

Contribution of Superoxide Dismutase and Catalase Activities to *Shigella flexneri* Pathogenesis

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A *Shigella flexneri* serotype 5 strain deficient in the production of the iron-containing superoxide dismutase FeSOD (*sodB*) and a catalase-negative (*katFG*) *S. flexneri* serotype 5 strain were isolated. Both strains were examined for increased sensitivity to oxygen stress by using assays involving killing by mouse peritoneal macrophages and human polymorphonuclear leukocytes as well as infection of rabbit ileal loops. The *sodB* mutant was extremely sensitive to killing by phagocytes when compared with the wild-type parent, M90T. The catalase mutant also showed an increased sensitivity to killing, but to a much lesser extent. Upon infection of rabbit ileal loops and subsequent histopathological examination, the *sodB* mutant caused very little detectable damage to intestinal villi. The pattern of infection was roughly similar to that of BS176, an avirulent plasmidless derivative of M90T. The *katFG* mutant, on the other hand, showed a high degree of destruction, similar to that caused by M90T. This evidence suggests that the superoxide dismutase encoded by *sodB* may play an important role in the pathogenesis of *S. flexneri*. In contrast, catalases appear to make a limited contribution to virulence.

The burst of respiratory activity that is concomitant with phagocytosis produces a number of toxic byproducts. These reactive oxygen intermediates include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (1O_2), and the hydroxyl radical (OH) (8, 15, 16, 32). Such compounds are part of the oxygen-dependent bactericidal mechanism that the phagocyte cell employs. Enzymes such as superoxide dismutase and catalase form part of a defense mechanism that helps protect enteric bacteria from oxygen toxicity and damage (18, 19, 21). The activities of such enzymes have been shown to be induced when bacteria shift from an anaerobic environment to an aerobic environment (19, 20). Superoxide dismutases are thought to help *Nocardia asteroides* (14) and *Listeria monocytogenes* (43) to thwart the killing process of phagocytosis, whereas catalase activity has been suggested to protect *Staphylococcus aureus* (28) and *Neisseria gonorrhoeae* (1). However the exact mechanisms by which bacteria combat oxidative burst during phagocytosis to enable intracellular survival remain poorly understood and warrant further investigation.

Superoxide dismutases are metalloenzymes that convert superoxide radicals into $H_2O_2 + O_2$ (33). *Escherichia coli* produces two such enzymes: an iron-containing superoxide dismutase and a manganese-containing superoxide dismutase, referred to as FeSOD and MnSOD, respectively (23, 45). FeSOD (*sodB*) is expressed under both anaerobic and aerobic conditions; however, MnSOD (*sodA*) is synthesized only after exposure to oxygen and is induced by the presence of superoxide radicals (19, 22). Cloning of the *E. coli* structural genes *sodA* and *sodB*, which encode MnSOD and FeSOD, respectively, has been reported previously (36, 40). By Mu transposition Carlouz and Touati (7) mutated each of these genes and then introduced them into the *E. coli* chromosome by allelic exchange.

Catalases also form part of the defense of the cells against oxidative stress. Three loci have been shown to affect the

synthesis of two independent catalases in *E. coli*. The *katG* locus encodes the structural gene for two isoenzymes that form catalase HPI (27), whereas the loci *katE* and *katF* are responsible for the production of a second catalase, HPIII (also referred to as HPII) (25, 26). Consequently, either a *katE katG* or a *katF katG* genotype results in total elimination of catalase activity. Loewen and co-workers have obtained Tn10 insertions in each of the loci *katE*, *katF*, and *katG*, giving rise to strains UM120, UM122, and UM202, respectively (25, 26).

Shigella flexneri could also be expected to possess superoxide dismutases and catalases to help deal with oxygen stress. This is of potential importance for when the *Shigella* bacteria pass from the anaerobic environment of the host intestine to macrophages and polymorphonuclear cells, where the bacteria are confronted with an instantaneous oxygen burst.

Hence, by P1 transduction, mutations were introduced into the chromosome of *S. flexneri*, which enhanced their sensitivity to oxygen radicals. These strains were examined by using mouse peritoneal macrophages and human polymorphonuclear leukocytes (PMNL) as well as more definitive assays such as guinea pig keratoconjunctivitis and rabbit ligated ileal loop assays to observe any changes in virulence that may have occurred and thereby might affect intracellular survival of the bacteria.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study and their relevant characteristics are listed in Table 1.

Medium and growth conditions. Bacteria were routinely grown in tryptic soy broth (Diagnostics Pasteur, Marnes la Coquette, France) or LB broth (34) at 37°C. When necessary, antibiotics were added at the following final concentrations: chloramphenicol, 30 µg/ml; kanamycin, 50 µg/ml; tetracycline, 12.5 µg/ml.

Transduction. The generalized transducing bacteriophage P1 *vir* was propagated on the appropriate strains, and lysates

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TABLE 1. Bacterial strains

Strain	Relevant characteristics	Reference or source
<i>E. coli</i> K-12		
UM120	<i>thi-1</i> HfrH <i>katE12::Tn10</i> Tet ^r	26
UM122	<i>thi-1</i> HfrH <i>katF13::Tn10</i> Tet ^r	26
UM202	<i>thi-1</i> HfrH <i>katG17::Tn10</i> Tet ^r	25
QC772	F ⁻ <i>Dlac-4169 rpsL (sodA-lacZ)</i> 49 cml ^r Lac ⁺	7
QC773	F- <i>Dlac-4169 rpsL (sodB-kan)</i> l-D2 Kan ^r	7
<i>S. flexneri</i> sero- type 5		
M90T	Harbors virulence plasmid pWR100	37
BS176	Derivative of M90T cured of pWR100	37
SC600	M90T <i>katE::Tn10</i> Tet ^r	This work
SC601	M90T <i>katF::Tn10</i> Tet ^r	This work
SC602	M90T <i>katG::Tn10</i> Tet ^r	This work
SC603	M90T <i>katF</i> , fusaric acid resistant, Tet ^r	This work
SC604	M90T <i>katF katG::Tn10</i> Tet ^r	This work
SC605	M90T <i>sodB</i> Kan ^r	This work

were used to transduce chromosomal DNA into *S. flexneri* M90T (34).

Selection for loss of tetracycline resistance. Elimination of the tetracycline-resistant transposon *Tn10* was achieved in the presence of fusaric acid (2 µg/ml) by direct plate selection on the medium of Bochner et al. (4).

Serological tests. The O-antigenic specificity of transductants was determined by slide agglutination with *S. flexneri* serotype 5 typing antiserum.

Preparation of superoxide dismutase crude extracts and assays. Cell extracts of late-logarithmic cultures were made as described by Carlioz and Touati (7, 41). Extracts were loaded on Nap-5 columns containing Sephadex G-25 (Pharmacia) and eluted with 50 mM KPO₄-0.1 mM EDTA (pH 7.8). Samples were stored at -70°C. Superoxide dismutase assays were performed by the method of Beauchamp and Fridovich (2), with minor alterations (29).

Gel electrophoresis of LPS. Extracts of lipopolysaccharide (LPS) were prepared as described elsewhere (44). LPS preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24) and transferred onto nitrocellulose filters (42). Filters were successively incubated with *Shigella* anti-LPS serum and ¹²⁵I-labeled protein A. The presence of LPS was then visualized by autoradiography.

In vitro assay for survival of bacteria within macrophages. Casein-elicited peritoneal macrophages were harvested from BALB/c mice by peritoneal lavage with RPMI 1640 (Flow laboratories, Inc., McLean, Va.). For casein elicitation, mice were injected with 1 ml of 6% sodium caseinate in 0.9% NaCl 4 days before sacrifice. Macrophages were maintained in RPMI 1640 supplemented with complement-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). Macrophages were incubated at 37°C in 5% CO₂ in 35-mm plastic tissue culture dishes for 18 h before bacterial infection. Then 7 × 10⁵ macrophages were allowed to adhere to each dish. Plates were washed three times with Earle balanced salt solution (GIBCO) before the addition of 1 ml of a bacterial suspension opsonized in 5% mouse serum, at a multiplicity of infection of 100. Plates were centrifuged for 10 min at 2,200 × g and incubated at 37°C for 15 min to allow entry. Plates were washed once with Earle balanced salt solution and covered with 1 ml of RPMI 1640 supplemented

with 2 mM glutamine and gentamicin (25 µg/ml). This point was defined as time zero (T₀). Plates were then sampled at various times (T₁ [5 min], T₂ [10 min], T₃ [15 min], and T₄ [30 min]) after infection by washing three times with Earle balanced salt solution, aspirating the medium, and lysing the macrophages with 0.5 ml of 1% sodium deoxycholate. Duplicate samples of each well were plated and counted. The mean of at least three experiments was calculated and graphed.

Preparation of PMNL and in vitro assay for survival of bacteria within PMNL. Blood (20 ml) was drawn from human volunteers in 20 ml of heparin and immediately placed on ice. After a dilution to 1/3 in Hanks buffer, PMNL were isolated by centrifugation on a Ficoll gradient followed by dextran sedimentation (5, 12). Residual erythrocytes were lysed by the addition of NH₄Cl. Cells were then washed and suspended in Hanks buffer. Bacterial suspensions opsonized in 5% human serum were added to 2 × 10⁶ PMNL at a multiplicity of infection of 1 in the same medium. Interaction was then allowed to proceed. Samples were taken at 5 and then every 15 min for a period of 65 min. Cells were then lysed as above, and samples were plated and counted. The mean of at least three experiments was calculated and graphed.

Keratoconjunctivitis assay. The keratoconjunctivitis assay was performed with guinea pigs as described by Sereny (39).

Rabbit ileal loop assay. Ligated ileal loops (10 cm) were prepared in rabbits (ca. 2 kg) anesthetized with 0.5 ml of 0.6% sodium pentobarbital per kg. Inocula of 10⁷ and 10⁹ CFU in 1 ml of tryptic soy broth were tested. Rabbits were sacrificed 18 h later. Fluid accumulation within loops was then recorded, and the volume/length ratio was calculated. Infected loops were then opened, the aspect of the mucosal surface was recorded, and portions were fixed in 10% buffered Formalin. Specimens were processed by standard histopathological procedures and stained with hematoxylin-eosin-safranin.

RESULTS

Isolation of catalase mutants. By P1 *vir* transduction each of the *Tn10* insertions was transferred separately to *S. flexneri* wild-type strain M90T, generating strains SC600 (*katE::Tn10*), SC601 (*katF::Tn10*), and SC602 (*katG::Tn10*), respectively. With the medium (with fusaric acid) developed by Bochner et al. (4), it was possible to select tetracycline-susceptible clones of SC601, hence generating a deletion around the *Tn10* insertion (SC603). SC603 was then retransduced with a P1 *vir* lysate prepared on strain UM202, thereby transferring the *katG::Tn10* region of UM202 into an *S. flexneri katF* background. Transductants were screened for catalase activity by applying a drop of 30% H₂O₂ to colonies and observing the presence of O₂ bubbles, which signifies catalase production. A complete catalase-deficient (i.e., no evolution of O₂ bubbles) *S. flexneri (katF katG::Tn10)* strain was identified and designated SC604.

Isolation of an *sodB* mutant. Again through P1 *vir* transduction, the mutated *sodA* and *sodB* loci were transferred to *S. flexneri* M90T. Although *S. flexneri sodB* transductants were obtained, several attempts to isolate *S. flexneri sodA* transductants proved unsuccessful. The reasons for this remain unclear. All transductants were confirmed by slide agglutination with antisera specific against *S. flexneri*. LPS preparations were made from each mutant strain, and each strain was seen to possess a complete O-polysaccharide side chain when compared with M90T. The ability to bind the dye

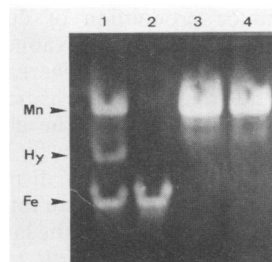


FIG. 1. Visualization of superoxide dismutase activity. Crude cell extracts (40 mg of protein) were loaded on a nondenaturing 10% polyacrylamide gel and then stained for superoxide dismutase activity. The following samples were run (lanes): 1, M90T; 2, QC772; 3, QC773; 4, SC605 (M90T *sodB*). Abbreviations: Mn, MnSOD; Hy, hybrid superoxide dismutase; Fe, FeSOD.

Congo red (Pcr^+) was also tested. Only transductants which were smooth and Pcr^+ were selected for further study, since rough strains and mutants no longer able to bind Congo red are avirulent in definitive assay systems such as the Sereny test and rabbit ileal loop assay (30, 31).

Superoxide dismutase activity. A number of transductants were analyzed on nondenaturing polyacrylamide gels for SOD activity (Fig. 1). The wild-type strain *S. flexneri* M90T showed three bands of activity. The upper band represents the MnSOD protein encoded by the *sodA* gene, and the bottom band represents the FeSOD protein encoded by *sodB*. A band of activity ran with an electrophoretic mobility between that of MnSOD and FeSOD. This represents a hybrid form of superoxide dismutase that contains one subunit of MnSOD and one subunit of FeSOD and has previously been observed and described in *E. coli* (10). In the isolated *sodB-kan Shigella* transductants there is the expected absence of the band that corresponds with FeSOD production.

Survival of *katFG* and *sodB* mutants within phagocytes. The ability of strains SC604 (*katFG*) and SC605 (*sodB*) to survive intracellularly within macrophages was assayed by using macrophages isolated from the peritoneal cavity of mice. Figure 2 shows survival curves of *S. flexneri* M90T, BS176, SC604, and SC605. M90T is an invasive isolate of *S. flexneri* serotype 5, with BS176 being a noninvasive derivative that does not harbor the 220-kilobase virulence plasmid pWR100. SC604 and SC605 are M90T with mutations in *katFG* and *sodB*, respectively. In the case of BS176 there was an initial killing period that resulted in a significant decrease in bacterial numbers, but this population then remained fairly constant over the range of time samples were taken. M90T also exhibited a substantial initial killing period; however, in contrast to BS176, this killing process remained moderately effective. In comparison, strain SC605 was immediately killed after phagocytosis, and within a short period of time the viable organisms remaining were insignificant in number if there were any at all. Strain SC604 initially exhibited a somewhat higher resistance to the killing effect of phagocytosis, but again numbers also rapidly declined with time.

When the phagocytes used in the assay were human PMNL, somewhat different results were obtained (Fig. 3). In this assay both M90T and BS176 only displayed a slight drop in viable numbers, and the ratio of surviving bacteria then remained constant. SC605 again was highly sensitive to the killing effects of phagocytes, regardless of whether the population was peritoneal macrophages or PMNL. SC604 in this system displayed a moderate resistance to killing some-

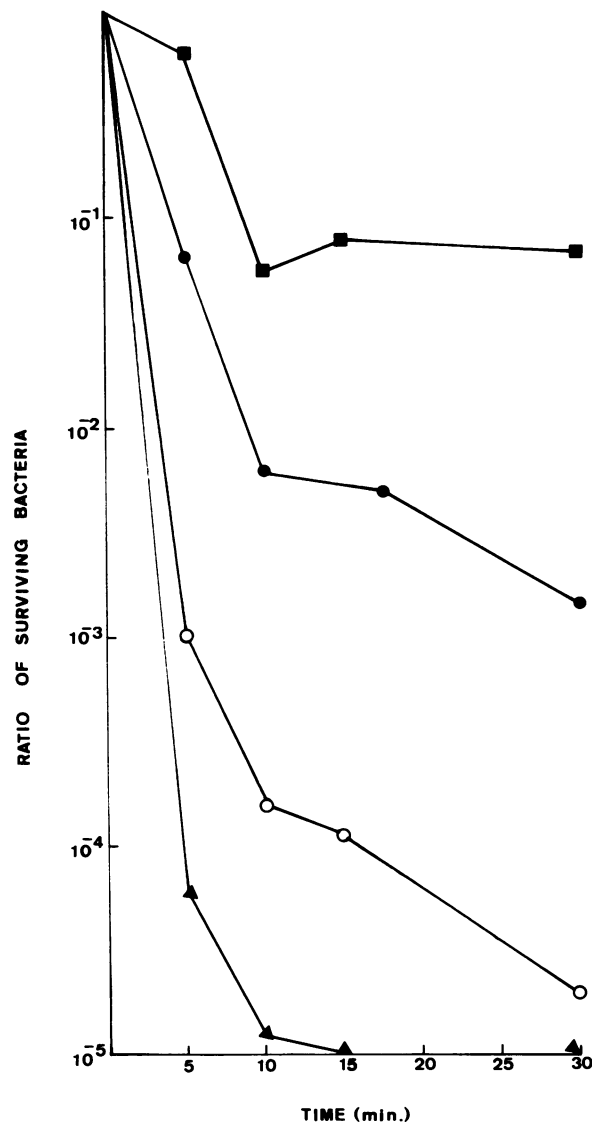


FIG. 2. Killing of *S. flexneri* in mouse peritoneal macrophages. Opsonized *S. flexneri* organisms (7×10^7 per well) were incubated with peritoneal macrophages (7×10^5 per well). Cells were then lysed after different periods of incubation, and the numbers of viable bacteria were counted. Each curve represents the ratio of the number of viable bacteria after a given time period to that number of bacteria at time zero. Symbols: (●) M90T, (■) BS176, (○) SC604 (M90T *katFG*), (▲) SC605 (M90T *sodB*).

where between that of the wild-type strain and that of the *sodB* mutant.

Keratoconjunctivitis production by *katFG* and *sodB* mutants. The *katFG* mutant of *S. flexneri* yielded a positive Sereny test, comparable to that obtained with the wild-type strain M90T. On the other hand, the *sodB* mutant elicited a slight transient conjunctivitis or no response at all.

Infection of rabbit ligated ileal loops. After 18 h of infection, rabbit ligated ileal loops infected by 10^9 bacteria of the wild-type strain M90T, its noninvasive plasmidless derivative BS176, the *katFG* double mutant, or the *sodB* mutant per ml were examined; dramatic differences in the appearance of such loops were visible. M90T and the *katFG* mutant induced production of a large volume of bloody and mucopu-

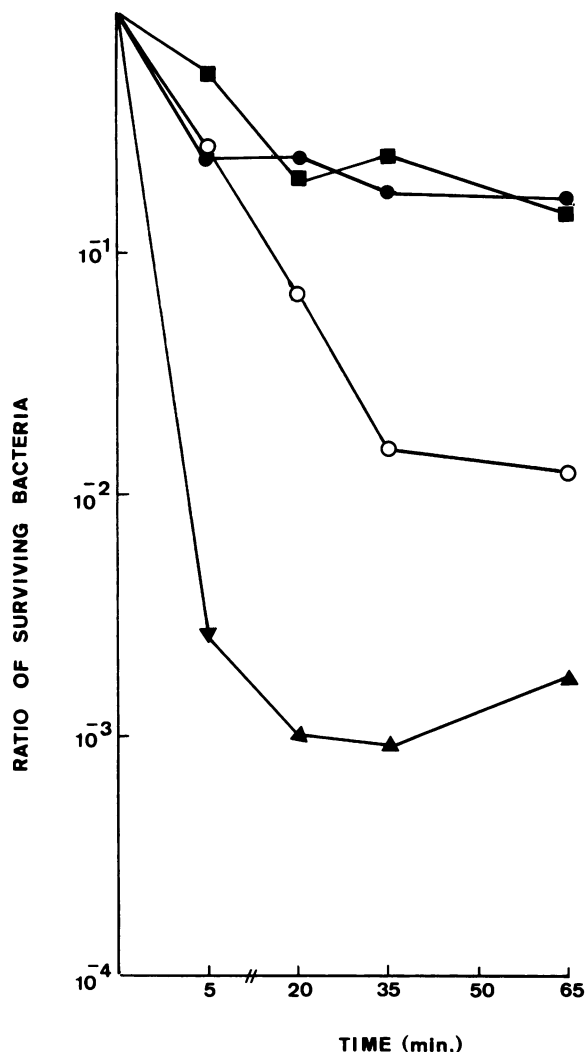


FIG. 3. Killing of *S. flexneri* in human PMNL. Opsonized *S. flexneri* organisms (7×10^7 per well) were incubated with PMNL (7×10^5 per well). Cells were then lysed after different periods of incubation, and the numbers of viable bacteria were counted. Each curve represents the ratio of the number of viable bacteria after a given time period to the number of bacteria at time zero. Symbols: (●) M90T, (■) BS176, (○) SC604 (M90T *katFG*), (▲) SC605 (M90T *sodB*).

purulent fluid (volume/length ratios of 1.46 and 1.21, respectively). Upon gross examination after longitudinal opening of the loop, the intestinal mucosa appeared destroyed by a purulent and bloody process of necrosis which left a thin, parchmentlike, hemorrhagic peritoneal serosa. The intensity of the lesion was maximum upon infection with M90T. Histopathological examination confirmed gross examination in the case of M90T; intestinal villi were no longer present due to total necrosis and replacement by a huge purulent hemorrhagic exudate (Fig. 4A). However, with the *katFG* double mutant, lesions did not appear as severe as expected upon gross examination (Fig. 4C), since villi were essentially conserved although dramatically shortened. The epithelial lining was often destroyed by ulcerated abscesses located both at the tip and in the crypts. In addition, important edema and polymorphic infiltration by inflammatory cells was observed within the lamina propria. BS176 and the *sodB*

mutant did not induce production of fluid. Upon gross examination of the mucosal surface, no abnormality could be observed with the former (Fig. 4B), whereas slight amounts of pus and rare patchy areas of hemorrhagic lesions could be observed with the latter (Fig. 4D). The absence of severe alterations was confirmed in both cases by histopathological examination. Loops infected by BS176 displayed a perfectly normal pattern, that is, long villi and an intact brush border (Fig. 4B). No significant infiltration of the lamina propria was observed. Loops infected by the *sodB* mutant showed a roughly similar pattern, although slight microabscesses and ulcerations (Fig. 4D, open arrowhead) could be observed at the tip of some villi with a bit of exudate within the lumen. Upon closer examination there was also evidence of brush border damage (closed arrowhead). In conclusion, a spectrum of severity of lesion damage could be observed between total purulent necrosis induced by M90T, slightly less severe lesions but the overall architecture of the intestinal tissue remaining with the *katFG* mutant, slight abscesses at the tip of the villi in the case of *sodB*, and no lesions at all upon infection with BS176.

DISCUSSION

Considering the physiopathology of bacillary dysentery, shigellae are confronted with oxygen stress in at least two situations: first, when passage from the anaerobic conditions of the human colonic lumen to the aerobic environment of the colonic epithelial cells occurs; second, upon the encounter of phagocytic cells (resident macrophages of the lamina propria, locally recruited monocytes, and PMNL). These cells produce oxygen radicals via the oxidative burst induced by bacteria during the phagocytic process (35).

To survive, shigellae must therefore be capable of overcoming such situations. Bacterial enzymes such as catalases and superoxide dismutases should play a major role in this process. Therefore, mutations obtained in the catalase (*kat*) or the superoxide dismutase (*sod*) genes should confirm this hypothesis as well as give some indication of the most efficient protective mechanism.

Most of the available data dealing with oxidative stress and bacteria have come from observations with gram-positive microorganisms. Such studies involved the use of non-characterized mutants or isolates that produced lower quantities of catalase or superoxide dismutase. On the other hand, *Salmonella typhimurium* Tn10 transposon mutants have been isolated on the basis of their hypersensitivity to killing by macrophages. Some of these have been mapped to the *oxyR* locus, which is known to be a positive regulator for the coordinate functions dealing with defense against oxygen stress (9, 13).

In this work, we decided to construct well-characterized mutants with mutations in the *kat* and *sod* genes by taking advantage of the close genetic homology existing between *E. coli* and *S. flexneri*, enabling transduction of mutations available within the former into the latter. This appears to be the first attempt at evaluating the role of these enzymes in the disease process of *S. flexneri*. By using this battery of mutants, virulence was studied at two levels: (i) interaction with phagocytic cells and (ii) a more definitive assay system, the rabbit ligated ileal loop.

The wild-type strain M90T and its noninvasive derivative BS176 appeared moderately sensitive to killing by PMNL and macrophages. We previously found (P. Sansonetti, unpublished results) that *S. flexneri* elicits 5 to 10 times less chemiluminescence than does *S. aureus* in infected PMNL,

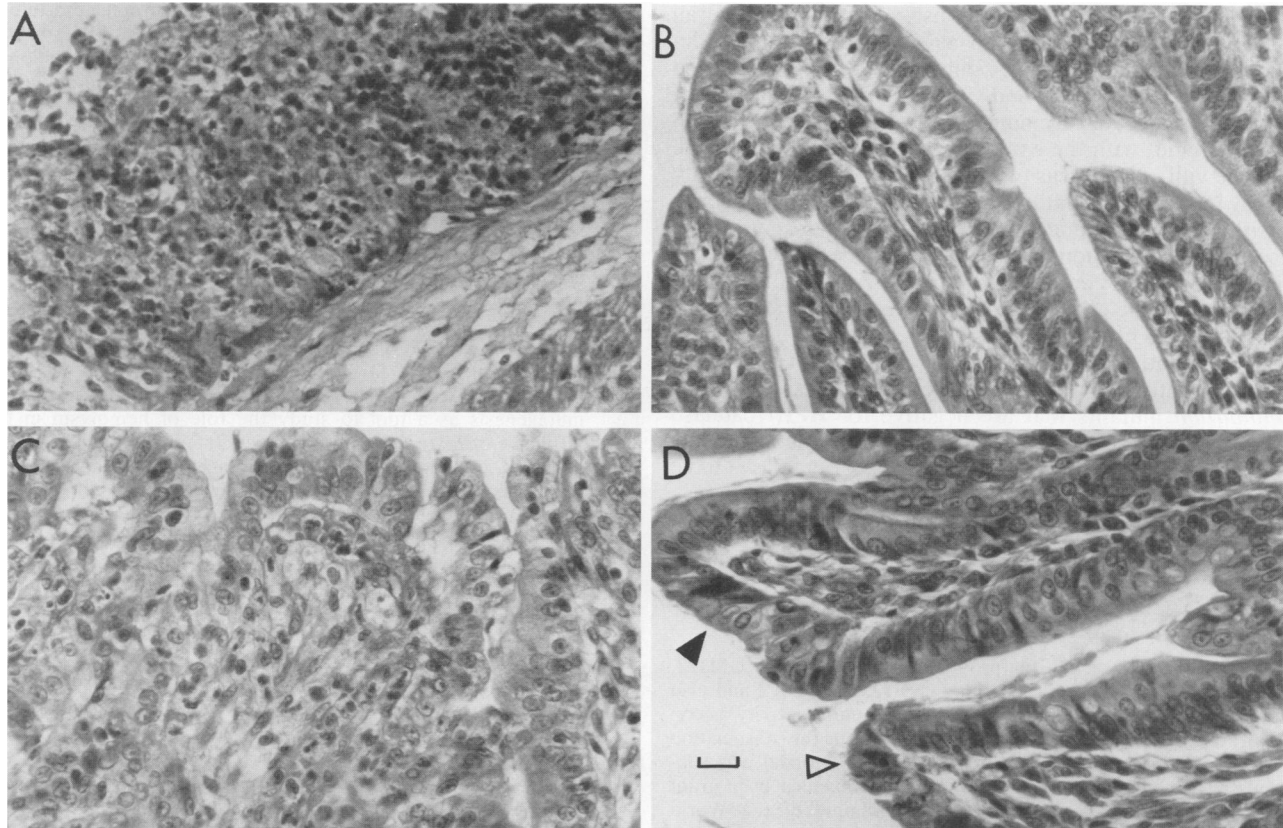


FIG. 4. Histopathological appearance of rabbit intestinal villi infected with (A) M90T, (B) BS176, (C) SC604 (M90T *katFG*), or (D) SC605 (M90T *sodB*). The closed arrowhead indicates dissolution of the brush border, and the open arrowhead indicates the presence of an abscess at the villi tip. Bar, 25 μ m for all panels.

thus suggesting that killing via release of superoxide radicals and their derivatives is more effective with gram-positive organisms such as *S. aureus*. However, things are even more complex, since in the case of infected macrophages M90T was killed more efficiently than its noninvasive mutant, BS176. Several possibilities could account for this unexpected observation. The *S. flexneri* virulence plasmid may encode a product that renders the bacterium more sensitive to killing by the oxidative burst. On the other hand, the invasive property of M90T may itself trigger a stronger oxidative burst due to an increase in the stimuli set onto the phagocyte membrane. *S. flexneri* produces a contact hemolytic activity that may mimic the stimulating effect observed with hemolytic *E. coli* (38). Although we would favor the latter hypothesis, it is not clear why this difference is not observed with PMNL. This is in contradiction with chemiluminescence data (P. Sansonetti, unpublished results) showing a higher response with M90T (i.e., fivefold) as compared with that of BS176 in PMNL.

The behavior of M90T in mouse peritoneal macrophages is somewhat inconsistent with previous observations showing that, unlike BS176, M90T killed phagocytes very rapidly (11). However, it should be emphasized that such experiments have been carried out in J774 continuous macrophage cell line, whereas the present experiments were carried out with opsonized bacteria in sodium caseinate-elicited peritoneal macrophages, both of which will enhance bacterial killing. It is not unusual to see different killing effects depending on the macrophage population. Buchmeier and Heffron (6), when assaying the intracellular survival of

wild-type *S. typhimurium* and macrophage-sensitive mutants in five different macrophage populations, observed that strains that survived well in splenic and bone-marrow macrophages survived poorly in peritoneal macrophages.

The *katFG* double mutant was much more efficiently destroyed by both types of phagocytes, although less than the *sodB* mutant. The *sodB* mutant was so rapidly and efficiently destroyed that within a few minutes after contact the bacterial inoculum was reduced by at least 3 log units. Therefore, the *sodB* mutant of *S. flexneri* appears to be extremely sensitive to oxygen stress. Although no stable *sodA* mutant could be obtained in this study, the data obtained with the *sodB* mutant indicate that this gene is most critical for virulence. Brisk changes in environmental conditions when going from the intestinal lumen to cells and then to phagocytes probably provides a selective advantage to a defense mechanism that is constitutively expressed. Recently Bloch et al. (3) demonstrated that a *sodA* mutation introduced into a pathogenic *E. coli* K1 strain in no way altered the ability of the strain to colonize the gastrointestinal tract or to cause bacteremia. A *sodB* mutation was not analyzed by these workers. Therefore it seems that *sodB* and not *sodA* may encode the superoxide dismutase that is most important in oxygen defense.

When strains were tested in a more definitive assay, such as the Sereny test, equivalent results were observed. In addition, in the rabbit ligated ileal loop assay, M90T induced complete destruction of the intestinal mucosa via a bloody, purulent necrosis, whereas BS176 did not cause any histopathological lesions at all. With the *katFG* double mutant,

slightly less severe lesions were observed, since the overall epithelial lining was conserved as compared with the destruction induced by M90T. Still, many surface ulcerated abscesses were observed, and villi were shortened, swollen, and largely infiltrated by numerous inflammatory cells. On the other hand, with the exception of microabscesses at the tip of the villi, some areas of brush border damage, and only a slight inflammatory infiltration within the lamina propria, the *sodB* mutant produced little detectable damage. Therefore, a good correlation exists between the sensitivity of the mutants to phagocytic cells and the severity of the lesions observed with the rabbit animal model.

We have also observed that the introduction of a catalase-negative mutation in *L. monocytogenes* (*kat::Tn1545*) did not significantly affect the virulence of this species (17). *S. typhimurium* mutants that are negative for HPIII catalase have also been shown to exhibit unaltered virulence (13).

Taken together, these results indicate that catalases have a limited role in the defense against oxidative stress, whereas superoxide dismutases could be critical. Whether such data could be utilized in the strategy for *S. flexneri* live attenuated vaccine construction remains to be demonstrated.

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