oxidative metabolic burst in these cells (17), this implies that this pathogen escapes the antibacterial products, especially the reactive oxygen metabolites, released into the phagosomal compartment within minutes following its internalization (3). One of these oxidizing agents, hydrogen peroxide, exhibits a potent antimicrobial activity when used either alone or in combination with the hydroxyl radical (OH · ) (3). Catalase produced by some aerobic microorganisms is able to convert hydrogen peroxide to nontoxic molecules ($H_2O$ and $O_2$) inside the phagosomal compartment. There is evidence that such a scavenger system might play an important role in the virulence of catalase-positive intracellular pathogens, including *Mycobacterium tuberculosis* and *Nocardia asteroides* (4, 13). With respect to *L. monocytogenes*, it has also been suggested that catalase and superoxide dismutase (SOD) might contribute to the pathogenicity of this bacterium by detoxifying the oxygen-dependent microbicidal products of phagocytic cells (5, 22, 23).

To study the role of catalase as a virulence factor during the process of intracellular growth of *L. monocytogenes*, we recently used transposon mutagenesis with the conjugative transposon Tn*1545* to construct a bank of mutants from strain EGD-SmR (10). A total of 2,500 mutants were screened for catalase activity by suspending bacterial colonies in hydrogen peroxide (10 volumes). This classic qualitative procedure allowed us to isolate two catalase-negative mutants, designated Cat-147 and Cat-160. By challenging mice intravenously (i.v.), we could not find any significant difference between the 50% lethal doses of these two mutants and those of the parental strain EDG-SmR; the 50% lethal doses ranged from $10^{6.2}$ to $10^{6.5}$ per mouse (10). The aim of the present work was to further evaluate the impact of this catalase defect on the ability of bacteria to multiply in host tissues.

The first step was to determine the level of catalase activity of these two mutants during the exponential phase of bacterial growth in vitro. This was done with a colorimetric assay derived from the method of Amin and Olson (1) modified by Sinha (21). Briefly, bacteria grown to the log phase in brain heart infusion broth (Diagnostics Pasteur, Marnes la Coquette, France) were washed three times in potassium phosphate buffer (50 mM, pH 7.0) and suspended in this buffer to an $A_{600}$ of 0.52. A 5-ml sample of this suspension was mixed with 5 ml of 0.2 M hydrogen peroxide in phosphate buffer. Samples (1 ml) were taken at intervals, added to 2 ml of a dichromate-acetic acid solution to stop the reaction, boiled for 10 min, and centrifuged for 15 min at 10,000 × *g*. The $A_{570}$ of the supernatants was determined, and the quantity of hydrogen peroxide remaining was calculated from a standard curve. The results are illustrated in Fig. 1. A suspension of $10^8$ cells of the parental strain EGD-SmR was capable of converting 30 μM hydrogen peroxide after 2.5 min of incubation with the substrate. No catalase activity could be detected from the same quantities of the two catalase-negative mutants under these experimental conditions, indicating that the two Tn*1545*-induced mutants actually express a genetic defect in the production of catalase.

Since it has been reported that catalase-negative strains of *L. monocytogenes* possess a higher level of SOD activity than do catalase-positive strains (22, 23), the next step was to determine the level of SOD activity in the two catalase-negative mutants. SOD assays were performed at 37°C with minor modifications of the procedure described by Sinet et al. (20). Bacteria grown at 37°C in brain heart infusion broth were harvested at the early stationary phase by centrifugation at 10,000 × *g* for 20 min at 4°C, washed twice, suspended in cold water to a density of about $10^{10}$ bacteria per ml, and disrupted by sonication. Bacterial debris was eliminated by two centrifugations at 20,000 × *g* and 4°C for 20 min. The supernatants were adjusted to a protein concentration of 1 mg/ml, as assessed by the method of Bradford (7), and tested for SOD activity. Reaction mixtures for the SOD assay contained 0.01 M $NaHCO_3$–0.01 M $Na_2CO_3$ (pH 10), $10^{-4}$ EDTA (Sigma Chemical Co., St. Louis, Mo.), 2.5 × $10^4$ M xanthine (Sigma), and about 2 × $10^{-4}$ M Nitro Blue Tetrazolium (NBT) (Sigma). The concentration of NBT was precisely adjusted to obtain an $A_{260}$ of 0.720 with NBT alone in 0.01 M $NaHCO_3$–0.01 M $Na_2CO_3$ (pH 10). Xanthine oxidase (grade III from buttermilk; Sigma) was progressively added to induce a rate of NBT reduction of 0.0230 absorbance units per min when measured at 540 nm. Under these conditions, one unit of SOD activity is defined as the amount of enzyme causing 50% inhibition of the rate of NBT reduction (20). Two separate determinations were made for each bacterial strain. SOD activities were expressed as units per milligram of protein. We found that the SOD activity reached 29 U/mg of protein for strain EGD-SmR and was

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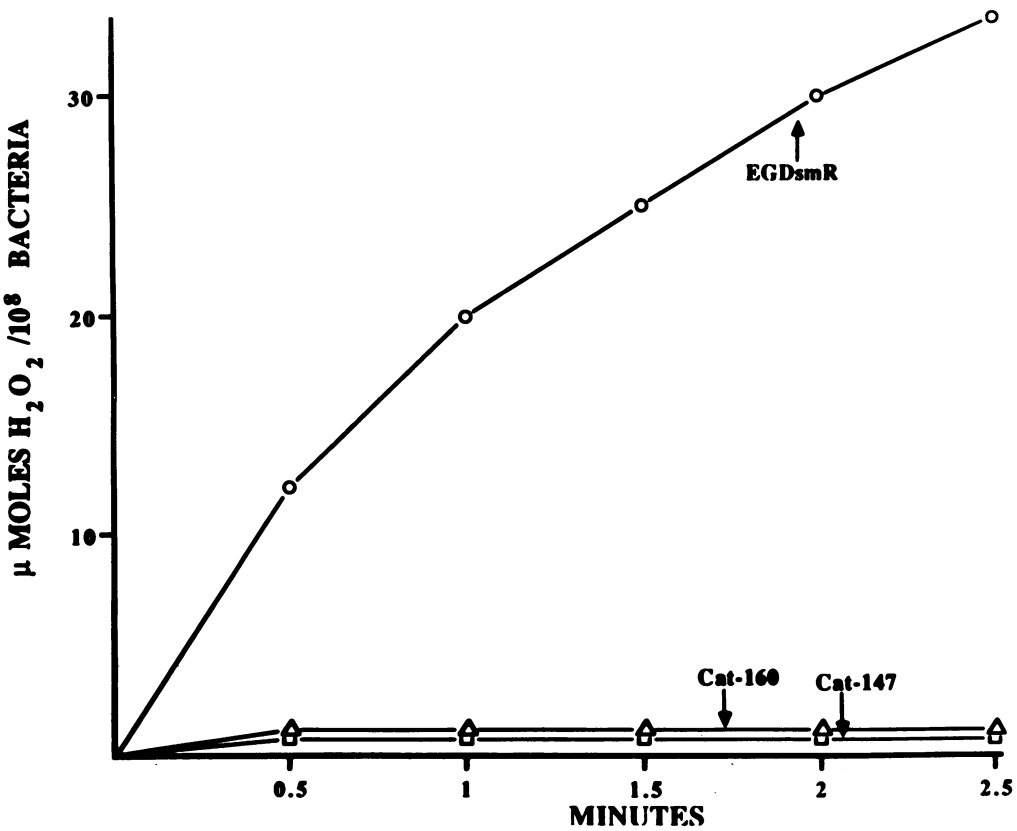


FIG. 1. Titration of catalase activity in the two catalase-negative Tn*1545*-induced mutants and in the parental strain EGD-SmR. Washed bacteria ($10^8$) in the log phase were mixed with hydrogen peroxide (0.2 M), and the quantity of hydrogen peroxide remaining at various times was assessed by a colorimetric assay. No catalase activity could be detected from suspensions ($10^8$ bacteria) of the two catalase-negative mutants. In contrast, strain EGD-SmR converted 30 μM hydrogen peroxide after 2.5 min of incubation with the substrate.

significantly increased for the catalase-negative mutants Cat-147 and Cat-160 to levels of 79 and 70 U/mg of protein, respectively.

If catalase represents a scavenger system protecting viable bacteria against oxygen derivatives, one might expect that catalase-negative bacteria would be exquisitely sensitive to hydrogen peroxide. This possibility was investigated by incubating at 37°C a washed (phosphate-buffered saline [pH 7.2]) suspension of exponentially growing bacteria (~$10^8$ bacteria per ml) in hydrogen peroxide at various concentrations (0.01 to 10 mM). Bacterial survival after hydrogen peroxide exposure was then determined by counting viable organisms at various times over a 3-h period. Bacterial survival was quite similar for the two catalase-negative mutants, as compared with that of the parental strain EGD-SmR (Fig. 2).

Direct evidence that catalase is not involved in the growth of *L. monocytogenes* in host tissues was found in in vivo experiments with 6- to 8-week-old female Swiss mice (Charles River Breeding Laboratories, Inc., St. Aubin Lès Elboeuf, France). The kinetics of bacterial survival in organs were determined after i.v. inoculation of a lethal challenge of ~$4 \times 10^6$ bacteria in 0.5 ml of 0.15 M NaCl per mouse. No significant difference could be found between the two mutants and strain EGD-SmR during the early phase of bacterial survival (48 h) in the spleen and liver (Fig. 3). When a sublethal dose (~$4 \times 10^3$ bacteria i.v. per mouse) was used, survival of the catalase-negative mutants in organs was again similar to that of strain EGD-SmR over a 7-day period, with an early phase of bacterial multiplication followed by a slow phase of bacterial elimination (Fig. 4). Although it could be speculated that another catalase gene not expressed in vitro might be turned on in vivo, our results strongly suggest that catalase does not represent a scavenger mechanism required to resist the macrophage oxidative burst, considering that bacterial growth in vivo is closely associated with intracellular multiplication in macrophages (15).

Aerobic microorganisms utilize catalase enzymes to limit the endogenous accumulation of hydrogen peroxide, which is an inevitable by-product of oxidative metabolism and which expresses a high cytotoxic potential (12). Hydrogen peroxide is also one of the microbicidal agents generated during the respiratory burst following phagocytosis (3). It has been postulated that the catalase activity exhibited by aerobic pathogens might detoxify this metabolite accumulating in the phagosomal compartment during the process of phagocytosis, enabling microorganisms to partially resist the microbicidal activity of phagocytes. This view is supported by many experimental data. As demonstrated for *N. asteroides* and *Staphylococcus aureus*, exogenous catalase added to bacteria of impaired virulence greatly enhances their resistance to the microbicidal effect of phagocytes (4, 16). Moreover, the virulence for humans or laboratory animals of some bacterial species, including *M. tuberculosis*, is apparently correlated with the relative amounts of catalase produced by these pathogens (3, 4, 8, 13, 16, 17). Such a relationship exists for *L. monocytogenes*, since it has been suggested that *L. monocytogenes* resists macrophage oxidative antibacterial agents by producing sufficient catalase to inactivate these products (6). Moreover, spontaneous cata-

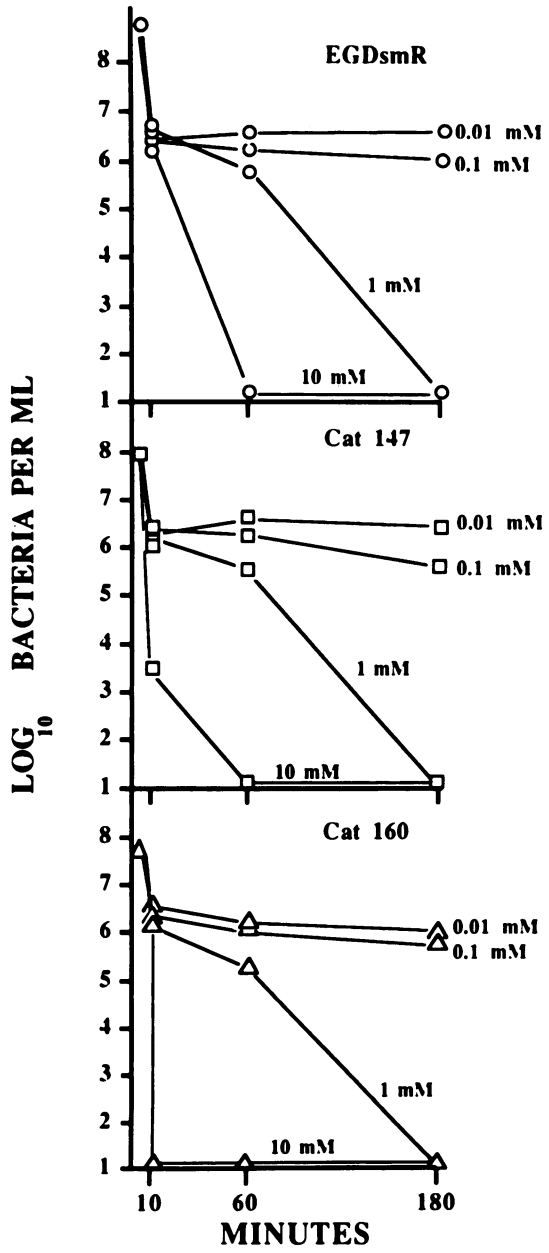


FIG. 2. Survival of the two catalase-negative mutants and of the parental strain EGD-SmR at various concentrations of hydrogen peroxide in vitro. Washed bacteria (~$10^8$/ml) in the log phase were incubated at 37°C with various concentrations of hydrogen peroxide (0.01 to 10 mM). Viable bacteria were counted at intervals by plating aliquots on tryptic soy agar.

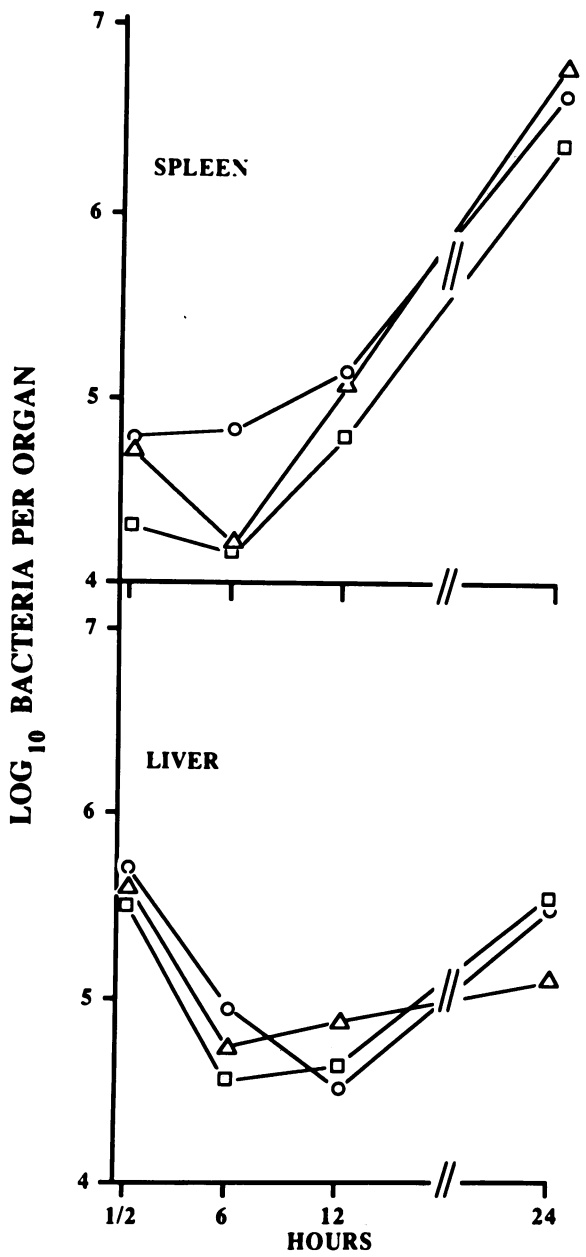


FIG. 3. Early bacterial multiplication in the spleens and livers of mice infected with a lethal challenge of the two catalase-negative mutants or of the parental strain EGD-SmR (~$4 \times 10^6$ bacteria i.v.). At intervals, groups of five mice were killed by cervical dislocation. The spleens and livers were aseptically removed and homogenized separately in sterile saline. Then, 0.1-ml volumes of serial 10-fold dilutions were surface plated on tryptic soy agar (minimal detection limit, $10^2$ bacteria per organ). No difference could be detected in early bacterial growth in host tissues over the first 24 h of infection between the two catalase-negative mutants and the parental strain.

lase-negative mutants of this species were found to be less virulent in mice than were catalase-positive strains (22, 23). However, the interpretation of these findings remains unclear for several reasons. First, the addition of exogenous catalase to bacteria prior to phagocytosis does not really mimic the conditions encountered in situ during the infectious process in the host. Second, some data are based on the use of genetically uncharacterized strains. Indeed, catalase-negative mutants isolated spontaneously or obtained after several in vitro passages may differ from virulent bacteria in many genes other than those responsible for catalase activity.

These difficulties were overcome in the present work by testing the virulence of catalase-negative mutants obtained from *L. monocytogenes* by chromosomal insertions of the conjugative transposon Tn*1545*, so that these mutants most likely differed from the parental strain in only a very limited number of genes (10). The loss of catalase activity was not associated with a restriction of bacterial replication in organs. This finding unambiguously demonstrates that catalase

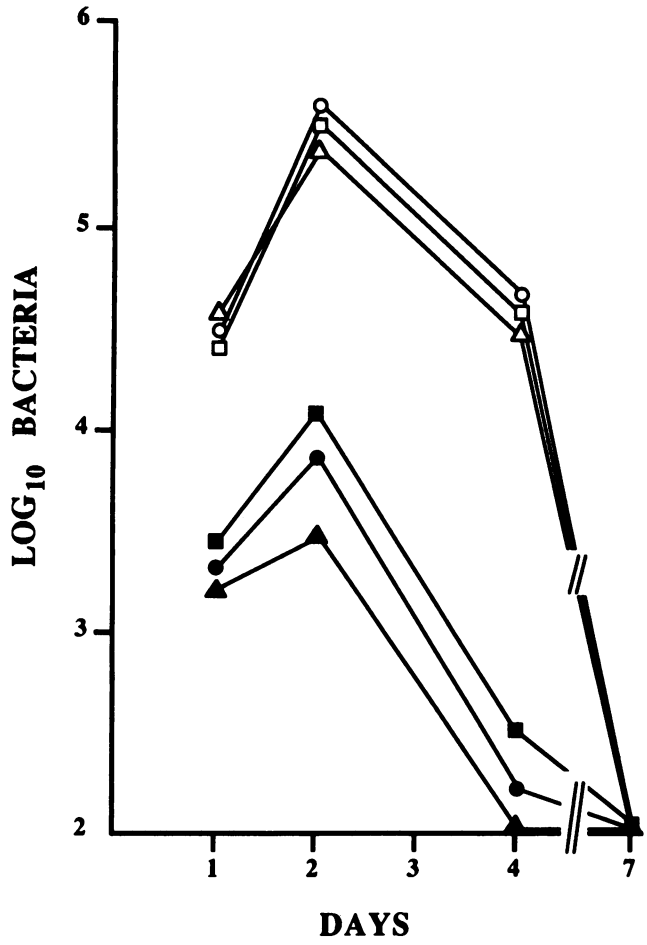


FIG. 4. Bacterial survival in the spleens and livers of mice infected with a sublethal challenge of the two catalase-negative mutants or of the parental strain EGD-SmR. Bacteria ($\sim 4 \times 10^3$) were inoculated i.v. into mice. Bacterial counts in organs were obtained as described in the legend to Fig. 3. The kinetics of elimination of the catalase-negative mutants were similar to those of the parental strain EGD-SmR. Symbols: ○ and ●, EGD-SmR in spleen and liver, respectively; □ and ■, Cat-147 in spleen and liver, respectively; △ and ▲, Cat-160 in spleen and liver, respectively.

does not play a critical role in the resistance of *L. monocytogenes* to macrophage killing. As already found by Welch et al. for catalase-negative *L. monocytogenes* strains (22, 23), the two mutants tested in our work expressed higher levels of SOD activity (about a twofold increase) than did the catalase-positive parental strain. Nevertheless, the relative high levels of SOD activity found in the catalase-negative mutants can not explain why the virulence of these mutants remained unchanged. Indeed, it was recently demonstrated that the killing of *L. monocytogenes* by hydrogen peroxide generated by the glucose oxidase-glucose system is unaffected by the level of SOD activity (6). The reason why the level of SOD activity was increased in the catalase-negative mutants remains obscure. There might exist some compensatory mechanisms which involve SOD activity and which would allow catalase-negative bacteria to rapidly multiply in vitro and in vivo.

That catalase-negative mutants from *L. monocytogenes* express the same level of virulence as does the parental strain is not really surprising. Although specific information about the catalase enzyme(s) of *L. monocytogenes* is lacking, it is assumed that the catalase activity of the bacteria is generally supported by intracytoplasmic enzymes (3). Consequently, as suggested by Beaman and Beaman (3), the bacterial catalase enzymes may not be readily available to convert hydrogen peroxide produced by the phagocytes through the metabolic respiratory burst. In this connection, it must be emphasized that *L. monocytogenes* does not resist the bactericidal activity of hydrogen peroxide in vitro, even at low concentrations of this oxidant (Fig. 2). Altogether, these data indicate that *L. monocytogenes* must use another strategy to avoid the microbicidal mechanisms of macrophages. We recently demonstrated in nonprofessional phagocytes that listeriolysin O, a sulfhydryl-dependent toxin fully active at a pH of ~5.5 (11), causes rapid destruction of vacuole membranes (9). A similar phenomenon might exist in professional phagocytes (2, 14, 19), enabling *L. monocytogenes* to escape from phagosomes and thus to avoid the antibacterial products delivered into these vacuoles. In this hypothesis, *L. monocytogenes* need not resist the reactive oxygen metabolites to grow intracellularly.

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