INFECTION AND IMMUNITY, July 1994, p. 2662–2668
0019-9567/94/$04.00+0

Vol. 62, No. 7

Comparison of the Sensitivities of *Salmonella typhimurium oxyR* and *katG* Mutants to Killing by Human Neutrophils

ERZSÈBET PAPP-SZABÒ,[1] MAX FIRTEL,[2]† AND P. DAVID JOSEPHY[1]*

Department of Chemistry and Biochemistry, Guelph-Waterloo Centre for Graduate Work in Chemistry,[1] and Department of Microbiology,[2] University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received 3 February 1994/Returned for modification 28 February 1994/Accepted 6 April 1994

The respiratory burst of neutrophils is believed to kill bacteria by generating oxidative species, such as superoxide anion, hydrogen peroxide, and oxidized halogen species. The *oxyR* gene of *Salmonella typhimurium* controls a regulon induced by oxidative stress, such as exposure to hydrogen peroxide. Some researchers have suggested that *oxyR* may play a key role in bacterial survival following phagocytosis. We have tested this possibility by comparing the survival, following exposure to human neutrophils, of isogenic strains bearing different *oxyR* alleles. Neither inactivation of the *oxyR* gene nor constitutive overexpression of the *oxyR*-regulated proteins (*oxyR1* allele) greatly alters bacterial resistance to neutrophils. The *katG* gene, encoding the *oxyR*-regulated enzyme hydroperoxidase I, was also without effect on survival following exposure to neutrophils. We conclude that the *oxyR* response does not play a significant role in the resistance of *S. typhimurium* to phagocytic killing in vitro.

Salmonella typhimurium and *Escherichia coli* can adapt to hydrogen peroxide toxicity. Challenging the cells with a low dose of hydrogen peroxide results in resistance to subsequent high doses of this agent and causes the induction of at least 30 proteins (7, 10, 29). Nine of these proteins are under the control of *oxyR* (7), and several have been identified, such as catalase (hydroperoxidase I [HPI]), encoded by *katG*; an alkyl hydroperoxide reductase, encoded by *ahpC* and *ahpF*; and glutathione reductase, encoded by *gorA* (7, 29). The oxidized form of the OxyR protein stimulates the transcription of these genes; thus, the protein acts as both sensor and transducer of the stress signal. OxyR also negatively regulates its own expression (44, 45).

Strains carrying null mutations of *oxyR*, being unable to induce the *oxyR*-controlled proteins, are highly sensitive to hydrogen peroxide and other oxidants relative to the wild-type cells. Strains carrying certain alleles at the *oxyR* locus (*oxyR1* in *S. typhimurium* and *oxyR2* in *E. coli*) constitutively overexpress the *oxyR*-regulated proteins and are relatively resistant to these oxidants and also to heat killing. Three of the nine identified *oxyR*-regulated proteins are heat inducible (7, 29).

Human neutrophils (polymorphonuclear leukocytes [PMN]) kill microorganisms by both oxygen-dependent and oxygen-independent mechanisms. In the oxygen-dependent respiratory burst, neutrophils generate superoxide anion ($O_2^{\cdot -}$) via a single-electron transfer from NADPH to molecular oxygen (41). The neutrophils in chronic granulomatous disease (CGD) are incapable of producing the respiratory burst. Individuals with CGD have high mortality rates due to bacterial infection (37), suggesting that oxygen-dependent killing mechanisms are crucial to survival. *Salmonella* spp. are a major cause of infection in CGD patients (30, 38). Superoxide and other reactive oxygen species derived from it, such as H_2O_2, oxidized halogen species formed by the action of myeloperoxidase, and hydroxyl radical, may contribute to bacterial killing (8). The critical cellular targets of oxidative killing mechanisms may include bacterial DNA, cell membranes, and proteins. Stimulated neutrophils can inactivate enzymes and oxidatively modify proteins (34). 6-Phosphogluconate dehydratase and other enzymes of *E. coli* are superoxide sensitive (19); the importance of such superoxide-dependent enzyme inactivation in bacterial cell death is unclear.

Following phagocytosis, in addition to the respiratory burst, the contents of the cytoplasmic granules are translocated into the phagosome and the phagosome is acidified. These processes are independent of the respiratory burst per se and contribute to oxygen-independent phagocytic killing (16). In vitro, human neutrophils can kill *S. typhimurium* LT2 under anaerobic conditions, but less effectively than in the presence of air (27, 33). Among the antimicrobial components of the granules are the defensins, cysteine-rich peptides which make up as much as half of the total protein in cytoplasmic granules from humans (18, 42). Purified defensins can kill bacterial cells in vitro (16, 24, 26). On the other hand, mouse neutrophils lack defensins but, nevertheless, have antimicrobial activity (13). In summary, the relative importance of the oxygen-dependent and -independent pathways of bacterial cell killing by neutrophils is not resolved and probably varies among different species of neutrophils and bacteria.

Several authors have hypothesized that the *oxyR* regulon plays a protective role in cell resistance to phagocytic killing (22, 23, 44). However, this hypothesis has not yet been tested by a direct comparison of the sensitivities to phagocytic killing of *oxyR* mutant and wild-type strains. In this paper, we have addressed this question by studying the response of *oxyR* mutants of *S. typhimurium* to human neutrophils.

MATERIALS AND METHODS

Bacterial strains. The genotypes and sources of the bacterial strains used in this study are listed in Table 1.

Medium and growth conditions. For strains LT2, TA4100, GSO14, DJ1414, and DJ110, cells were grown in Oxoid nutrient broth no. 2 (Oxoid Ltd., Nepean, Canada), at 37°C, with shaking at 120 rpm, to an optical density at 650 nm (OD_{650}) of about 1.0.

* Corresponding author. Phone: (519) 824-4120, ext. 3833. Fax: (519) 766-1499. Electronic mail address: JOSEPHY@CHEMBIO.UOGUELPH.CA.

† Present address: Department of Microbiology, University of Toronto, Toronto, Ontario, Canada.

TABLE 1. Genotypes and sources of bacterial strains used

Strain	Genotype	H_2O_2 sensitivity (diam, mm)[a]	Cells per ml, 10^8 (OD_{650})	Source (reference)
LT2	Wild type	23.4 ± 0.5	12.7 (1.0)	B. N. Ames
TA4126	*hisG46 zii614*::Tn*10* Δ*uvrB* pKM101	23.3 ± 0.5	1.5 (0.4)	Storz et al. (43)
TA4127	*hisG46 zii614*::Tn*10* Δ*uvrB oxyR1* pKM101	18.5 ± 0.8	1.0 (0.5)	Storz et al. (43)
TA4128	*hisG46 zii614*::Tn*10* Δ*uvrB oxyR*Δ2 pKM101	39.3 ± 1.0	1.7 (0.4)	Storz et al. (43)
TA4100	*oxyR1*	12.3 ± 0.5	4.8 (1.0)	Christman et al. (7)
GSO14	LT2 *oxyR*::Tn*10*	42.0 ± 1.3	20.0 (1.0)	G. Storz
DJ1414	Independent construction of LT2 *oxyR*::Tn*10*	39.2 ± 1.5	13.0 (1.0)	This paper
DJ110	LT2 *katG1*::Tn*10*	33.5 ± 0.5	11.1 (1.0)	McGowan-Jordan and Josephy (28)

[a] Hydrogen peroxide sensitivities were measured as described in the text; data represent average diameters ± standard deviations (six measurements) of the zones of growth inhibition.

For strains TA4126, TA4127, and TA4128, the extent of growth of overnight cultures was variable. For the neutrophil killing assay, these strains were grown in nutrient broth with tetracycline (10 μg/ml) and ampicillin (25 μg/ml), at 37°C, with shaking overnight at 120 rpm. Following incubation, the cultures were diluted 25-fold into fresh nutrient broth (minus antibiotics) and were grown for 190 min at 37°C, with shaking at 180 rpm, to a final OD_{650} of about 0.5.

***oxyR* and *katG* mutants.** *oxyR* mutants (TA4128, GSO14, and DJ1414) grow poorly when inoculated at low cell densities. Therefore, for reisolation and subsequent frozen storage of these strains, our standard protocol was modified. Single colonies were picked from plates and grown overnight in nutrient broth (75 ml) to which sterile catalase solution (bovine liver [Sigma Chemical Co., St. Louis, Mo.]; 1,600 U/mg; 150 μg) was added, as well as tetracycline and/or ampicillin, as appropriate. Catalase was not added when overnight cultures were grown for use in neutrophil killing experiments; however, for clonogenic survival assays, catalase was added to the top agar in which the *oxyR* or *katG* mutants were plated (2 mg of sterile catalase per 100 ml of top agar). In each experiment comparing these strains with others, catalase-containing top agar was used for all strains.

Preparation of bacteria for serum sensitivity and neutrophil killing assays. Methods for preparation of bacteria for serum sensitivity and neutrophil killing assays were those previously described (35), with the following modifications. The relationship between OD and viable cell concentration was determined for each strain by plating on nutrient agar plates. Large differences among the strains were observed (Table 1), presumably due to differences in cell size (see below).

For the killing assay, bacterial cells were pelleted in a low-speed clinical centrifuge and washed twice in KRP buffer (Krebs original Ringer phosphate, without Ca^{2+} and Mg^{2+}). The cells were then diluted into the assay buffer (KRP buffer with $CaCl_2$ [1.69 mM], $MgSO_4$ [1.18 mM], gelatin [0.1%, wt/vol], and glucose [0.16%, wt/vol]). Cell concentrations were adjusted to give either 5×10^6 or 10^7 cells per ml.

Serum and neutrophil killing assays. Opsonization was performed with 5% pooled normal human serum stored at −70°C for no longer than 3 months. Methods were as described previously (35). In brief, for serum sensitivity assays, bacteria (2×10^6 cells per ml) and serum were combined in buffer. Samples were withdrawn and diluted; bacteria were overlaid in top agar onto nutrient broth plates. Colonies were counted after overnight incubation. For neutrophil phagocytic killing assays, fresh human blood samples were obtained from healthy adult volunteers (with informed consent). The killing assay was carried out by exposing bacteria to serum and neutrophils. Tubes were rotated in LabQuake rotators in a 37°C incubator. Samples were withdrawn at intervals, added to chilled (4°C) lysis buffer, and then allowed to stand at room temperature for at least 20 min. Aliquots were withdrawn, mixed with top agar, overlaid onto nutrient broth plates, and incubated overnight. (In the experiments shown in Fig. 6, fresh autologous serum [5%] was used, and the assay buffer contained 0.8% [wt/vol] bovine serum albumin, instead of gelatin.)

For each time point, the percent cell survival value was determined, relative to 100% survival at time (t) = 0 for the given strain. Log(survival) [log(S)] values were calculated. The data points represent the means ± standard errors of these log(S) values.

Significance testing. Upper and lower 95% confidence intervals for each survival curve were used to assess statistical significance. All regression lines were fitted to the data, using the second-degree polynomial, which was found to fit the data satisfactorily (regression coefficient, >97% in all fittings).

Respiratory burst. The respiratory burst was measured by assaying hydrogen peroxide production, as described previously (31). Neutrophils (2×10^6 cells) were stimulated with phorbol myristate acetate (Sigma Chemical Co.) or *E. coli* (4×10^7 cells).

Motility. Cells of strain GSO14 (LT2 *oxyR*::Tn*10*) could not be pelleted satisfactorily by low-speed centrifugation (1,000 × *g*). This unexpected behavior prompted us to examine the motility of all of the strains used in our experiments. Visual inspection under the phase-contrast microscope and growth on semisolid nutrient agar plates (0.2 or 0.3% agar) were used. An aliquot (2 μl) of a late-log-phase culture was placed onto the surface of the semi-solid agar plate; the plates were incubated at 37°C for 5 h, or overnight, and the ring of bacterial growth was inspected visually.

Optical microscopy and staining. Morphological features were examined by bright-field microscopy. Smears of each strain were prepared on acetone-cleaned glass slides, heat fixed, and stained for 1 min with crystal violet stain. The stained smears were washed under gently flowing tap water until the water ran clear, dried, and viewed with a ×40 objective.

Catalase and peroxidase activities. Catalase (negative stain with horseradish peroxidase, hydrogen peroxide, and diaminobenzidine) and peroxidase (positive stain with hydrogen peroxide and diaminobenzidine) activities were detected on 7% nondenaturing polyacrylamide gels (9). Protein contents of the bacterial extracts were measured with the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, Ill.).

H_2O_2 sensitivity test. The H_2O_2 sensitivity test was performed as previously described (7), except that the cells were

plated on nutrient agar plates. Sterile paper disks were treated with 10 μl of 3% hydrogen peroxide solution.

RESULTS

Strain characteristics. In this work, we used two sets of isogenic strains of *S. typhimurium*, differing only at the *oxyR* locus. The first set consists of strains TA4126, TA4127, and TA4128. These strains are *oxyR*$^+$ (wild type), *oxyR1* (constitutive), and *oxyRΔ2* (deletion), respectively; the background for these strains is *hisG46 zii614*::Tn*10* Δ*uvrB* pKM101, an Ames mutagenicity tester strain phenotype. (Transposon insertion *zii614*::Tn*10* is linked to the *oxyR* gene and was used in the construction of the strains.)

To confirm that any effects observed with the first set of strains were related to the *oxyR* genotype (and not peculiar to a particular genetic background), a second set of strains was also studied. This second set consists of strains LT2, TA4100, and DJ1414. These strains are *oxyR*$^+$ (wild type), *oxyR1* (constitutive), and *oxyR*::Tn*10* (null), respectively; the background for these strains is the wild-type strain LT2. DJ1414 was constructed in our laboratory by P22-mediated transduction of *oxyR*::Tn*10* from strain GSO14 into strain LT2 (see below).

The effect of *katG* was examined by comparison of strains LT2 and DJ110, an isogenic *katG* null mutant (HPI$^-$).

The *oxyR* phenotypes of all strains were confirmed by testing hydrogen peroxide sensitivity on nutrient agar plates and by native polyacrylamide gel electrophoresis (PAGE), stained for catalase and peroxidase activities. The hydrogen peroxide sensitivity data are shown in Table 1. In both sets of strains, the constitutive mutant was highly resistant and the null mutant (transposon insertion or deletion) was highly sensitive to hydrogen peroxide, relative to the corresponding wild-type strains, in agreement with previous observations (7). Overall, the sensitivities observed in the two backgrounds were similar.

The *katG* mutant DJ110 was also sensitive to hydrogen peroxide, as observed previously (28; note that Table 2 of the earlier paper gives an inhibition zone of 10.5 mm; this measurement refers to the annular width of the inhibition ring around the filter disk [diameter, 6.5 mm] and is thus equal to a zone diameter of 27.5 mm).

On nondenaturing polyacrylamide gels, the *oxyR1* strains both showed great elevations in HPI catalase and peroxidase levels, and the *oxyR* null mutants showed normal levels of both HPI and HPII (Fig. 1).

Motility and morphology. The number of motile cells was assessed by inspection of wet mounts prepared for phase-contrast microscopy. TA4126, TA4127, and TA4128 were completely nonmotile. Nevertheless, upon examination by transmission electron microscopy, these strains were observed to be flagellated (data not shown). LT2 and TA4100 cells, in contrast, were motile. GSO14 cells were much more motile than these two strains.

The motilities of the strains were also measured by examining the spread of growth on semisolid agar plates. The differences noted under the optical microscope were also obvious in these tests. Strains TA4126, TA4127, and TA4128 showed no detectable motility; i.e., no expanding ring of cells was visible, even following overnight growth. LT2 cells were motile, reaching a diameter of 17 mm after 5 h of incubation (37°C; 0.2% agar). Strains TA4100 and GSO14, grown simultaneously on the same plate, reached diameters of 28 and 64 mm, respectively.

We attempted to pinpoint the gene responsible for the lack of motility in strains TA4126, TA4127, and TA4128. The following strains were examined, using the semisolid agar plate assay: TA1530 (*hisG46* Δ*uvrB* Δ*gal*); TA100 (*hisG46* Δ*uvrB* Δ*gal rfa* pKM101); TT2385 (*zii614*::Tn*10*), obtained from John Roth; and our own construction of LT2 *zii614*::Tn*10*, by P22-mediated transduction of the transposon from TA4126 to LT2. All of these strains were motile (data not shown). Several spontaneous *his*$^+$ revertants from TA4126, selected according to standard Ames test protocols, were examined, and all were nonmotile. In summary, none of the known mutations in strains TA4126, TA4127, and TA4128 appeared to be responsible for their nonmotile phenotypes.

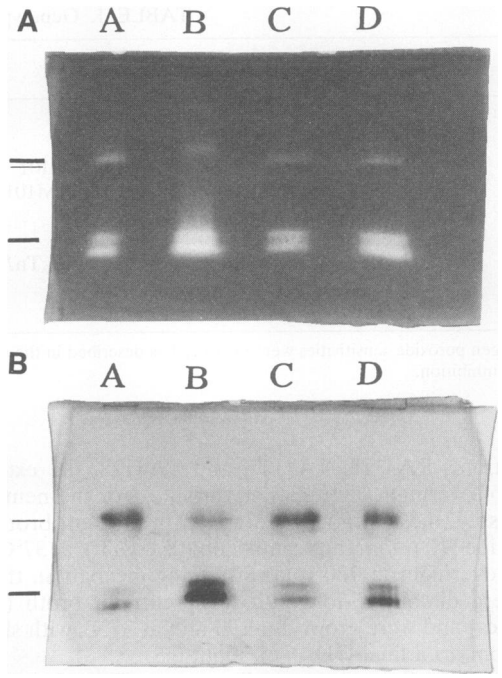

FIG. 1. PAGE separation of the *S. typhimurium* HPs. (A) Gel stained for catalase activity. (B) Gel stained for peroxidase activity. Samples applied to the gels were as follows: lanes A, LT2, 66 μg of protein; lanes B, TA4100 (*oxyR1*), 19 μg; lanes C, GSO14 (*oxyR*::Tn*10*), 58 μg; lanes D, DJ1414 (*oxyR*::Tn*10*), 66 μg. On the catalase gel (negative stain), the top band is HPII and the bottom (double) band is HPI. The dark band under HPII (thin tick mark) is an unidentified peroxidase. On the peroxidase gel (positive stain), the top band (thin tick mark) is an unidentified peroxidase (does not require H_2O_2; appears on gel after incubation with 3,3′-diaminobenzidine); the bottom double band is HPI.

Strain GSO14 was, unexpectedly, hypermotile. We constructed strain DJ1414 by transducing (via P22) the Tn*10* insertion in *oxyR* from GSO14 back to LT2. DJ1414 was phenotypically *oxyR* (Table 1), but its motility, behavior upon centrifugation, and size were similar to those of LT2. Therefore, we carried out phagocytic killing experiments with DJ1414 instead of GSO14.

We conclude that strains TA4126, TA4127, and TA4128 all bear an uncharacterized mutation which eliminates motility, without affecting flagellum synthesis. GSO14 bears an uncharacterized mutation leading to hypermotility.

Cells of strain TA4100 (*oxyR1*) were much longer than cells of strain LT2 when examined by light microscopy (Fig. 2). This difference accounts for the very different relationship between cell number and optical density observed for the strains (Table 1).

Killing by neutrophils. The human neutrophil preparation

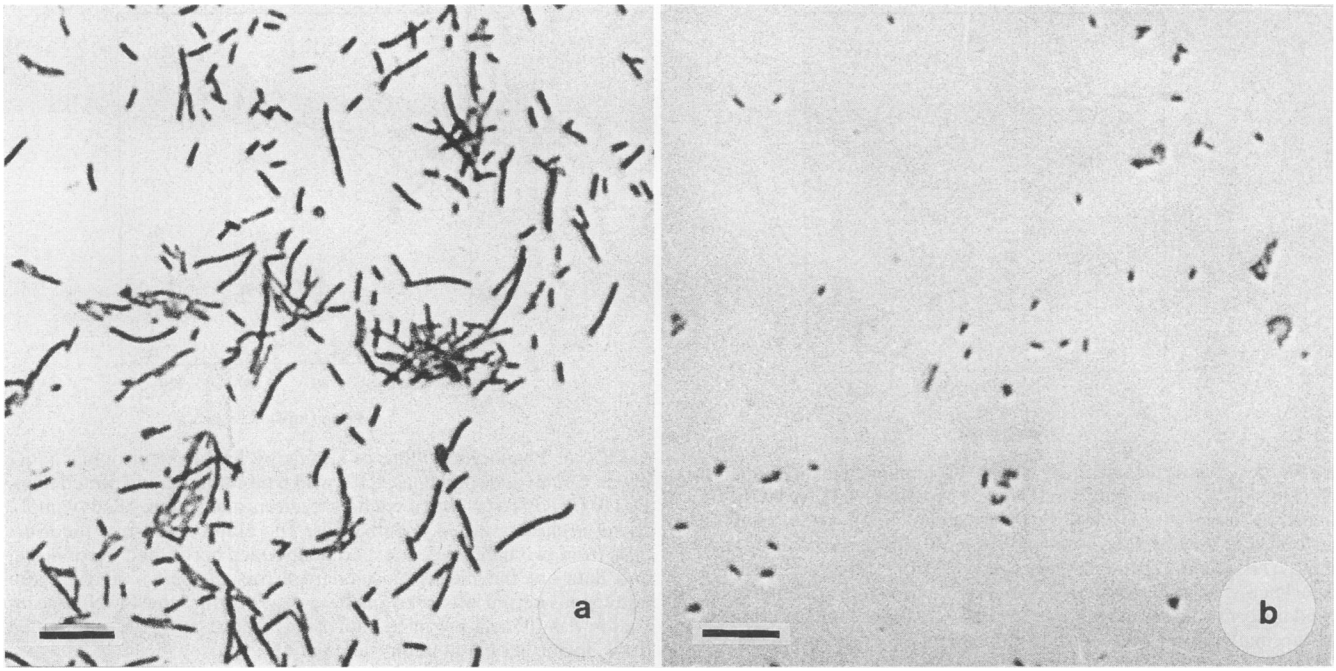

FIG. 2. Light microscopy (bright-field micrographs) of *S. typhimurium* strains stained with crystal violet dye. (a) Strain TA4100 (*oxyR1*); (b) strain LT2. For both panels a and b, bar = 12.5 μm. Cultures were grown overnight to OD_{650} = 1.0.

exhibited a respiratory burst response. Hydrogen peroxide production increased almost 10-fold (from a baseline value of about 1 nmol/min; 2×10^6 cells) upon stimulation with either phorbol myristate acetate or bacterial cells (data not shown).

We examined the role of the *oxyR* gene in resistance to neutrophil killing. First, we studied TA4126, TA4127, and TA4128 (Fig. 3). All strains grew in 5% serum (in the absence of neutrophils); the *oxyR1* strain TA4127 grew slightly faster than the other two strains. Upon exposure to neutrophils, the *oxyRΔ2* strain was more resistant than the other two strains. The data points for the *oxyRΔ2* strain generally lay outside the 95% confidence limits of the other two strains.

Next, we studied the same *oxyR* genes in the wild-type strain LT2 background (Fig. 4 and 5). The *oxyR1* strain grew faster than the wild type in the presence of 5% serum. In the

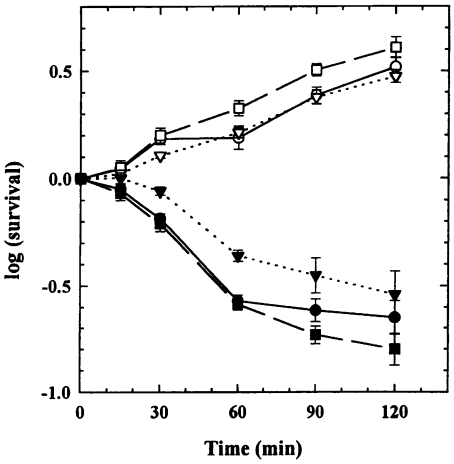

FIG. 3. Phagocytic killing of strains by human neutrophils: *oxyR* effect in TA4126 background. Circles and solid lines, TA4126 (wild type); squares and long dashed lines, TA4127 (*oxyR1*); inverted triangles and dotted lines, TA4128 (*oxyRΔ2*); open symbols, bacteria plus serum; filled symbols, bacteria plus serum plus neutrophils. The averages and standard errors were calculated from three independent experiments, each performed in triplicate. Serum used was normal human pooled serum. PMN concentrations in the different experiments were between 1.2×10^6 and 2.0×10^6 cells per ml.

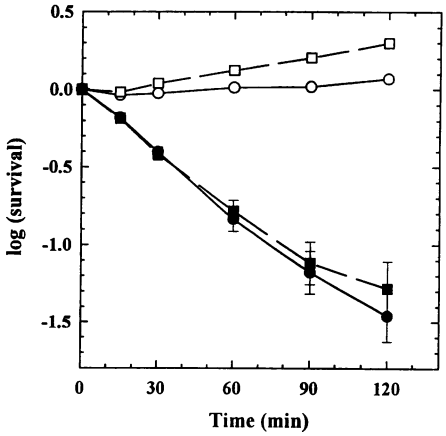

FIG. 4. Phagocytic killing of strains by human neutrophils: *oxyR1* effect in LT2 background. Circles and solid lines, LT2 (wild type); squares and long dashed lines, TA4100 (*oxyR1*); open symbols, bacteria and serum; filled symbols, bacteria plus serum and neutrophils. The graph represents the averages from four independent experiments, each performed in triplicate, and data are the means ± standard errors of log(S) values. Serum used in all four experiments was normal human pooled serum. All experiments were performed at a PMN concentration of 2×10^6 cells per ml.

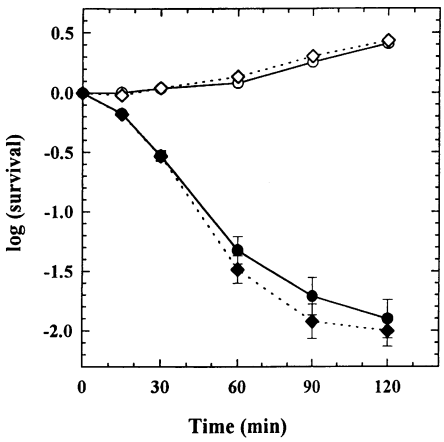

FIG. 5. Phagocytic killing of strains by human neutrophils: *oxyR* effect in LT2 background. Circles and solid lines, LT2 (wild type); diamonds and dotted lines, DJ1414 (*oxyR*::Tn*10*); open symbols, bacteria and serum; filled symbols, bacteria plus serum and neutrophils. The graph represents the averages from three independent experiments, each performed in triplicate, and data are the means ± standard errors of log(S) values. Serum used in all four experiments was normal human pooled serum. All experiments were performed at a PMN concentration of 2×10^6 cells per ml. In these experiments, the bacterial strains were not washed with KRP buffer.

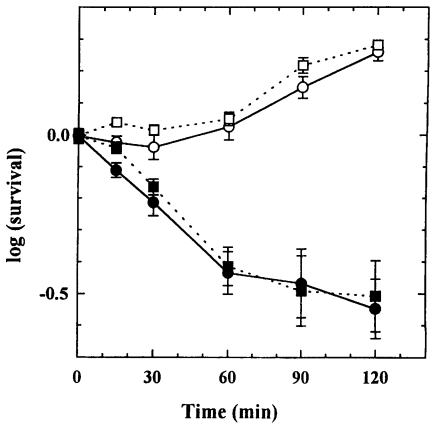

FIG. 6. Phagocytic killing of strains by human neutrophils: *katG* effect. Circles and solid lines, LT2 (wild type); squares and dotted lines, DJ110 (*katG*::Tn*10*); open symbols, bacteria and serum; filled symbols, bacteria plus serum and neutrophils. The graph represents the averages from two independent experiments, each performed in triplicate, and data are the means ± standard errors of log(S) values. Fresh autologous serum was used in these experiments; the PMN concentration was 10^6 cells per ml in both assays. The assay mixture contained 0.8% bovine serum albumin (no gelatin).

presence of neutrophils, the two strains showed very similar survival values (Fig. 4).

As discussed above, strain GSO14 behaved anomalously, in terms of motility and morphology, as well as growth, compared with strain LT2. We constructed a new strain, DJ1414, by P22-mediated transduction of the transposon mutation in the *oxyR* gene from GSO14 back to LT2 (Table 1). We verified that DJ1414 bears the *oxyR* null phenotype: the strain was highly sensitive to hydrogen peroxide, as measured by the disk growth inhibition assay, and had levels of HP activities comparable to those of LT2 (Fig. 1; Table 1). Figure 5 shows a comparison of strains DJ1414 and LT2. No significant differences between the responses of the two strains, in either the presence or the absence of neutrophils, were observed.

One of the genes regulated by *oxyR* is *katG*, which encodes HPI. Since this enzyme has catalase activity (detoxication of hydrogen peroxide), we tested the possible role of this gene in phagocytic killing (Fig. 6). We compared LT2 and the *katG1*::Tn*10* strain, DJ110. No significant differences between the strains were observed.

DISCUSSION

Motility. Although the main objective of our studies was to elucidate the role of the *oxyR* gene in resistance to phagocytic killing, the unexpected behavior of the mutant strains with respect to centrifugation and enumeration (viable cells per OD unit) prompted us to examine their morphology and motility. The *oxyR* null mutant GSO14 was extremely motile compared with strain LT2. These differences were not related to the *oxyR* mutation; reconstruction of this strain gave a strain (DJ1414) with the *oxyR* null phenotype but which was otherwise similar to LT2 (size, cell number per OD_{650} unit, flagella, and motility). It seems probable that significant variations in the characteristics of LT2-derived strains used in different laboratories, not associated with known genes, are responsible for these effects.

Bacterial resistance to phagocytic killing. In our earlier paper, we reported that genetic elimination of the endogenous Fe-superoxide dismutase (SOD) enzyme of *E. coli* does not affect survival following exposure to human neutrophils (35). In this study, we have carried out phagocytic killing assays with *S. typhimurium* wild-type, *oxyR1*, *oxyRΔ2*, and *oxyR* transposon null mutants, as well as with a *katG* null mutant, to test the effects of these genes on survival following exposure to human neutrophils. Our results show that the *oxyR1* strains did not have any selective advantage. The *oxyR* null mutants did not show greater sensitivity. In the Ames test strain background, the deletion mutant showed somewhat higher survival (Fig. 3), but in the wild-type background, no difference was observed between LT2 and DJ1414 (LT2 *oxyR*::Tn*10*). The presence of the *katG* mutation made no difference in terms of survival.

Bacterial cells which are recognized and ingested by neutrophils have multiple survival strategies. Some *Legionella* strains produce acid phosphatases which inhibit the respiratory burst and block the production of $O_2^{\cdot-}$; these strains can survive and multiply in PMN (39). *Nocardia asteroides* SOD, which is secreted into the growth medium and is also cell surface associated, protects this species from oxidative killing by neutrophils (2, 3). Antioxidant enzymes are probably most effective as defenses against the respiratory burst when secreted into the phagosome.

DNA damage may cause bacterial death following phagocytosis; several lines of evidence support this hypothesis. For example, the *recA* and *recBC* gene products are necessary for full virulence in *S. typhimurium* (5). The products of the respiratory burst ($O_2^{\cdot-}$, H_2O_2) are mutagenic in some assay systems and evoke inducible DNA repair (6, 20). Indeed, phagocytic cells induce mutations in bacteria (1, 17, 46). The DNA repair enzyme endonuclease IV (*nfo*) is induced by oxidative stress, under the control of *soxRS*. Exonuclease III (*xth*) is not induced (6), but strains bearing *xth* mutations are extremely sensitive to H_2O_2 (11).

Oxidative damage to plasma or outer membranes may also contribute to bacterial cell death. The Ahp alkyl HP (encoded by *ahpC* and *ahpF*) plays an important role in membrane

protection by reducing fatty acid hydroperoxides (14). Ahp is *oxyR* regulated. Farr et al. (15) investigated the effect of oxidative stress on membrane functions; the *oxyR* regulon was reported to play a crucial role in protecting the cells from oxidative damage. Hydrogen peroxide-pretreated, wild-type cells rapidly recovered guanosine and α-methylglucopyranoside transport activity following subsequent treatment with hydrogen peroxide, while strains bearing either an *oxyR* deletion or *katG* null mutation did not.

Our results indicate that the *oxyR* response does not protect *S. typhimurium* against killing by neutrophils. The chain of oxidative events initiated by the respiratory burst, including membrane damage, ΔpH, decrease in ATP synthesis, increase in permeability to toxic compounds, oxidative DNA (21), and protein damage (14), may overwhelm the defensive capacity of the *oxyR*-regulated enzymes, including HPI. Overproduction of antioxidant enzymes can also have unexpected or "paradoxical" consequences. SOD overproduction results in higher sensitivity to the redox-cycling agent paraquat (4, 40). The basis for this effect is not clear; possibly, overexpression of SOD prevents up-regulation of the expression of other proteins required for paraquat resistance. Perhaps the *oxyR1* gene has an analogous effect: enhanced expression of those proteins which are induced early following hydrogen peroxide exposure might delay or suppress the induction of other proteins involved in stress resistance.

The *soxRS* genes control a regulon induced by exposure of *E. coli* to superoxide or nitric oxide. Mn-SOD expression is one of the enzymes under the control of the *soxRS* response. Demple and colleagues (32) recently reported that Δ*soxRS* mutants are more sensitive to killing by murine peritoneal macrophages than are parental *soxRS*$^+$ cells. In the macrophage study, the difference between wild-type and Δ*soxRS* mutants only became apparent following long exposure (greater than 4 h) of the bacteria to the macrophages. The more rapid killing kinetics obtained in our system probably afford less opportunity for the induction of bacterial stress responses. Deletion of the regulatory gene, under these circumstances, would be without effect, as we observed.

Catalase HPI also failed to play a major role in cell survival. As with SOD, *E. coli* and *S. typhimurium* have two distinct catalases, and deletion of both may be required for sensitivity to neutrophil killing. Studies of HPI$^-$ HPII$^-$ mutants are planned. $O_2^{\cdot -}$ inhibits catalase (25), and this effect could mitigate the protective value of catalase for the phagocytosed bacterium. However, the steady-state concentration of $O_2^{\cdot -}$ within the bacterial (SOD$^+$) cell, even following phagocytosis, probably remains very low, at least until membrane integrity is disrupted. If the lethal injury to the bacterial cell is inflicted by attack on the membrane, responses to oxidative conditions within the cellular milieu may be of little relevance.

The importance of *Salmonella* infections in CGD patients (see above) suggests that oxygen-dependent killing is an important defense against this bacterium. Experimentally, the differentiation of oxygen-dependent and -independent mechanisms is difficult. CGD neutrophils can be tested in vitro to examine the role of the respiratory burst response in bacterial cell killing, but such neutrophils are not readily obtained. Normal neutrophils can be tested under anoxic conditions, but the removal of oxygen affects all respiratory processes of the cells, not just the respiratory burst. Another possibility is the use of NADPH oxidase inhibitors, such as diphenylene iodonium (12). Studies with this inhibitor are under way in our laboratory and will be reported separately.

Since the respiratory burst of neutrophils kills bacteria (at least in part) by exposing them to oxidants, many researchers have suggested that bacterial regulatory responses to oxidative stress play a role in defense against phagocytic killing. However, these regulatory systems (such as those controlled by *oxyR* and *soxRS*) may have evolved under the selective pressure of very different oxidative conditions. Phagocytic killing plays a critical role in immune defense but may have been of little significance to the evolution of enteric microorganisms. The oxidative stress faced by a bacterium passing from the intestinal tract into the aerobic external environment is, perhaps, a lesser threat than the intense and acute oxidative attack following phagocytosis. Our results suggest that the *oxyR* system is not capable of defending the *S. typhimurium* cell from this attack.

ACKNOWLEDGMENTS

We thank Margaret Berry and Premila Sathasivam for taking the blood samples; Dan McManus for performing the measurements of the respiratory burst; Terry Beveridge, Department of Microbiology, for helpful discussions; and Gisela Storz for providing bacterial strains.

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

ADDENDUM IN PROOF

In a recent report, the sensitivity of a double catalase mutant (*katE katG*) of *S. typhimurium* 14028 to killing by murine macrophages was found to be not significantly different from that of the wild-type parent strain (N. Buchmeier, S. Libby, and F. Fang. Abstr. 94th Gen. Meet. Am. Soc. Microbiol. 1994, abstr. B-331, 1994).

REFERENCES

1. **Barak, M., S. Ulitzur, and D. Merzbach.** 1983. Phagocytosis-induced mutagenesis in bacteria. Mutat. Res. **121**:7–16.
2. **Beaman, B. L., C. M. Black, F. Doughty, and L. Beaman.** 1985. Role of superoxide dismutase and catalase as determinants of pathogenicity of *Nocardia asteroides*: importance in resistance to microbicidal activities of human polymorphonuclear neutrophils. Infect. Immun. **47**:135–141.
3. **Beaman, L., and B. L. Beaman.** 1990. Monoclonal antibodies demonstrate that superoxide dismutase contributes to protection of *Nocardia asteroides* within the intact host. Infect. Immun. **58**:3122–3128.
4. **Bloch, C. A., and F. M. Ausubel.** 1986. Paraquat-mediated selection for mutation in the manganese-superoxide dismutase gene *sodA*. J. Bacteriol. **168**:795–798.
5. **Buchmeier, N. A., C. J. Lipps, M. Y. H. So, and F. Heffron.** 1993. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. Mol. Microbiol. **7**:933–936.
6. **Chan, E., and B. Weiss.** 1987. Endonuclease IV of *Escherichia coli* is induced by paraquat. Proc. Natl. Acad. Sci. USA **84**:3189–3193.
7. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. Cell **41**:753–762.
8. **Cohen, M. S., B. A. Britigan, D. J. Hassett, and G. M. Rosen.** 1988. Do human neutrophils form hydroxyl radical? Evaluation of an unresolved controversy. Free Radical Biol. Med. **5**:81–88.
9. **DeBruin, L. S., and P. D. Josephy.** 1989. Dichlorobenzidine-DNA binding catalyzed by peroxidative activation in *Salmonella typhimurium*. Arch. Biochem. Biophys. **269**:25–31.
10. **Demple, B., and J. Halbrook.** 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature (London) **304**:466–468.
11. **Demple, B., J. Halbrook, and S. Linn.** 1983. *Escherichia coli xth* mutants are hypersensitive to hydrogen peroxide. J. Bacteriol. **153**:1079–1082.
12. **Doussière, J., and P. V. Vignais.** 1992. Diphenylene iodonium as an inhibitor of the NADPH oxidase complex of bovine neutrophils. Factors controlling the inhibitory potency of diphenylene

iodonium in a cell-free system of oxidase activation. Eur. J. Biochem. **208:**61–71.
13. **Eisenhauer, P. B., and R. I. Lehrer.** 1992. Mouse neutrophils lack defensins. Infect. Immun. **60:**3446–3447.
14. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
15. **Farr, S. B., D. Touati, and T. Kogoma.** 1988. Effects of oxygen stress on membrane functions in *Escherichia coli*: role of HPI catalase. J. Bacteriol. **170:**1837–1842.
16. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
17. **Fulton, A. M., S. E. Loveless, and G. H. Heppner.** 1984. Mutagenic activity of tumor-associated macrophages in *Salmonella typhimurium* strains TA98 and TA100. Cancer Res. **44:**4308–4311.
18. **Ganz, T., M. E. Selsted, D. Szklarek, S. S. Harwig, K. Daher, and R. I. Lehrer.** 1985. Defensins: natural peptide antibiotics of human neutrophils. J. Clin. Invest. **76:**1427–1435.
19. **Gardner, P. R., and I. Fridovich.** 1991. Superoxide sensitivity of the *Escherichia coli* 6-phosphogluconate dehydratase. J. Biol. Chem. **266:**1478–1483.
20. **Imlay, J. A., and S. Linn.** 1987. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **169:**2967–2976.
21. **Imlay, J. A., and S. Linn.** 1988. DNA damage and oxygen radical toxicity. Science **240:**1302–1309.
22. **Johnson, K., I. Charles, G. Doudan, D. Pickard, P. O'Gaora, G. Gosta, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
23. **Johnson, S. R., B. M. Steiner, D. D. Cruce, G. H. Perkins, and R. J. Arko.** 1993. Characterization of a catalase-deficient strain of *Neisseria gonorrhoeae*: evidence of the significance of catalase in the biology of *N. gonorrhoeae*. Infect. Immun. **61:**1232–1238.
24. **Kohashi, O., T. Ono, K. Ohki, T. Soejima, T. Moriya, A. Umeda, Y. Meno, K. Amako, S. Funakosi, M. Masuda, and N. Fujii.** 1992. Bactericidal activities of rat defensins and synthetic rabbit defensins on *Staphylococci*, *Klebsiella pneumoniae* (Chedid, 277, and 8N3), *Pseudomonas aeruginosa* (mucoid and nonmucoid strains), *Salmonella typhimurium* (Ra, Rc, Rd, and Re of LPS mutants) and *Escherichia coli*. Microbiol. Immunol. **36:**369–380.
25. **Kono, Y., and I. Fridovich.** 1982. Superoxide radical inhibits catalase. J. Biol. Chem. **257:**5751–5754.
26. **Lehrer, R. I., A. Barton, K. A. Daher, S. S. L. Harwig, T. Ganz, and M. E. Selsted.** 1989. Interaction of human defensins with *Escherichia coli*. J. Clin. Invest. **84:**553–561.
27. **Mandell, G. L.** 1974. Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. Infect. Immun. **9:**337–341.
28. **McGowan-Jordan, I. J., and P. D. Josephy.** 1990. Hydroperoxidase I catalyses peroxidative activation of 3,3′-dichlorobenzidine in *Salmonella typhimurium*. Arch. Biochem. Biophys. **282:**352–357.
29. **Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz, and B. N. Ames.** 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. Proc. Natl. Acad. Sci. USA **83:**8059–8063.
30. **Mouy, R., A. Fischer, E. Vilmer, R. Seger, and C. Griscelli.** 1989. Incidence, severity, and prevention of infections in chronic granulomatous disease. J. Pediatr. **114:**555–560.
31. **Negri, M., P. Bellavite, C. Lauciello, P. Guzzo, and M. Zatti.** 1991. A photometric assay for hydrogen peroxide production by polymorphonuclear leucocytes. Clin. Chim. Acta **199:**305–310.
32. **Nunoshiba, T., T. deRojas-Walker, J. S. Wishnock, S. R. Tannenbaum, and B. Demple.** 1993. Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. Proc. Natl. Acad. Sci. USA **90:**9993–9997.
33. **Okamura, N., and J. K. Spitznagel.** 1982. Outer membrane mutants of *Salmonella typhimurium* LT2 have lipopolysaccharide-dependent resistance to the bactericidal activity of aerobic human neutrophils. Infect. Immun. **36:**1086–1095.
34. **Oliver, C. N.** 1987. Inactivation of enzymes and oxidative modification of proteins by stimulated neutrophils. Arch. Biochem. Biophys. **253:**62–72.
35. **Papp-Szabò, E., C. L. Sutherland, and P. D. Josephy.** 1993. Superoxide dismutase and the resistance of *Escherichia coli* to phagocytic killing by human neutrophils. Infect. Immun. **61:**1442–1446.
36. **Rajkovic, I. A., and R. Williams.** 1985. Inhibition of neutrophil function by hydrogen peroxide. Effect of SH group-containing compounds. Biochem. Pharmacol. **34:**2083–2090.
37. **Royer-Pokora, B., L. M. Kunkel, A. P. Monaco, S. C. Goff, P. E. Newburger, R. L. Baehner, F. S. Cole, J. T. Curnutte, and S. H. Orkin.** 1986. Cloning the gene for an inherited human disorder—chronic granulomatous disease—on the basis of its chromosomal location. Nature (London) **322:**32–38.
38. **Safe, A. F., R. T. Maxwell, A. J. Howard, and R. C. Garcia.** 1991. Relapsing *Salmonella enteritidis* infection in a young adult male with chronic granulomatous disease. Postgrad. Med. J. **67:**198–201.
39. **Saha, A. K., J. N. Dowling, K. L. LaMarco, S. Das, A. T. Remaley, N. Olomu, M. T. Pope, and R. H. Glew.** 1985. Properties of an acid phosphatase from *Legionella micdadei* which blocks superoxide anion production by human neutrophils. Arch. Biochem. Biophys. **243:**150–160.
40. **Scott, M. D., S. R. Meshnick, and J. W. Eaton.** 1987. Superoxide dismutase-rich bacteria. Paradoxical increase in oxidant toxicity. J. Biol. Chem. **262:**3640–3645.
41. **Segal, A. W., and A. Abo.** 1993. The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem. Sci. **18:**43–47.
42. **Selsted, M. E., S. S. L. Harwig, T. Ganz, J. W. Schilling, and R. I. Lehrer.** 1985. Primary structures of human defensins: three antimicrobial peptides from blood neutrophils. J. Clin. Invest. **76:**1436–1439.
43. **Storz, G., M. F. Christman, H. Sies, and B. N. Ames.** 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **84:**8917–8921.
44. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science **248:**189–194.
45. **Tartaglia, L. A., C. J. Gimeno, G. Storz, and B. N. Ames.** 1992. Multidegenerate DNA recognition by the OxyR transcriptional regulator. J. Biol. Chem. **267:**2038–2045.
46. **Weitzman, S. A., and T. P. Stossel.** 1981. Mutation caused by human phagocytes. Science **212:**546–547.