The synthesis of manganese-superoxide dismutase in response to hydrogen peroxide and to pararquat was examined in strains of *Escherichia coli* with different mutations in the *oxyR* gene. Hydrogen peroxide treatment did not induce manganese-superoxide dismutase, but did induce the *oxyR* regulon. Pararquat induced this enzyme in a strain compromised in its ability to induce the defense response against oxidative stress (*oxyR* deletion) as well as in a strain that is constitutive and overexpresses the *oxyR* regulon. Catalase (HPI), but not manganese-superoxide dismutase, was overexpressed under anaerobic conditions in a strain harboring a constitutive *oxyR* mutation. The data clearly demonstrate that the induction of manganese-superoxide dismutase is independent of the *oxyR*-controlled regulon.

Superoxide dismutases (EC 1.15.1.1) are metalloenzymes that are widely distributed among living organisms. They provide an essential defense against oxygen toxicity, which is mediated by the partially reduced oxygen intermediates (i.e., superoxide radical (O\textsubscript{2}\textsuperscript{-}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and hydroxyl radical (OH\textsuperscript{-})) generated during normal biological reduction of dioxygen (1, 2). The levels of these reactive oxygen intermediates can be exacerbated by the presence of many environmental factors such as higher pO\textsubscript{2} (3), ionizing radiation (4), redox-active compounds (5), ozone (6), and other oxidants (7). In *Escherichia coli*, three isozymic forms of superoxide dismutase are found: manganese, iron, and hybrid isozymes (8). Currently, the iron-superoxide dismutase is thought to be constitutive with respect to oxygen, whereas manganese-superoxide dismutase is under rigorous control in terms of turbidity measured at 600 nm (OD\textsubscript{600}). The enzyme is inducible by oxygen (8), redox-active compounds that generate O\textsubscript{2}\textsuperscript{-} in the presence of oxygen (5, 9), ferrous iron chelators under both aerobic and anaerobic conditions (10, 14, 15), nitrate during anaerobic growth (11), and several strong oxidants such as ferricyanide, ammonium persulfate, and copper-cyanide complex (12). A model has been proposed for the regulation of manganese-superoxide dismutase in which the *sodA* gene, the structural gene for the enzyme, is thought to be negatively regulated by an iron-containing redox-sensitive repressor protein (10, 11). Recent findings (11, 12, 17) support the proposed model and suggest the possibility of more layers of control elements (17).

The presence of a global regulatory mechanism for coordinate expression of enzymes and proteins needed for cellular protection against hydrogen peroxide and oxidative stress is well documented (18-20). The induction of the oxidative response regulon is under positive control by the *oxyR* gene product, whose expression or activity is H\textsubscript{2}O\textsubscript{2}-inducible (18). It has been suggested that manganese-superoxide dismutase is part of the *oxyR*-controlled regulon (18-20). The data to support this conclusion came from the fact that its activity is about 2-fold higher in an *oxyR*\textsuperscript{mut} mutant strain of *Salmonella typhimurium* relative to a wild-type parental strain (18). *oxyR*\textsuperscript{mut} is a dominant mutation that confers resistance to hydrogen peroxide and causes constitutive overexpression of nine H\textsubscript{2}O\textsubscript{2}-inducible proteins; manganese-superoxide dismutase is believed to be one of these constitutive proteins (18-20).

The aim of this study was to explore the role of *oxyR* in the regulation of manganese-superoxide dismutase in *E. coli*. We examined the induction of this enzyme in wild-type strains (oxyR\textsuperscript{+}) and compared it with that of mutants affected in the expression of the *oxyR* locus. The data clearly indicate that the expression of the manganese-superoxide gene (sodA) is not regulated by *oxyR*.

**MATERIALS AND METHODS**

**Bacterial Strains**—The *E. coli* strains used in this study are listed in Table I.

**Media and Growth Conditions**—A minimal salts medium (21) supplemented with 0.5% glucose, 0.05% yeast extract, and 10 µg/ml each arginine and methionine was used and is designated as GY medium. The rich medium, TSY, contained 5% Trypticase soy broth plus 0.5% yeast extract. Addition of the amino acids and yeast extract to the glucose minimal medium was required to support the growth of the *oxyR* deletion mutant and was used to grow all four strains. Overnight cultures grown in GY medium at 37°C on a rotary shaker at 200 rpm were used to inoculate fresh GY and TSY media. Growth was estimated in terms of turbidity measured at 600 nm (OD\textsubscript{600}). The experimental cultures were allowed to grow for about two generations from an initial OD\textsubscript{600} of 0.03 or 0.05 before addition of hydrogen peroxide or pararquat from sterile stock solutions. Anaerobic growth conditions were maintained by growing the slant cultures, the overnight cultures, and the experimental cultures in the designated media equilibrated in an anaerobic environment maintained in a Coy anaerobic chamber. At the end of the specified growth period, chloramphenicol (150 µg/ml) was added, and the cultures were allowed to stand for 15 min before removing from the anaerobic chamber (10).

**Assay**—Cell-free extracts were prepared by sonication followed by dialysis (22). Protein was estimated by method of Lowry et al. (23) using bovine serum albumin as a standard. Superoxide dismutase (24) and catalase (25) were assayed as described previously. Superoxide dismutase isozymes, i.e., manganese, iron, and hybrid, were separated

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1 H. M. Hassan and D. Touati, unpublished data.
by electrophoresis in 10% polyacrylamide gels (26), visualized by the
nitro blue tetrazolium activity stain (27), and quantitated by linear
scanning densitometry (8). Catalase activity was localized in 10%
polyacrylamide gels and stained as described previously (28). Western
blotting was done according to the method described by Towbin et
al. (29). Antisera prepared against pure manganese-superoxide dis-
mutase was a kind gift of Dr. D. Clare (Department of Animal Science,
North Carolina State University). The peroxidase conjugate of goat
anti-rabbit immunoglobulin was purchased from Cappel/Cooper-
Biomedical. Sensitivity of each strain to hydrogen peroxide and
paraquat stock solutions were applied to the discs, giving final
concentrations of 60 and 300 μg of H₂O₂ or 38 and 190 μg of paraquat.
The diameter of the zone of inhibition was measured after 24 h at
37°C.

Results

Effects of Hydrogen Peroxide on Catalase and Superoxide
Dismutase Biosynthesis—Exposure of E. coli and S. typhi-
murium to 60 μM hydrogen peroxide for 60 min has been shown to
induce the oxidative stress regulon via the activation of oxyR (18, 19).
Fig. 1 compares the catalase and superoxide dismutase activities of the four test strains growing in GY
medium 1 h after initial exposure to hydrogen peroxide (60 μM).
Superoxide dismutase biosynthesis in each of the four
cells was unaffected by H₂O₂ treatment, whereas catalase
levels, in agreement with findings by Christman et al. (18),
increased 3.5-fold in oxyR⁺ strains RK4936 and K12, but  not
in the oxyA3 deletion strain TA4112. In TA4110,  which
contains the dominant oxyR2 allele, catalase levels  were 15-
fold higher than those seen in the parental strain, K12.
Furthermore, in this mutant strain, catalase was uninducible
by H₂O₂, as previously reported (18). Fig. 2 shows the gels
developed for catalase after aliquots of the extracts from the
H₂O₂-treated cultures (oxyR⁺ and oxyA3) were electropho-
resed. The induction pattern of the two bands shows that
HPI, the faster mobility band, was the catalase induced by
hydrogen peroxide in strains with a functional oxyR locus.
The superoxide dismutase isozymes in these extracts were
also separated by electrophoresis, and the gels were stained
for superoxide dismutase activity. Densitometric scans of
these gels revealed no significant differences in manganese-
containing superoxide dismutase in the four test strains fol-
lowing exposure to hydrogen peroxide (Table II). Similar
results were seen after 2 h of exposure (data not shown). The
activity of the iron- and hybrid-superoxide dismutase was not
affected by hydrogen peroxide treatment (Table II; the differ-
ence between the total and Mn-superoxide dismutase repre-
sents the iron-plus-hybrid form). About 5 mM H₂O₂ is nor-
ma\textsuperscript{mally required} to inactivate the iron-isozyme (30).

Induction of Manganese-Superoxide Dismutase by Paraquat
in oxyA3 and oxyR2 Mutants—Paraquat has been shown to
increase the intracellular flux of O₂⁻; and in turn, the cells
induce manganese-superoxide dismutase to scavenge O₂⁻ (5, 9,
10, 13, 14). As expected, the synthesis of this enzyme in the
wild-type (oxyR⁺) strains, RK4936 and K12, was inducible by
the presence of paraquat in the growth medium (Table III).

![Fig. 1. Expression of total superoxide dismutase and cata-
lase upon exposure to 60 μM hydrogen peroxide in GY me-
dium. The four test strains in late logarithmic phase in GY medium
were inoculated into fresh GY medium to an initial OD₅₅₀ of 0.03.
After cells had reached an OD₅₅₀ of 0.3, hydrogen peroxide was added
at a final concentration of 60 μM. The incubation was allowed to
continue for 1 h before cell-free extracts were prepared and assayed
for catalase and superoxide activity as described in the text. SOD,
superoxide dismutase.

![Fig. 2. Nondenaturing polyacrylamide gel of catalase from
samples exposed (lanes b) and not exposed (lanes a) to hydro-
gen peroxide. Aliquots of extracts containing 50 μg of protein from
the H₂O₂-treated deletion strain (TA4112) and its parent (RK4936)
(Fig. 1) were loaded onto a 10% polyacrylamide gel which was dev-
eoped and stained for activity as described under "Materials and
Methods." HPI and HPII, hydroperoxidase isozymes.

### Table I

| Strain       | Genotype                  | Relevant characteristics | Source/Ref. |
|--------------|----------------------------|--------------------------|-------------|
| RK4936       | araD139/argF              | oxylR⁺                    | B. N. Ames/18 |
|              | lac2:35/70B5301/          |                          |             |
|              | non-9gag-A219/            |                          |             |
|              | relA1/rpsL150/            |                          |             |
|              | MetE70/                   |                          |             |
|              | bta:Tn10                  |                          |             |
| TA4112       | oxyA3[oxyA(oxy-          | oxyl deletion             | B. N. Ames/18 |
|              | Rbtu][3)] (derived from    |                          |             |
|              | RK4936)                   |                          |             |
| TA4110       | oxyR2 (derived from       | oxyl overproducer        | B. N. Ames/18 |
|              | oxyR (constitutive)       | K12)                     |             |
| K12          | Wild type                 | oxylR                    | B. N. Ames/18 |
If induction of the manganese-containing superoxide dismutase is a function of the oxidative stress regulon, then a mutation altering the expression of the regulatory gene, oxyR, or its product should prevent or alter its induction by paraquat. This was not the case. The data (Table III) clearly showed that it was induced by paraquat in the oxyA3 mutant (strain TA4112) as well as in the oxyR2 mutant (strain TA4110). Thus, strain TA4112, which lacks the functional oxyR gene, did not appear to be hindered in its ability to induce manganese-superoxide dismutase as compared to the parental strain, RK4936. TA4110 contained greater amounts of this activity at both paraquat levels compared to its parent, K12. However, the percentage of manganese-isozyme of the total activity was about the same in both strains, indicating a similar level of induction. 

Effect of oxyR2 on Anaerobic Expression of Manganese-Superoxide Dismutase and HPI—Manganese-superoxide dismutase is not expressed in anaerobically grown cultures (8) except when iron chelators (10, 14, 15) or oxidants (11, 12) are added to such cultures. Therefore, it was of interest to see if the dominant oxyR2 mutation plays a role under anaerobic conditions. For this experiment, strain TA4110 and its parent, K12, were grown anaerobically in GY and TSY media, and cell-free extracts were prepared as described under “Materials and Methods.” Analysis of superoxide dismutase revealed that only the iron form was synthesized anaerobically. This was not the case. The data (Table I) indicated that the activities of superoxide dismutase isozymes were not affected by this treatment. These observations are in agreement with Demple and Harwood (31), who also observed no change in superoxide dismutase activity and only a moderate 2-4-fold increase in catalase activity in extracts from wild-type E. coli exposed to hydrogen peroxide. The concentration of H$_2$O$_2$ used in this study (60 mM) was very low and did not cause significant inhibition of the iron- or hybrid-superoxide dismutase (Table II and data not shown), nor did it cause a significant change in the redox potential of the cells. Recently, we have shown that 0.5 mM H$_2$O$_2$ can induce manganese-superoxide dismutase only in a catalase-deficient mutant of E. coli due to a positive change in the cells’ redox potential (12). The observation that catalase was induced by H$_2$O$_2$ treatment whereas manganese-superoxide dismutase was not affected suggests that sodA is probably not a part of the oxyR regulatory network. However, in agreement with the findings by Christman et al. (18), that the oxyR2 mutant (overproducer strain) was the most resistant to killing by hydrogen peroxide (60 and 300 µg/disc), whereas the oxyA3 mutant (deletion strain) was the least resistant. On the other hand, all four strains were equally sensitive to paraquat (38 and 190 µg/disc).

**DISCUSSION**

In enteric bacteria, hydrogen peroxide adaptation (31) has been shown to coincide with the induction of 30 proteins (18). Nine of these H$_2$O$_2$-inducible proteins are coordinately and positively regulated by the oxyR gene (18). Catalase, alkylhydroperoxide reductase, and the manganese-containing superoxide dismutase are three of the enzymes identified to be regulated by the oxyR locus (18–20). Furthermore, these three antioxidant enzymes are reported to be overexpressed in oxyR constitutive mutants (18–20). The manganese-superoxide dismutase is known to be induced by oxygen (3, 8), reoxy-active compounds (5, 9), and many oxidants (6, 11, 12). Therefore, the goal of this study was to elucidate the role of the oxyR locus in the regulation of this enzyme.

**Superoxide Dismutase Is Not Induced during H$_2$O$_2$ Adaptation**—In this part of the study, we examined the biosynthesis of superoxide dismutase in oxyR$^+$ (wild type), oxyA3 (deletion) and oxyR2 (constitutive) strains growing in GY medium in the presence of 60 mM H$_2$O$_2$. This concentration of H$_2$O$_2$ was found, as expected (18), to induce catalase activity 2.5-3.5-fold in the oxyR$^+$ strain, but had no effect on the oxyA3 or the oxyR2 strain. The data, however, clearly showed that the activities of superoxide dismutase isozymes were not affected by this treatment. These observations are in agreement with Demple and Harwood (31), who also observed no change in superoxide dismutase activity and only a moderate 2-4-fold increase in catalase activity in extracts from wild-type E. coli exposed to hydrogen peroxide. The concentration of H$_2$O$_2$ used in this study (60 mM) was very low and did not cause significant inhibition of the iron- or hybrid-superoxide dismutase (Table II and data not shown), nor did it cause a significant change in the redox potential of the cells.

**TABLE II**

*Expression of manganese-superoxide dismutase after exposure to 60 µM hydrogen peroxide*

| Strain   | Phenotype | 0 µM H$_2$O$_2$ | 60 µM H$_2$O$_2$ |
|----------|-----------|-----------------|------------------|
|          |           | Mn-SOD $^a$     | Total SOD        |
|          |           | units/mg        | units/mg         |
| RK4936   | oxyR$^+$  | 2.6             | 5.4              |
| TA4112   | oxyA3     | 2.4             | 4.6              |
| K12      | oxyR$^+$  | 2.9             | 6.6              |
| TA4112   | oxyR2     | 6.0             | 9.9              |

$^a$ SOD, superoxide dismutase.

**TABLE III**

*Induction of manganese-superoxide dismutase by paraquat*

| Strain   | Phenotype | 0 mM | 0.1 mM | 0.5 mM |
|----------|-----------|------|--------|--------|
|          |           | Mn-SOD $^a$ | Total SOD | Mn-SOD | Total SOD | Mn-SOD | Total SOD |
|          |           | units/mg |           | units/mg |           | units/mg |           |
| RK4936   | oxyR$^+$  | 7.8      | 10.5    | 16.5    | 18.8    | 31.4    | 34.5    |
| TA4112   | oxyA3     | 6.7      | 10.2    | 28.0    | 32.0    | 33.8    | 38.8    |
| K12      | oxyR$^+$  | 8.9      | 12.3    | 14.1    | 16.8    | 17.3    | 20.1    |
| TA4110   | oxyR2     | 8.2      | 14.6    | 21.2    | 25.8    | 26.8    | 32.5    |

$^a$ SOD, superoxide dismutase.

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pared to the wild-type strain when both were grown in minimal media (see further discussion below).

**Induction of Manganese-Superoxide Dismutase Is Not Influenced by Mutations in oxyR Locus**—In this part of the study, we used paraquat to induce manganese-superoxide dismutase in the different oxyR mutants (Table III). It is clear that the oxyR deletion strain as well as the oxyR overproducer strain were not affected in their abilities to induce manganese-superoxide dismutase in response to added paraquat. These results, together with the findings that all the test strains were equally sensitive to paraquat, as determined by disc inhibition assays, clearly suggest that the oxyR locus is not involved in the induction of manganese-superoxide dismutase by paraquat or in providing enhanced resistance to this compound.

**oxyR Locus Is Functional during Anaerobiosis but Does Not Control Manganese-Superoxide Dismutase**—It was of interest to observe that the oxyR2 mutant possessed higher levels of catalase compared to oxyR” when grown in the absence of oxygen. This suggested that the oxidative stress regulon is anaerobically overexpressed in this strain, TA4110. However, if oxyR2 in TA4110 also affects the synthesis of manganese-superoxide dismutase, then we should have seen the expression of sodA in anaerobic cultures of this strain. This was not the case. We were unable to detect any manganese-superoxide dismutase activity or antigen in cell-free extracts prepared from anaerobic cultures of K12 or TA4110.

**Why Does oxyR Constitutive Mutant Have Slightly Higher Basal Level of Manganese-Superoxide Dismutase Than Its Parent Strain?**—As indicated above, we found that strain TA4110 contained about 2-fold more manganese-superoxide dismutase than strain K12 when grown in GY medium (Table II). This difference was not seen, however, when these two strains were grown in rich TSY medium (Table III, 0 mM paraquat). The increased level of the enzyme seen in the oxyR constitutive strain (TA4110) in GY (but not in TSY) medium may be explained by some differences in their metabolism in the minimal medium. We examined the rate of oxygen uptake and the rate of endogenous O$_2^-$ generated (32) by cells grown in GY and TSY media and found no significant differences. The possibility still remains that TA4110 may have a lower internal concentration of iron when grown in a microaerobic medium, and/or other nutritional factors may also be involved.

In conclusion, the data demonstrate that the biosynthesis and induction of manganese-superoxide dismutase in *E. coli* are independent of the oxyR-controlled regulon. Recent work using sodA::lacZ protein fusions (17) is in agreement with our conclusion. We have previously reported (33) that this enzyme is not part of the inducible DNA repair (SOS) system. Furthermore, recent studies (17) have shown that the sodA gene is not part of the heat shock regulon. The possibility of a special superoxide-inducible (sod) regulon being examined (34).

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3 S. W. Bowen and H. M. Hassan, unpublished data.

4 F. T. Lee and H. M. Hassan, unpublished data.

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