Cell, Vol. 41, 753–762, July 1985, Copyright © 1985 by MIT 0092–8674/85/070753–10 $02.00/0

# Positive Control of a Regulon for Defenses against Oxidative Stress and Some Heat-Shock Proteins in Salmonella typhimurium

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## Summary

**S. typhimurium become resistant to killing by hydrogen peroxide and other oxidants when pretreated with nonlethal levels of hydrogen peroxide. During adaptation to hydrogen peroxide, 30 proteins are induced. Nine are constitutively overexpressed in dominant hydrogen peroxide-resistant *oxyR* mutants. Mutant *oxyR1* is resistant to a variety of oxidizing agents and overexpresses at least five enzyme activities involved in defenses against oxidative damage. Deletions of *oxyR* are recessive and uninducible by hydrogen peroxide for the nine proteins overexpressed in *oxyR1*, demonstrating that *oxyR* is a positive regulatory element. The *oxyR1* mutant is also more resistant than the wild-type parent to killing by heat, and it constitutively overexpresses three heat-shock proteins. The *oxyR* regulatory network is a previously uncharacterized global regulatory system in enteric bacteria.**

## Introduction

All organisms that use molecular oxygen must defend themselves from the toxic byproducts of oxygen metabolism. In addition to the complete four electron reduction of molecular oxygen to water, reactive species such as hydrogen peroxide, superoxide anion, singlet oxygen, and the hydroxyl radical can be generated in vivo during respiration (Fridovich, 1978; Chance et al., 1979). These reactive oxygen species can oxidize membrane fatty acids initiating lipid peroxidation (Mead, 1976), oxidize proteins (Brot et al., 1981), and damage DNA (Demple and Linn, 1982; Levin et al., 1982; Hollstein et al., 1984; Cathcart et al., 1984).

Enteric bacteria have several enzyme activities that may protect the cells from oxidative damage. These include superoxide dismutase and catalase (Fridovich, 1976). In addition, other enzymes such as exonuclease III (Demple et al., 1983) and recA protein (Carlsson and Carpenter, 1980) appear to be important in repairing DNA lesions resulting from oxidative damage.

The induction of catalase and superoxide dismutase in E. coli during anaerobic to aerobic shifts has been studied extensively by Hassan and Fridovich (Hassan and Fridovich, 1977a; 1977b; 1977c; 1978). Treatment with hydrogen peroxide has been shown to induce catalase activity both in S. typhimurium (Finn and Condon, 1975) and in E. coli (Richter and Loewen, 1981). Despite the inducibility of these activities, little is known about the regulatory mechanisms involved. Although mutants with altered levels of catalase activity have been described in E. coli (Loewen, 1984) and in S. typhimurium (Levine, 1977), none have been demonstrated to be regulatory. No mutants altered in superoxide dismutase activity have been described.

In previous studies, we have shown a variety of oxidative stresses and heat-shock to induce rapidly the accumulation of AppppA and of a series of related adenylylated nucleotides (Bochner et al., 1984; Lee et al., 1983). These dinucleotides may be alarmones (Stephens et al., 1975) the function of which is to redirect cellular metabolism to cope with oxidative stress. Hydrogen peroxide induces the synthesis of adenylylated nucleotides and is a useful compound for studying cellular responses to reactive oxygen species because it is more stable than superoxide anion, singlet oxygen, and the hydroxyl radical. E. coli is capable of adapting to hydrogen peroxide (Demple and Halbrook, 1983) and, recently, it has been reported that S. typhimurium also can adapt to hydrogen peroxide (Winquist et al., 1984). As a first step toward understanding the regulation of defenses against oxidative stress, we present here a genetic and biochemical analysis of the adaptation to hydrogen peroxide in S. typhimurium.

## Results

### Adaptation to Hydrogen Peroxide in S. typhimurium

Exponentially growing cells of Salmonella typhimurium treated with 60 μM hydrogen peroxide for 60 min are resistant to killing by 10 mM hydrogen peroxide when compared with cells that have not been pretreated (Figure 1). Cells pretreated with 60 μM hydrogen peroxide in the presence of chloramphenicol do not acquire the resistance to killing by 10 mM hydrogen peroxide, indicating a requirement for de novo protein synthesis for the adaptation. The adaptation to hydrogen peroxide in S. typhimurium occurs under conditions similar to those previously described for Escherichia coli (Demple and Halbrook, 1983). As in E. coli, the adaptation is *recA*-independent (data not shown). Hydrogen peroxide-damaged P22 bacteriophage were not reactivated in S. typhimurium cells adapted to hydrogen peroxide (data not shown), in contrast to the reported finding of reactivation of P1 bacteriophage in adapted E. coli (Demple and Halbrook, 1983). It has been observed previously that S. typhimurium cells induce catalase activity upon exposure to low levels of hydrogen peroxide (Finn and Condon, 1975) in agreement with our results, which show that catalase activity in exponential cultures is induced 4- to 5-fold (from 0.09 units/$A_{650}$ to 0.44 units/$A_{650}$) after 1 hr of exposure in minimal glucose medium to 60 μM hydrogen peroxide.

### Hydrogen Peroxide Adaptation Induces Resistance to Killing by Other Stresses

S. typhimurium cells that have been exposed to the adaptive dose of hydrogen peroxide (60 μM) for 1 hr are resis-

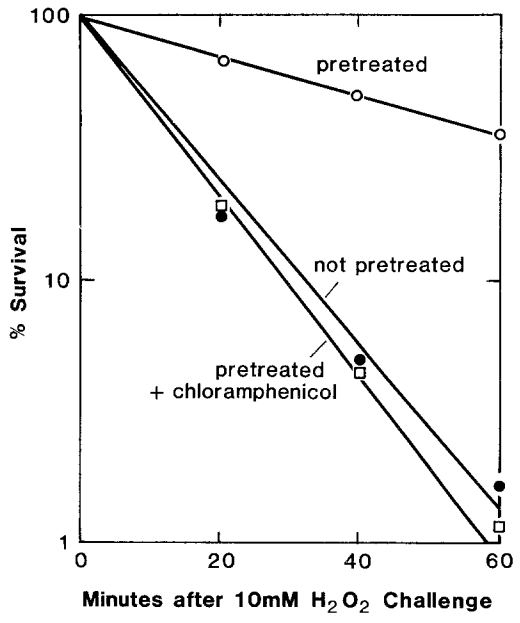



Figure 1. Adaptation to Hydrogen Peroxide in Salmonella typhimurium

Wild-type (S. typhimurium LT2) cells were grown in minimal glucose medium at 37°C with shaking to an $A_{650}$ of 0.2 and were then treated with 60 μM hydrogen peroxide for 60 min in the presence or absence of 100 μg/ml chloramphenicol. After 60 min, 10 mM hydrogen peroxide was added to all cultures. Aliquots were withdrawn, diluted in minimal salts, and plated on nutrient broth plates at 37°C to monitor cell viability. Cells pretreated with 60 μM hydrogen peroxide in the presence (□), and absence (○), of 100 μg/ml chloramphenicol. No treatment: (●).

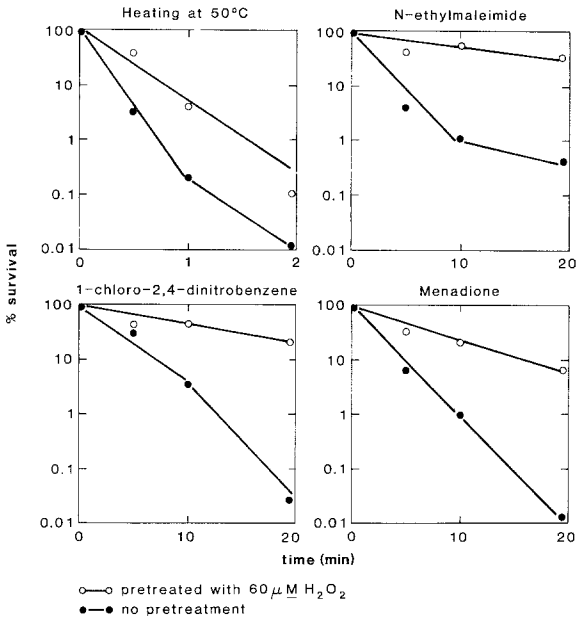



Figure 2. Hydrogen Peroxide-Induced Resistance to Other Stresses

Wild-type cells were grown in VBC salts containing 0.4% glucose at 37°C with shaking to an $A_{650}$ of 0.2 and were then treated with N-ethylmaleimide (1 mM), 1-chloro-2,4-dinitrobenzene (10 mM), or menadione (50 mM). Aliquots were withdrawn at the indicated times, diluted in VBC salts, and plated on nutrient broth plates at 37°C to monitor cell viability. For heat killing, an aliquot (0.2 ml) was withdrawn and heated at 50°C in a heating block for the indicated times. Heated samples were withdrawn, cooled on ice, diluted and plated as described above.

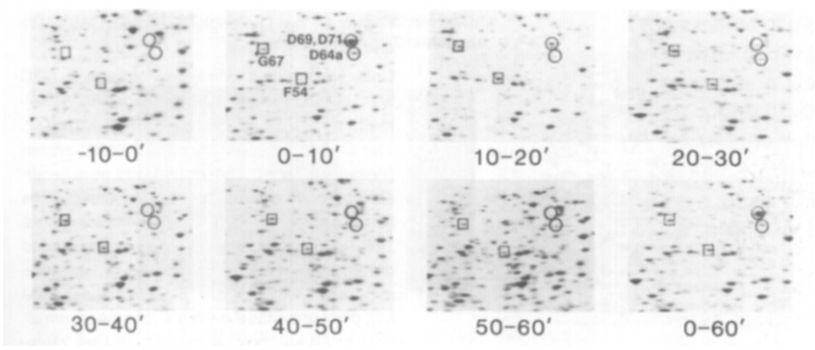
tant to killing both by a variety of chemical oxidants and by heating at 50°C (Figure 2). N-ethylmaleimide is a nonspecific thiol inactivating reagent, chlorodinitrobenzene depletes glutathione, and quinones such as menadione cause redox cycling, which can generate oxygen radicals (Chesis et al., 1984). Heat shock and these chemical oxidants all elevate levels of AppppA and other dinucleotides in S. typhimurium (Bochner et al., 1984). Although the mechanism of bacterial heat killing is not known, our results suggest a connection between oxidative stresses and heat stress (Bochner et al., 1984). The responses to these stresses are not identical, however, as heat shock induces thermotolerance (Yamamori and Yura, 1982) but not resistance to hydrogen peroxide (data not shown).

### Proteins Induced by Hydrogen Peroxide

Two-dimensional protein gel analysis of S. typhimurium cells treated with 60 μM hydrogen peroxide revealed that the rate of synthesis of 30 proteins is elevated in the 60 min following the addition of hydrogen peroxide (Morgan et al., unpublished data). This dramatic change in the pattern of protein synthesis has been consistently observed through many different experiments. Cells were pulse-labeled with L-[$^{35}$S]methionine for 10 min periods beginning at various times after the addition of hydrogen peroxide. These time-course experiments revealed two major temporal classes of hydrogen peroxide-inducible proteins (Figure 3). The first class consists of the "early" proteins; those for which synthesis is maximal during the first 10 min interval following the addition of hydrogen peroxide. For eight of the early proteins the rate of synthesis has returned to normal within 30 min after hydrogen peroxide addition. A second class of hydrogen peroxide-inducible proteins, the "late" proteins, is synthesized at a maximal rate starting 10–30 min after hydrogen peroxide addition. A number of the late proteins are induced to some extent before 20 min, and most continue to be synthesized at an elevated rate between 50 and 60 min after hydrogen peroxide addition. There are 12 early and 18 late proteins. The fact that 60 μM hydrogen peroxide induces the synthesis of 30 proteins illustrates that the adaptation response is complex, and that it involves more than the induction of catalase.

### A Hydrogen Peroxide Resistant Mutant Constitutively Expresses Nine of the Twelve Early Proteins

We have isolated a large number of hydrogen peroxide-resistant mutants following mutagenesis with diethylsulfate. The mutants arise at a frequency of about $1 \times 10^{-6}$ per survivor following mutagenesis. No spontaneous hydrogen peroxide-resistant mutants have been isolated, indicating a spontaneous frequency to hydrogen peroxide resistance of less than $1 \times 10^{-8}$. Only one major class of hydrogen peroxide-resistant mutants arise under the conditions used, as determined by two-dimensional protein gel analysis, enzyme activities (see below), and a variety of other phenotypes, such as colony morphology and resistance to alkyl hydroperoxides (data not shown). There-




○:"Early" $H_2O_2$-Inducible Proteins
□:"Late" $H_2O_2$-Inducible Proteins

Figure 3. Two Major Temporal Classes of Hydrogen Peroxide Inducible Proteins

Wild-type cells were pulse-labeled with L-[$^{35}$S]-methionine for 10 min intervals before and after the addition of 60 μM hydrogen peroxide as described in Experimental Procedures. (0–60') cells labeled for the entire pretreatment period. The same small region of the 2D protein gels is shown at each time interval. Times listed are minutes after addition of 60 μM hydrogen peroxide.

fore, one of the mutants was chosen for further characterization, and its mutation has been designated *oxyR1* (strain TA4100). *oxyR1* is highly resistant to killing by hydrogen peroxide compared with the wild-type (LT2) parent strain (Table 1) and constitutively expresses 9 of the 12 early hydrogen peroxide-inducible proteins (Figure 4). For some of these proteins, the relative abundance in the mutant is different from their relative abundance in hydrogen peroxide-adapted LT2. For instance, the C22 protein is the most abundant protein in *oxyR1* extracts, but it is not the protein most highly induced by hydrogen peroxide treatment in LT2 (data not shown). We have identified enzyme activities for the proteins D69, D71, F52a, and C22 (see below).

### Map Location of *oxyR1*

We have isolated a strain carrying a Tn*5* insertion element linked to *oxyR1*. In transductional crosses using strain TA4101 (*oxyR1*/zhh116::Tn*5*) as a donor and LT2 as a recipient, 85% of kanamycin-resistant transductants are resistant to hydrogen peroxide. When strain TA4102 (*oxyR*$^+$/zhh116::Tn*5*) is used as the donor in a transductional cross and TA4100 (*oxyR1*) as the recipient, 85% of kanamycin-resistant transductants acquired the wild-type sensitivity to hydrogen peroxide.

The Tn*5* insertion element near *oxyR* was transduced into HfrK4, and a kanamycin-resistant, hydrogen peroxide-sensitive (i.e., *oxyR*$^+$) transductant (TA4103) was used as a donor in Hfr mapping experiments (see Experimental Procedures). The Tn*5* insertion mutation was localized to the region near *argE* at 88′ on the S. typhimurium genetic map (data not shown). Transduction mapping using P22 demonstrated that the Tn*5* is 50% linked to *argE94* in TA4104. *oxyR1* was subsequently shown to be transductionally linked to *argE94* (50%) and to Tn*10* insertion mutations in *argH* (70%, strain TT137) and near *tyrU* (25%, strain TT2385). One hydrogen peroxide-resistant transductant, obtained in a transductional cross using TA4105 (*oxyR1*/argH1823::Tn10) as the donor and LT2 as the recipient, was analyzed by two-dimensional protein gel electrophoresis. It was found to overexpress the nine characteristic *oxyR1* proteins constitutively. Thus, the proteins overproduced by *oxyR1* are coordinately controlled by a single genetic locus.

Table 1. Sensitivity to Killing by Oxidants of Wild-Type Versus *oxyR1* Cells

| Oxidant | Diameter of Killing Zone (mm) | |
|---|---|---|
| | Wild-Type | *oxyR1* |
| Hydrogen Peroxide (300 μg) | 21.0 | 13.5 |
| Cumene Hydroperoxide (300 μg)* | 21.5 | 13.5 |
| t-butyl Hydroperoxide (70 μg) | 23.5 | 15.0 |
| Heating at 50°C for 1 min (% Killing) | 95% | 16% |
| 1-chloro-2,4-dinitrobenzene (200 μg)* | 15.5 | 19.5 |
| 6-amino-7-chloro-5,8-dioxoquinoline (10 μg)* | 16.5 | 20.5 |
| Menadione (1 mg)* | 14.5 | 18.0 |
| Paraquat (190 μg) | 30.5 | 28.0 |
| Cadmium chloride (1 mg) | 7.5 | 7.5 |
| N-ethylmaleimide (5 mg) | 42.0 | 42.5 |
| Diamide (170 μg) | 13.5 | 13.5 |

Cells were grown overnight at 37°C in nutrient broth prior to testing. Aliquots (0.1 ml) of cultures were then added to soft agar and were plated on VBC plates containing 2% glucose. Ten microliters of solutions containing inhibitors (dissolved in either DMSO or water) were applied to BBL filter discs, and the discs were placed in the center of the agar. The diameter of the zone of killing (clear area) was measured after 24 hr at 37°C. Heat killing was performed as described in Experimental Procedures.
* Dissolved in DMSO.

Introduction of an E. coli episome covering the *argE* region (F′14) into *oxyR1* has no apparent effect on the degree of hydrogen peroxide resistance or on the elevated catalase levels (see below). Therefore, *oxyR1* is dominant, assuming that the heterologous complementation by the E. coli episome is efficient. It is reasonable to assume that the complementation would be efficient, since similar hydrogen peroxide-resistant mutants that we have isolated in E. coli (see below) also map near *argE* (P. Loewen, personal communication), and deletions of *oxyR* in S. typhimurium are efficiently complemented by the same E. coli episome (see below).

### Resistance of *oxyR1* to a Variety of Oxidants

*oxyR1* is resistant to hydrogen peroxide, organic hydroperoxides, and to heat killing, compared with the wild-type parent strain (Table 1). However, *oxyR1* is more sensitive to killing by quinones such as menadione, 6-amino-7-

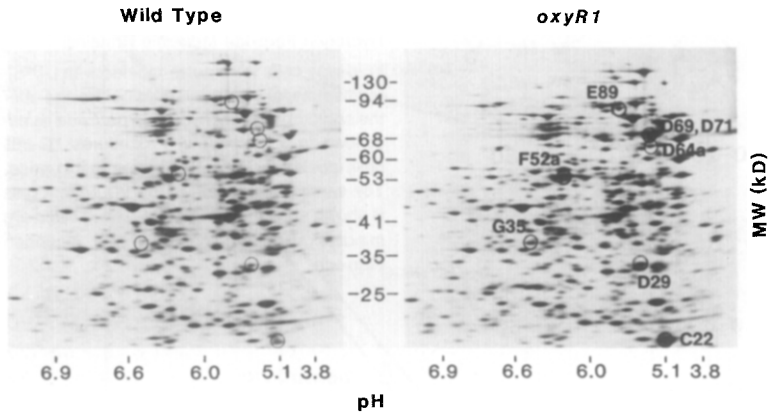



Figure 4. Two-Dimensional Protein Gels of LT2 and *oxyR1*

Cells were grown in minimal glucose at 37°C with shaking and were labeled with L-[$^{35}$S]-methionine for 1 hr, starting with an $A_{650}$ of 0.2. The circled proteins are constitutively overexpressed in *oxyR1*. The protein gels were generated by equilibrium isoelectric focusing in the first dimension and by electrophoresing on a 10% SDS-polyacrylamide gel, in the second dimension. Standards used to estimate molecular weights are β-galactosidase (130 kd), phosphorylase A (94 kd), bovine serum albumin (68 kd), bovine catalase (60 kd), glutamate dehydrogenase (53 kd), alcohol dehydrogenase (41 kd), lactate dehydrogenase from beef heart (35 kd), and histidine transport J protein (25 kd). *oxyR1* protein G18 is not visible on a 10% gel.

Table 2. Enzyme Activities Elevated in *oxyR1*

| | Minimal Glucose | | | LB | | |
|---|---|---|---|---|---|---|
| Enzyme Assayed* | LT2 | *oxyR1* | Fold Induction | LT2 | *oxyR1* | Fold Induction |
| Catalase | | | | | | |
| Whole Cells† | 0.44 | 20 | 45 | 4 | 40 | 10 |
| Cell Extract‡ | 2.2 | 110 | 50 | 28 | 490 | 17 |
| Superoxide Dismutase§ | | | | | | |
| Manganese | 1.0 | 2.2 | 2.2 | 1.0 | 1.8 | 1.8 |
| Iron | 0.0 | 0.0 | NA | 0.8 | 0.4 | 0.5 |
| Glutathione Reductase | | | | | | |
| Cell Extract | 0.29 | 1.11 | 3.8 | 0.23 | 0.93 | 4.0 |
| Alkylhydroperoxide Reductase | | | | | | |
| Whole Cells | 0.0036 | 0.072 | 20 | 0.0075 | 0.036 | 5.0 |
| Cell Extract | ND | ND | ND | 0.22 | 0.89 | 4.0 |

* All assays were performed on stationary-phase cells as described in Experimental Procedures.
† Activities are expressed as μmoles/min/$A_{650}$.
‡ Activities are expressed as μmoles/min/mg protein.
§ Relative activities were estimated from gel densitometer tracings of gels like the one shown in Figure 5.
ND, not determined.
NA, not applicable.

chloro-5,8-dioxoquinoline (ACDQ) and 1-chloro-2,4-dinitrobenzene (CDNB). A third group of oxidants, including cadmium chloride, diamide, paraquat, and N-ethylmaleimide, are equally toxic to *oxyR1* and LT2. Thus, *oxyR1* mutants are resistant to a subset of the oxidants to which hydrogen peroxide-adapted cells are resistant. All of the oxidative agents listed in Table 1 induce the accumulation of adenylylated nucleotides in S. typhimurium.

## *oxyR1* Overproduces Four Enzyme Activities Involved in Defenses Against Oxidative Stress

### *Catalase/Peroxidase*

The specific activity of catalase in VBCG grown *oxyR1* is 45–50 times that of the parent wild-type (LT2) strain (Table 2). The previously described inhibitory effect in E. coli B of glucose on catalase synthesis (Hassan and Fridovich, 1978) is diminished in *oxyR1* (compare LT2 with *oxyR1* grown in minimal glucose and LB in Table 2).

More than 95% of the catalase activity in *oxyR1* extracts is precipitated by 0–25% ammonium sulfate. In contrast, catalase activity in wild-type extracts is precipitated by 25–55% ammonium sulfate (Figure 5A). *oxyR1* overproduces two major catalase activities of similar mobility in native polyacrylamide gels, and it produces a minor, slower moving band corresponding to the catalase activity band above HPIII (Figure 5A). All three of the overproduced catalase activity bands precipitate from 0–25% ammonium sulfate. Each of these bands also has an associated peroxidase activity (data not shown). We have isolated deletions extending from *argH* through *metB* that eliminate HPI-II catalase activity (and the induced band that runs above HPIII), as well as eliminating proteins D69 and D71 (M. F. Christman and B. N. Ames, unpublished). This deletion should eliminate the *katG* gene, which is the structural gene for the HPI-II catalases (Loewen et al., 1985). The HPI-II catalase/peroxidases are concluded to be *oxyR1* proteins D69 and D71 (see Figure 4). Thus, *oxyR1* overexpresses two major catalase/peroxidases that appear to be analogous to those termed HPI and HPII in E. coli (Claiborne and Fridovich, 1979; Claiborne et al., 1979). The catalase activity referred to as HPIII (Richter and Loewen, 1981) does not appear to be affected by the *oxyR1* mutation.

### *Manganese Superoxide Dismutase*

Physiological studies in E. coli B have shown that catalase and superoxide dismutase (SOD) activities are coin-

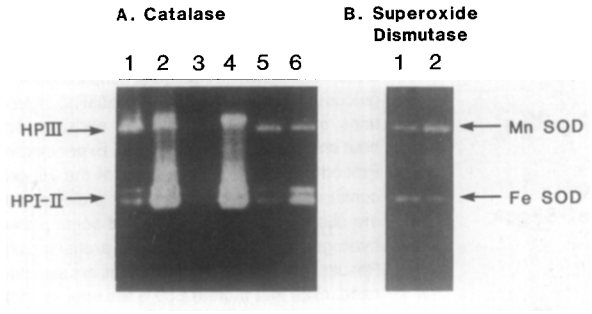



Figure 5. Catalase and Superoxide Dismutase Activity Stains of LT2 and *oxyR1* Extracts

Cells were grown to stationary phase in Luria Broth, and extracts were prepared by sonication. For both catalase and superoxide dismutase activity stains, 9% acrylamide gels were run as described previously (Davis, 1964). Catalase activity staining gels were slightly modified, such that the running gel buffer was at pH 8.0. The activity stains are described in Experimental Procedures. (A) 1, 30 μg LT2 crude extract; 2, 30 μg *oxyR1* crude extract; 3, 4 μg LT2 0–25% ammonium sulfate; 4, 4 μg *oxyR1* 0–25% ammonium sulfate; 5, 25 μg LT2 25–55% ammonium sulfate; and 6, 25 μg *oxyR1* 25–55% ammonium sulfate. (B) 1, 100 μg LT2 crude extract; and 2, 100 μg *oxyR1* crude extract.

duced after an anaerobic to aerobic shift (Gregory and Fridovich, 1973). Native gels stained for SOD activity on wild-type and *oxyR1* extracts are shown in Figure 5B. Integrations of the gel densitometer tracings show that the activity band of slower mobility is increased about 2.2-fold in *oxyR1*, whereas the faster mobility band is half as abundant in *oxyR1* as in the wild type. The induction of the slower SOD activity band and the repression of the faster activity band has been observed in E. coli B and K12 following anaerobic to aerobic shifts (Hassan and Fridovich, 1977a). The slower band has been identified as the manganese containing SOD, and the faster form, as an iron-containing SOD in E. coli (Hassan and Fridovich, 1977a). In S. typhimurium and in E. coli, activity of the faster band is inhibited by hydrogen peroxide and is not produced during growth on minimal glucose. Therefore, we presume that the slower mobility band in S. typhimurium represents the manganese SOD, and the faster band, the iron SOD.

***Glutathione Reductase***

Glutathione is present at levels of 5–10 mM in E. coli (Meister and Anderson, 1983). It can be easily oxidized to the intermolecular disulfide, which subsequently becomes reduced by glutathione reductase (Meister and Anderson, 1983). Extracts of *oxyR1* contain 4-fold higher levels of glutathione reductase than do wild-type extracts (Table 2). Reduced glutathione is probably important for preventing excessive damage from toxic oxygen species (Penninckx and Jaspers, 1982; Morse and Dahl, 1978), and its coregulation with catalase and superoxide dismutase supports this proposed function.

***NAD(P)H-Dependent Alkyl Hydroperoxide Reductase***

*oxyR1* is resistant to growth inhibition by cumene hydroperoxide and t-butyl hydroperoxide (Table 1). In an attempt to determine the mechanism of resistance to these organic hydroperoxides (which are not catalase substrates), we assayed the ability of extracts of *oxyR1* to degrade cumene hydroperoxide using a reverse phase HPLC assay (see Experimental Procedures). Either whole cells in the presence of glucose or extracts of *oxyR1* in the presence of NAD(P)H will reduce cumene hydroperoxide to the corresponding alcohol at 5–20 times the rate that wild-type cells or extracts catalyze the reaction (Table 2). To our knowledge, this is a novel activity in enteric bacteria, although NADH-dependent hydrogen peroxide reductases have been described in Streptococcus faecalis (Dolin, 1957) and in Lactobacillus casei (Mizushima and Kitahara, 1962). The activity has been purified to near homogeneity from *oxyR1* extracts (Jacobson et al., unpublished data), and has been shown to consist of two proteins, both of which are required for activity. One component binds to 5′ AMP nucleotide affinity columns, contains FAD, and has a 52 kd subunit molecular weight. The other component, which does not bind to a 5′ AMP column, has a 22 kd subunit molecular weight. Two-dimensional electrophoresis of the purified components with wild-type cell extracts allows assignment of the proteins with those shown in Figure 4. The 52 kd protein is *oxyR1* protein F52a, and the 22 kd protein is *oxyR1* protein C22 (see Figure 4). This represents a fourth activity related to protection against oxidative damage that is overexpressed in *oxyR1* mutants. In addition to the activities described above, *oxyR1* extracts produce 2-fold higher levels of glucose-6-phosphate dehydrogenase than do wild-type extracts. This enzyme, which produces NADPH, may serve to provide the reducing equivalents needed for glutathione reductase and the alkyl hydroperoxide reductase. There is no difference between the levels of hexokinase and lactate dehydrogenase in *oxyR1* and LT2 extracts.

### *oxyR1* Mutants Overexpress Three Heat-Shock Proteins

Both *oxyR1* and hydrogen peroxide-treated wild-type cells are resistant to heat killing (Table 1 and Figure 2). Heat induces production of the family of dinucleotides that are also induced by oxidants (Bochner et al., 1984; Lee et al., 1983). This led us to investigate possible overlaps between proteins induced by a temperature shift and overexpressed proteins in *oxyR1* mutants. *oxyR1* proteins F52a, D64a, and E89 are also heat-shock proteins in wild-type S. typhimurium (Figure 6). Two of these three proteins (D64a and E89) can be induced further by heat shock in *oxyR1*, indicating the existence of at least two levels of control for these proteins in S. typhimurium. We do not know whether the high-level expression of these heat-shock proteins in *oxyR1* is responsible for the resistance to heat killing. This establishes that a single regulatory locus controls the expression of some heat-shock proteins and of proteins involved in defenses against oxidative damage.

### Deletions of *oxyR* Are Recessive and Uninducible by Hydrogen Peroxide for the Nine Proteins Overexpressed by *oxyR1* Mutants

Tn*10*-mediated deletions have been obtained from both wild-type and *oxyR1* strains carrying a Tn*10* in *argH* using a positive selection for tetracycline sensitivity (Bochner et al., 1980). Thirty tetracycline-sensitive isolates from TA4106

A

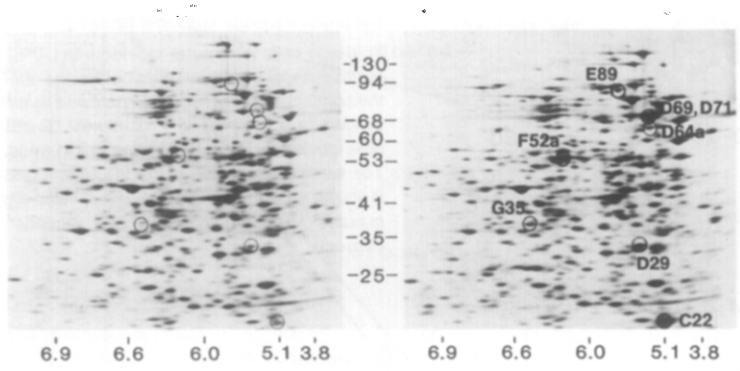



Figure 6. Hydrogen Peroxide and Heat-Shock Induction of Proteins in oxyΔ2

Cells were grown in VBC salts containing 0.4% glucose at 37°C with shaking. Labeling of proteins after hydrogen peroxide addition and heat shock were as described in Experimental Procedures. (1) A small region of the 2D gel containing the three heat-shock proteins that are overexpressed in *oxyR1* and some of the hydrogen peroxide-inducible proteins. (2) Results for all of the *oxyR1* proteins are summarized. Note that protein E89 is the smaller spot in the center of the circle labeled E89.

B

| Proteins Overproduced in *oxyR1* | Wild Type | | *oxy△2* | |
|---|---|---|---|---|
| | Inducing Condition | | | |
| | $H_2O_2$ | Heat-Shock | $H_2O_2$ | Heat-Shock |
| G18 | + | − | − | (−) |
| C22 | + | − | − | (−) |
| D29 | + | − | − | (−) |
| G35 | + | − | − | (−) |
| F52a | + | + | − | − |
| D64a | + | + | − | + |
| D69 | + | − | − | (−) |
| D71 | + | − | − | (−) |
| E89 | + | + | − | + |

(LT2/argH1823::Tn*10*) and 25 isolates from TA4105 (*oxyR1*/argH1823::Tn*10*) were screened for sensitivity to hydrogen peroxide. Two isolates from each group were extremely sensitive to hydrogen peroxide (diameter of killing zone with 300 μg of hydrogen peroxide: TA4106, 25mm; TA4105, 16mm; and hypersensitive deletions, 45mm).

Two-dimensional protein gels of TA4105 and one of the hydrogen peroxide-hypersensitive deletions derived from it, oxyΔ1 (TA4107), indicate that the nine proteins overexpressed in TA4105 are only present at wild-type levels in oxyΔ1. Furthermore, oxyΔ1 has roughly wild-type levels of catalase and of the alkyl hydroperoxide reductase.

One of the hydrogen peroxide-hypersensitive deletions derived from TA4106, oxyΔ2 (TA4108), was tested for induction of adaptation proteins by 60 μM hydrogen peroxide, and the results are shown in Figure 6. All nine of the *oxyR1* proteins are uninducible in oxyΔ2 by 60 μM hydrogen peroxide. Most of the 21 hydrogen peroxide-inducible proteins that are not overexpressed in *oxyR1* are induced normally in oxyΔ2 (data not shown). Most of the heat-shock proteins can be induced normally by a temperature shift in oxyΔ2, including *oxyR1* proteins D64a and E89 (Figure 6). The only exception is protein F52a, which can no longer be heat-induced in oxyΔ2. However, the F52a protein spot is visible on two-dimensional gels of oxyΔ2, and the alkyl hydroperoxide reductase activity, of which F52a is an essential component, is present at wild-type levels in oxyΔ2.

Introduction of an E. coli episome covering the *argH* region (F'14) into oxyΔ1 and oxyΔ2 restores their resistance to hydrogen peroxide to the wild-type level. The inducibility of the nine proteins overexpressed in *oxyR1* by 60 μM hydrogen peroxide is restored in oxyΔ2 strains containing F'14 (TA4109).

## Discussion

We have observed that S. typhimurium is capable of adapting to hydrogen peroxide in a manner similar to that previously described for E. coli (Demple and Halbrook, 1983). Adapted cells are resistant to a variety of other agents causing oxidative damage, as well as to heat killing. Two-dimensional protein gels show that S. typhimurium cells induce the synthesis of 30 proteins when exposed to 60 μM hydrogen peroxide. A class of hydrogen peroxide-resistant mutants, represented by *oxyR1*, has been isolated and has been shown to overexpress nine of the twelve most rapidly induced hydrogen peroxide adaptation proteins constitutively. *oxyR1* mutants have elevated levels of catalase/peroxidases, Mn superoxide dismutase, glutathione reductase, and a novel alkyl hydroperoxide reductase. In addition, *oxyR1* mutants constitutively overexpress three heat-shock proteins. A deletion generated from a Tn*10* that is closely linked to *oxyR1* is thought to remove the *oxyR* gene, as it is hypersensitive to hydrogen peroxide, recessive, and uninducible by hydrogen peroxide for the nine *oxyR1* proteins. Heat-shock protein induction is normal in the oxyΔ2 deletion for

Table 3. Bacterial Strains

| Strain | Genotype | Source |
|---|---|---|
| TA4100 | *oxyR1* | This study |
| TA4101 | *oxyR1*/zhh116::Tn*5* | " |
| TA4102 | oxyR⁺/zhh116::Tn*5* | " |
| TA4103 | HfrK4/zhh116::Tn*5* | " |
| TA4104 | argE94/zhh116::Tn*5* | " |
| TA4105 | *oxyR1*/argH1823::Tn*10* | " |
| TA4106 | LT2/argH1823::Tn*10* | " |
| TA4107 | oxyΔ1[oxyΔ(oxyRargH)1] | " |
| TA4108 | oxyΔ2[oxyΔ(oxyRargH)2] | " |
| TA4109 | oxyΔ2/F′14 | " |
| TA4110 | *oxyR2* (E. coli) | " |
| TA4112 | oxyΔ3[oxyΔ(oxyRbtuB)3] (E. coli, derived from RK4936) | " |
| TA4113 | katΔ1[katΔ(metBkatGargH)1] | " |
| TT137 | argH1823::Tn*10* | John Roth |
| TT2385 | zii614::Tn*10* | " |
| RK4936 | araD139/(argF-lac)205/flbB5301/non-9gyrA219/relA1/rpsL150/metE70/btuB::Tn*10* (E. coli) | R. Kadner |

most proteins with the exception of F52a (the alkyl hydroperoxidase flavoprotein component), which is not heat inducible in oxyΔ2. We have isolated and characterized mutants similar to *oxyR1* and oxyΔ2 in E. coli (Table 3).

### The *oxyR* Gene Product Is a Positive Regulator of Defenses Against Oxidative Stress and Some Heat-Shock Proteins

Deletion oxyΔ2 is presumed to inactivate the *oxyR* gene, since it is uninducible by hydrogen peroxide for the nine *oxyR1* proteins, and an E. coli episome restores the ability of oxyΔ2 to induce these proteins. The most reasonable interpretation is that the *oxyR* gene product is a positive effector of gene expression, the activity or expression of which is hydrogen peroxide-inducible. *oxyR1* mutants may either overproduce the *oxyR* gene product or produce an inducer-independent product. Such mutants are predicted to be dominant, as is observed for *oxyR1*.

The map positions of three of the genes controlled by *oxyR* are known in E. coli. Since the genetic maps of these two closely related bacteria are almost identical, these genes probably map in the same region in S. typhimurium. They are *sodA* at 89.5 min. (Mn SOD, Touati, 1983), *gor* at 77 min. (glutathione reductase, Davis et al., 1982), and *katG* at 88 min. (HPI-II catalases, Loewen et al., 1985). Since *sodA* and *gor* are unlinked to the regulatory gene, *oxyR* must act in *trans* to affect gene expression. Deletions extending from *argH* to *metB* (e.g., TA4113) abolish HPI-II activity but do not delete *oxyR*. Therefore, *oxyR* maps to the opposite side of *argH* from *katG*, indicating that these two genes are not part of the same operon.

The genes for proteins D64a and E89 are subject to multiple controls, since their hydrogen peroxide induction is *oxyR*-dependent but their basal level of expression and their heat induction are *oxyR*-independent. The expression of protein F52a appears to be different. Its hydrogen peroxide and heat induction are *oxyR*-dependent, but its basal level of expression is *oxyR*-independent.

The *oxyR* gene product could function in a manner analogous to other positive regulatory proteins in bacteria such as, an alternate sigma factor for RNA polymerase (Grossman et al., 1984), a nucleotide binding protein that interacts with RNA polymerase at certain promoters, or an indirect-acting positive regulator such as the recA protein. Substantial homology in primary amino acid sequence has been observed between the *htpR* sigma factor and the normal sigma protein (Landick et al., 1984) as well as between positive regulatory proteins CRP and Fnr (Shaw et al., 1983). Thus, the DNA sequence of the *oxyR* gene may shed light on the mechanism of action of the gene product.

### Purpose of an Adaptive Response to Oxidative Stress

Oxygen is highly toxic to anaerobic organisms, and its metabolic products are toxic to aerobes as well (Fridovich, 1978). The major toxic products that arise from oxygen metabolism are superoxide anion, hydrogen peroxide, singlet oxygen, and the hydroxyl radical (Fridovich, 1978). The existence of an adaptive response to oxidative agents such as hydrogen peroxide, therefore, might be expected in facultative anaerobes such as S. typhimurium and E. coli, which undergo shifts from anaerobic to aerobic environments.

Hydrogen peroxide adaptation may also be useful as a means for potential pathogens, such as S. typhimurium and E. coli, to survive respiratory bursts of hydrogen peroxide and superoxide anion associated with phagocytosis of bacteria by activated granulocytes. The respiratory bursts are capable of generating millimolar concentrations of hydrogen peroxide and are thought to facilitate killing of engulfed bacteria (Root and Cohen, 1981). In addition, studies with catalase deficient mutants of Staphylococcus aureus have demonstrated a direct correlation between catalase activity and pathogenicity (Mandell, 1975).

### *oxyR* Controls a Fourth Global Response to a DNA-Damaging Agent

Hydrogen peroxide has been shown to damage DNA in vitro (Demple and Linn, 1982) and cause mutations in vivo (Levin et al., 1982). Thus, in addition to the SOS response (Walker, 1984), the adaptive response to alkylating agents (Samson and Cairns, 1977) and heat-shock (Krueger and Walker, 1984), the *oxyR* regulatory network is a fourth major regulon in enterics that can be induced by an agent that damages DNA. The SOS and adaptive responses involve the induction of DNA repair systems that repair DNA damage caused by the inducing agent. Hydrogen peroxide adaptation in E. coli may involve the induction of DNA repair specific for oxidative damage (Demple and Halbrook, 1983; Hollstein et al., 1984), although the gene products involved have not been identified. Each of these four systems is activated by a positive effector of gene expression (Walker, 1984; Krueger and Walker, 1984).

### The Relationship between Dinucleotides, Heat Shock, and Oxidative Stress

There is evidence that heat shock and oxidative stress may be related phenomena (Lee et al., 1983). The heat-shock response in eukaryotic cells can be induced by a variety of oxidizing agents, including hydrogen peroxide,

menadione (Ashburner and Bonner, 1979), and diamide (Levinson et al., 1980). These agents, like heat shock, elevate levels of the AppppA family of dinucleotides in S. typhimurium and E. coli that may be alarmones for oxidative stress (Bochner et al., 1984; Lee et al., 1983). Exposure to oxygen after anoxia induces the heat-shock proteins in Drosophila (Ashburner and Bonner, 1979). Heat shock or exposure to ethanol increases the level of oxidized glutathione in bacteria (Bochner et al., 1984). The finding that a single regulatory mutation alters expression of three heat-shock proteins, as well as four enzyme activities that provide defenses against oxidative damage, lends support to this theory.

The *oxyR1* mutation is dominant, which suggests that it affects the activity or expression of a positive regulatory element rather than that of a repressor. If AppppA and the other adenylylated nucleotides are involved in the induction of genes during adaptation to hydrogen peroxide, then perhaps the *oxyR* gene product is a protein that binds one of a set of dinucleotide alarmones and thereby activates transcription of a specific set of genes. This situation would be analogous to the interaction of cAMP with its receptor protein CRP. Mutant alleles of *oxyR* might be inducer-independent, as is the case for one class of *crp* mutants (Sabourin and Beckwith, 1975). Since each oxidant induces a different pattern of dinucleotides, the complexities of the involvement of each in gene regulation remain to be elucidated.

## Experimental Procedures

### Bacterial and Phage Stocks

Wild-type Salmonella typhimurium LT2 or Escherichia coli K12 were the parents for most strains used in this study. *oxyR1* (TA4100, derived from LT2), *oxyR2* (TA4110, derived from K12), *katR1* (TA4111, derived from K12) were isolated as resistant to 300 μM hydrogen peroxide on minimal glucose after mutagenesis with diethylsulfate. Auxotrophic strains used in the Hfr mapping were derived from LT2. HfrK4 was used as the conjugal donor in Hfr crosses. The tail-dependent Tn*5* vector of M. Susskind (unpublished) was used to generate a random pool of 10,000 Tn*5* insertions. P22 HT105/1 int-201 (Anderson and Roth, 1978) was used in all transductions.

### Culture Methods for Adaptation Experiments

S. typhimurium LT2 was grown routinely in minimal VBC salts (Vogel and Bonner, 1956) containing 0.4% glucose as a carbon source at 37°C with shaking. For adaptation experiments, overnight cultures of LT2 were used to inoculate fresh 3 ml cultures of VBC glucose to an initial $A_{650}$ of 0.02. When cells had reached an $A_{650}$ of 0.2, hydrogen peroxide was added to a final concentration of 60 μM. After the pretreated cells had reached an $A_{650}$ of 0.4, the challenge was administered (hydrogen peroxide, 10 mM; N-ethylmaleimide, 1 mM; menadione, 50 mM; or heating to 50°C). Heat killing was performed by placing 0.2 ml aliquots of cultures at an $A_{650}$ of 0.4 in a heating block at 50°C. Samples were withdrawn from the heating block and diluted immediately to determine viable cells. Dilutions were done in VBC salts, and aliquots of diluted cells were plated on nutrient broth plates and incubated overnight at 37°C for viable cell counts.

### Cell Labeling and Preparation for Two-Dimensional Gel Electrophoresis

Cells were grown overnight in VBC glucose at 37°C in 2 ml culture tubes prior to labeling. Exponentially growing cultures in VBC glucose were used for labelings. The labeling reaction consisted of 0.4 ml of culture, 200 μCi/ml L-[$^{35}$S]methionine, and unlabeled L-methionine, such that the final methionine concentration was $1 \times 10^{-5}$ M. The labeling mixture was incubated with shaking for 10 to 60 min, depending on the sample, and quenched with 10 μl of 0.1 M L-methionine. Samples undergoing hydrogen peroxide adaptation were labeled for 60 min immediately following addition of hydrogen peroxide to a final concentration of 60 μM, and samples undergoing heat shock were labeled for 10 min, 1 min after a temperature shift from 28°C to 42°C. Cells were harvested by centrifugation for 5 min in a Beckman microfuge at 9K and were resuspended in two-dimensional gel sample buffer. Two-dimensional gel electrophoresis was done as described previously (O'Farrell, 1975). Proteins were classified according to an alphanumeric system based on isoelectric point and molecular weight similar to that used by Neidhardt and coworkers (Pedersen et al., 1978).

### Enzyme Assays

#### *Catalase/Peroxidase*

Catalase activity in whole cells and in extracts was determined as described previously (Beers and Sizer, 1952). Catalase activity in polyacrylamide gels was localized as described previously (Harris and Hopkinson, 1976), and peroxidase activity was stained using tetramethylbenzidine as the electron acceptor (Liem et al., 1979).

#### *Superoxide Dismutase*

SOD activity in solution was measured as described previously (McCord and Fridovich, 1969), using its ability to compete with ferricytochrome C for enzymatically generated superoxide anion. SOD in nondenaturing polyacrylamide gels (Davis, 1964) was detected as described previously (Beauchamp and Fridovich, 1971). Iron and manganese forms of SOD were distinguished by soaking the gels in hydrogen peroxide prior to activity staining (Clare et al., 1984).

#### *Glutathione Reductase*

Enzyme activity in crude extracts was assayed as described previously (Lopez-Barea and Lee, 1979).

#### *NAD(P)H-Dependent Alkyl Hydroperoxide Reductase*

Cells grown overnight in LB or VBCG were centrifuged and then were resuspended in VBC salts to an $A_{650}$ of 4.0. A stock suspension (0.05%) of cumene hydroperoxide (CHP) was made in VBC salts by brief sonication with the micro-probe of a Branson sonicator. The reaction was initiated by mixing equal volumes of cells and CHP along with enough 40% glucose to bring the final concentration to 0.4%. Aliquots (0.5 ml) were removed at 5, 10, and 30 min and were immediately vortexed with 0.5 ml of HPLC-grade ethyl acetate and 0.2 g NaCl in a 3 ml conical centrifuge tube. Following centrifugation (10 min at 4000 rpm), a 10 μl aliquot was analyzed for CHP and cumene alcohol (CA) using a Supelco LC18 reverse phase HPLC column. Separations were done isocratically using 35% acetonitrile: 65% 10 mM KPi (pH 7.0) at 3 ml/min. Peak areas were determined using a Waters model 440 detector (254 nm) interfaced with an HP 3390A recording integrator.

Enzyme activity in cell-free extracts was determined by a modification of the above procedure. Stock CHP suspension was prepared in 50 mM KPi (pH 7.0) containing 15 mg/ml BSA. The final reaction mixture contained 0.025% (1.6 mM) CHP, 7.5 mg/ml BSA, 5 mM MgCl, 1 mM NADP, 20 mM glucose-6-phosphate, and 1 unit/ml Torula yeast glucose-6-phosphate dehydrogenase (Sigma type XII). Following addition of cell extract to a final concentration of 2 mg/ml, CA production was assayed as described above.

#### *Hexokinase, Lactate Dehydrogenase, and Glucose-6-Phosphate Dehydrogenase*

These enzymes were assayed as controls using the protocols outlined in the Worthington manual (Decker, 1977).

### Mutagenesis with Diethylsulfate

S. typhimurium LT2 or Escherichia coli K12 were grown overnight in NB to $2 \times 10^9$ cells/ml and were then diluted in 10 ml of VBC salts to $1 \times 10^8$ cells/ml. Then, 0.1 ml of diethylsulfate was added to the 10 ml culture, and the tube was vortexed vigorously for 20 sec. After standing at room temperature for 20 min, 1.0 ml of the mutagenized culture was withdrawn from the top of the tube and used to inoculate 10 ml of nutrient broth. This culture was grown for 6 hr at 37°C and was then plated on minimal glucose in the presence of 300 μM hydrogen peroxide.

### Isolation of a Tn*5* Insertion Mutation Linked to *oxyR1*

One milliliter of log-phase culture of LT2 in LB broth was mixed with $1 \times 10^9$ pfu of a tail-dependent P22 bacteriophage containing a Tn*5* insertion in the *sieA* gene of P22 and a deletion (Δ3Ap68tpfr 251) that

removes both *att* and *int* (Miriam Susskind, unpublished data). The mixture was allowed to stand at 37°C for 1 hr. Cells were then concentrated, resuspended in saline, and spread onto LB plates containing 75 μg/ml kanamycin. 18,000 kanamycin-resistant colonies were pooled together in LB kanamycin. P22 HT105 int-201 was grown on the pool of kanamycin-resistant colonies.

LT2 was transduced to kanamycin-resistance using the pooled lysate at a multiplicity of infection of 0.5, such that roughly 500 transductants/plate were obtained. After 24 hr at 37°C, the plates were replica-plated onto LB plates containing 500 μM cumene hydroperoxide. Colonies that grew on the cumene hydroperoxide-containing plates after 24 hr were purified and tested for *oxyR1* phenotypes. Apparent positives were then tested for linkage to *oxyR1* in TA4100.

### Hfr Mapping

The Tn*5* insertion mutation near *oxyR1* was transduced into a series of auxotrophic mutants spanning the S. typhimurium genetic map. Cultures of the Tn*5* auxotrophs were grown in NB to stationary phase and were mixed with log-phase cultures of HfrK4. The mixtures were diluted in VBC salts and were plated immediately on minimal glucose to select for prototrophic recombinants. Prototrophs were purified and scored for kanamycin-resistance.

### Acknowledgments

The authors would like to thank Helmut Sies and Giovanna Ferro-Luzzi Ames for critical reviews of the manuscript. This work was supported by National Institutes of Health (NIH) grant GM19993 to B. N. A. and by National Institute of Environmental Health Sciences Center Grant ES01896. M. F. C. was supported by NIH training grant GM07232, R. W. M. was supported by NIH postdoctoral fellowship GM08864, and F. S. J. was supported by NIH postdoctoral fellowship ES05237.

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Received March 1, 1985; revised April 16, 1985

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