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A Global Response Induced in *Escherichia coli* by Redox-Cycling Agents Overlaps with That Induced by Peroxide Stress

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***Escherichia coli* treated with nontoxic levels of the superoxide-generating redox-cycling agents menadione and paraquat showed dramatic changes in protein composition as monitored by two-dimensional gel analysis. The distribution of proteins synthesized after treatment with these agents overlapped significantly with that seen after hydrogen peroxide treatment, and it included all the proteins in the *oxyR* regulon. The redox-cycling agents also elicited the synthesis of at least 33 other proteins that were not seen with hydrogen peroxide, including three heat shock proteins, the Mn-containing superoxide dismutase, the DNA repair protein endonuclease IV, and glucose-6-phosphate dehydrogenase. At least some of these redox-inducible proteins appear to be part of a specific response to intracellular superoxide. *E. coli* is thus equipped with a network of inducible responses against oxidative damage, controlled in multiple regulatory pathways.**

Many organisms are able to survive harmful changes in the environment by elevating the levels of specific cellular proteins. These responses have been observed for such disturbances as heat shock (30), nutrient starvation (5), and treatment with DNA-damaging agents (9, 41). The enteric bacteria *Escherichia coli* and *Salmonella typhimurium* can stimulate the production of enzymes that scavenge the superoxide radical (O_2^-) or various peroxides of DNA repair enzymes and of other proteins that mitigate the toxic effects of oxidative mutagens (6, 7, 10, 11, 15). These protective inductions represent adaptive responses to oxidative stress because they are triggered by nontoxic levels of oxidizing agents and protect cells against a subsequent challenge with otherwise lethal levels of those oxidants.

Thus far, only the bacterial *oxyR* gene has been identified as a regulator of acquired resistance to oxidative stress (7, 29). The *oxyR* gene is required for the expression of a portion of the many stress proteins that are induced in response to sublethal levels of hydrogen peroxide (7). The proteins under *oxyR* control include the scavenging enzymes catalase-hydroperoxidase I (HPI; encoded by the *katG* gene) and an alkylhydroperoxide reductase (Ahp; encoded by the *ahp* operon) (7, 19, 29). In addition to providing inducible protection against exogenous hydrogen peroxide and other agents, the *oxyR*-dependent induction of these peroxide-scavenging enzymes also limits spontaneous mutagenesis under aerobic conditions (13, 38). Since some groups of these inducible polypeptides appear to be products of the same gene or operon (13, 29), *oxyR* probably regulates 10 or fewer different promoters.

The *E. coli* $oxyR^+$ gene is also required for normal resistance to the redox-cycling drugs paraquat (PQ) and menadione (MD) (13). The mechanism of action of these drugs involves the one-electron reduction of MD and PQ at the expense of NADPH or NADH, followed by one-electron oxidation of the reduced forms by O_2, generating O_2^- (17, 22). Because MD and PQ can be repeatedly reduced and oxidized to form a flux of superoxide, they are called redox-cycling drugs. The O_2^- produced by these drugs can be converted to H_2O_2 by nonenzymatic disproportionation or by superoxide dismutases (12). The sensitivity of Δ*oxyR* mutant cells to MD and PQ, as well as to the thiol reagent *N*-ethylmaleimide (NEM), is suppressed by constitutively high levels of HPI or Ahp (13), which indicates that intracellular peroxides are critical in mediating cell killing by these agents.

It thus seemed likely that redox-cycling agents would elicit changes in cell physiology similar to those made by direct exposure to H_2O_2. However, redox-cycling drugs might also generate signals for novel inductions that are not triggered by H_2O_2. Such signals could arise, for example, from the flux of superoxide caused by these drugs (17, 22) or from the possible depletion of cellular NADPH or NADH pools. Indeed, manganese-containing superoxide dismutase (2, 39) and the DNA repair enzyme endonuclease IV (6) are induced by MD or PQ but not by H_2O_2. This work examines the nature of the cellular response of *E. coli* to redox-cycling agents that generate the oxygen radical superoxide and the overlap of this inducible system (11, 15) with that switched on by hydrogen peroxide (7, 10, 29, 40).

MATERIALS AND METHODS

Bacterial strains and growth media. *E. coli* K-12 JTG100 has been described previously (13). Bacteria were grown, unless noted otherwise, at 37°C with shaking at 200 rpm in M9 medium (27) supplemented with 0.4% glucose, 0.1% casamino acids, 0.1 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 1 μg of thiamine hydrochloride per ml.

Chemicals. H_2O_2 (30%) was purchased from Fluka Chemical Corp., Hauppage, N.Y. [^{35}S]methionine (1,072 Ci/mmol) was from Dupont, NEN Research Products, Boston, Mass. PQ, MD, and NEM were from Sigma Chemical Co., St. Louis, Mo.

Labeling of bacteria and two-dimensional gel analysis. Bacteria were labeled with [^{35}S]methionine as previously described (13). For the heat treatment experiment, cells were shifted from 28 to 50°C and labeled for 30 min. For other treatments, cells were usually labeled for 20 min after the addition of the indicated agent. Extracts were prepared, and gels were run as described by Phillips (32). Equal amounts of trichloroacetic acid-precipitable radioactivity were always loaded on the gels for control samples and the

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corresponding treated samples. Molecular weight standards were as follows: phosphorylase B, 92,000; bovine serum albumin, 66,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,000. The positions of the *oxyR*-induced proteins were deduced by comparing gel patterns of MD-treated bacteria that harbored the *oxyR*$^+$, Δ*oxyR*, and *oxyR2* (*oxyR*-constitutive) genes (7). The identities of the heat shock proteins were deduced from their reported gel positions (30) and from a gel of heat-shocked bacteria. Polypeptide 40 is the 50S ribosomal subunit L12, as deduced from a reference gel (31).

Adaptation of bacteria by MD treatment. Early-log-phase bacteria (2×10^7 cells/ml) cultured at 37°C with shaking at 200 rpm were pretreated with 1.45 mM MD for 1 h. The bacteria were pelleted, washed with M9 salts (27), suspended in prewarmed M9 salts containing 0.4% glucose, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$, and challenged for 30 min at 37°C with various amounts of MD in the presence of 50 μM $CuCl_2$. Samples were diluted and plated on LB agar. $CuCl_2$ alone did not affect cell viability in the absence of MD (data not shown). Since MD was not bactericidal towards *E. coli* during transient exposures in the absence of added metals (data not shown), the transition metal $CuCl_2$ was included during high-level MD treatments. Such metals are known to enhance the toxicity of the redox-cycling agent PQ, presumably by enhancing the production of OH · (25).

Preparation of crude extracts and enzyme assays. Bacteria grown to an optical density at 600 nm of 0.3 were aliquotted into 250-ml flasks, each with 50 ml of culture, and treated with various oxidants for 45 min. The cultures were then chilled and centrifuged at 10,000 × *g* for 10 min, and the pellets were washed with 1 ml of M9 salts and frozen at −80°C. The frozen pellets were thawed on ice for 1 h, suspended in 1 ml of buffer containing 50 mM Tris hydrochloride (pH 7.5) and 0.2 M NaCl, and passed twice through a French press at 12,000 lb/in^2. After centrifugation at 14,500 × *g* for 45 min, the supernatants were collected. Glucose-6-phosphate dehydrogenase (G6PD; 1) and catalase (13) were assayed as described previously.

RESULTS

Most H_2O_2-inducible proteins are inducible by redox-cycling agents. Large-scale physiological changes can be probed by monitoring the induction of individual polypeptides by two-dimensional gel electrophoresis after cell treatment with different oxidants. Figure 1 shows such an experiment with MD. A summary of the proteins that were elevated by different levels of MD, PQ, H_2O_2, or NEM is shown in Fig. 2. Treatment with H_2O_2, MD, or PQ was repeated with at least three different strains, while treatment with NEM was done only with JTG100 (see legend to Fig. 2). (The alphanumeric designations used for individual polypeptides in Fig. 2 are given only for cross-referencing to Fig. 1, except for the heat shock proteins and RecA, which were assigned in a standardized scheme by Neidhardt et al. [30, 31].) Of the conditions employed, only the higher H_2O_2 concentration and the NEM treatment caused significant growth inhibition (see below).

Several striking features are evident from the analysis of these two-dimensional gels. Both MD and PQ stimulated the synthesis of most of the proteins that were elevated by H_2O_2, including all of those under the control of *oxyR* (Fig. 1 and 2). NEM seems also to produce a significant amount of its toxicity by generating cellular peroxides (13), but this compound stimulated the synthesis of only some of the *oxyR*-controlled proteins. Various protein spots exhibited diminished intensity after treatment with the oxidants, but it is unclear whether these represent specific responses and are not further considered here.

The induction by all of these agents of proteins in the *oxyR* regulon was dependent upon the *oxyR*$^+$ gene, since Δ*oxyR* mutants treated with H_2O_2, MD, PQ, or NEM did not show induction of any proteins in the *oxyR* regulon (data not shown). These results extend the previous observations of elevated catalase activity after treatment of *E. coli* with MD (16) or PQ (20). These overlapping patterns of protein induction make it likely that the various oxidants used here produce common gene-activating signals via the intracellular formation of H_2O_2 (13).

The intracellular production of H_2O_2 by PQ or MD is apparently not strictly dependent on enzymatic disproportionation of superoxide. Both bacterial superoxide dismutases can be removed (in an *E. coli sodA sodB* mutant [4]) without preventing the induction of the proteins of the *oxyR* regulon by PQ or MD. For example, treatment of a *sodA sodB* mutant with 0.145 or 1.45 mM MD resulted in an approximately twofold or fivefold induction, respectively, of the HPI polypeptides. The *sod*$^+$ parent induced HPI approximately fivefold in response to the lower amount of MD.

Redox-cycling agents stimulate the synthesis of many proteins not elicited by H_2O_2. The proteins induced by redox-cycling agents can be only partly explained by hydrogen peroxide generation. MD and PQ induced 33 polypeptides that were not elicited by H_2O_2, including three heat shock proteins (C41.7, C62.5, and GroES) and the DNA repair enzyme endonuclease IV (6), which acts on oxidative damages in DNA (26). The identities of the heat shock proteins were confirmed by examining gels from bacteria shifted from 28 to 50°C. The role of the *rpoH* gene in the induction of heat shock proteins by MD and PQ was not investigated here. As reported by others (16), the level of the *sodA* Mn-superoxide dismutase (SodA) was also elevated by MD or PQ (data not shown). The SodA protein was not resolved in the gel system shown in Fig. 1, but substantial induction of this protein was easily detected when a broader isoelectric gradient (37) was employed (data not shown).

The treatments with different levels of a given oxidant generally elicited qualitatively similar patterns of protein induction, but the higher-level treatments sometimes resulted in the production of additional proteins (Fig. 2) or induced some species to still higher levels (data not shown). In addition, H_2O_2, MD, and PQ each induced a few unidentified proteins unique to the agent (Fig. 2). The identities of most of the polypeptides induced in response to these various stresses have not yet been determined.

Enzyme activities induced by redox-cycling agents. As reported previously by others, the cellular levels of active SodA (16) and endonuclease IV (6) were elevated three- to sixfold in PQ- or MD-treated *E. coli* (data not shown). We also confirmed and extended the report of Kao and Hassan (21) that the level of the enzyme glucose-6-phosphate dehydrogenase (G6PD) is elevated by PQ treatment. The G6PD level was increased twofold by 1.3 mM PQ and more than threefold by 1.45 mM MD but <1.2-fold by 60 μM H_2O_2 in wild-type cells grown in supplemented M9 medium (Table 1). A fivefold elevation of G6PD by PQ was seen in extracts from cells treated in LB medium (data not shown). Catalase activity in the same extracts (Table 1) was regulated differently, being induced more than twofold by H_2O_2, as well as more than twofold by PQ and eightfold by MD as expected from the two-dimensional analysis (Fig. 1 and 2).

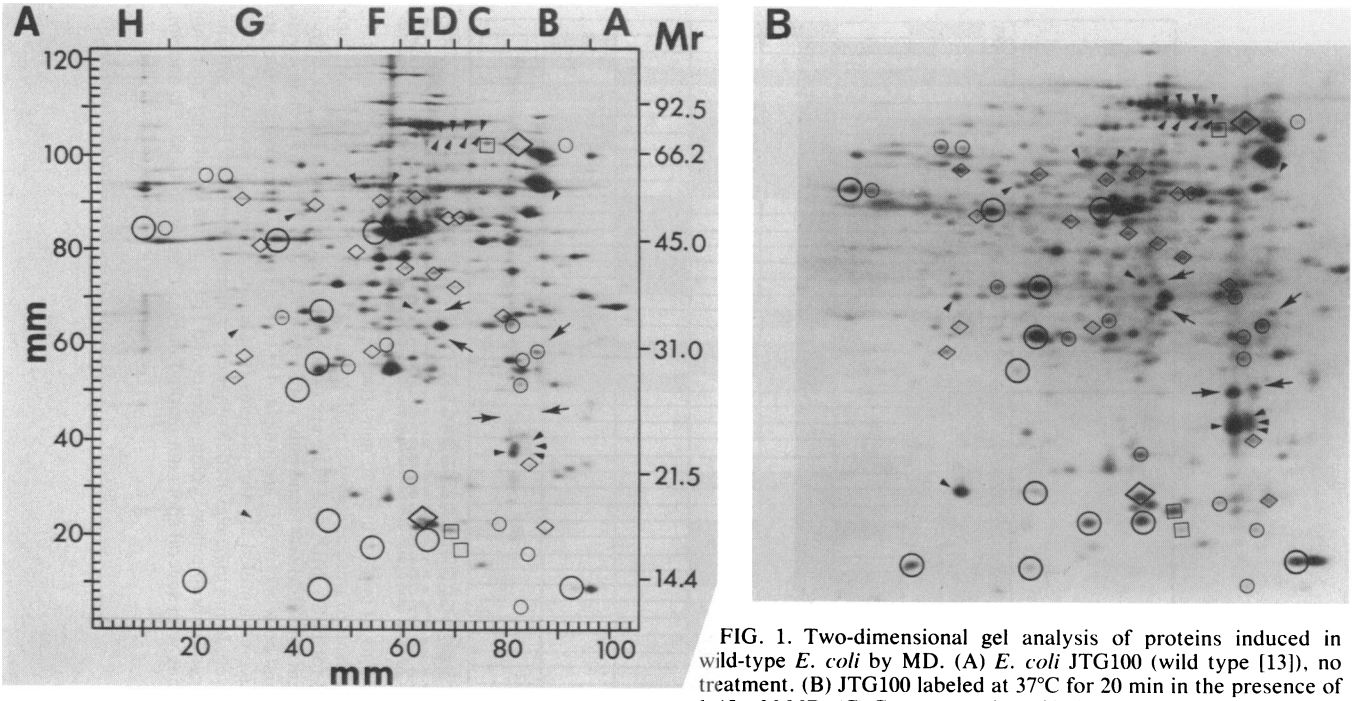

FIG. 1. Two-dimensional gel analysis of proteins induced in wild-type *E. coli* by MD. (A) *E. coli* JTG100 (wild type [13]), no treatment. (B) JTG100 labeled at 37°C for 20 min in the presence of 1.45 mM MD. (C) Cartoon tracing of induced proteins. Arrowheads indicate proteins under *oxyR* control. Symbols: ○, locations of *oxyR*-independent proteins that are inducible by H_2O_2 and redox-cycling agents; ◇, proteins that are inducible by the redox-cycling agents but not efficiently inducible by H_2O_2; □, proteins that are reported to be under the control of the heat shock regulatory gene *rpoH* (30). Arrows indicate proteins that are inducible by MD only. M_rs (in thousands) are located on the sides of panels A and C. The pH gradient runs in the horizontal direction, with acidic pH on the right and the letters at the top given in accordance with nomenclature of Neidhardt and co-workers (31). Polypeptides 1 through 8 are HPI subunits (13). Polypeptides 9, 10, and 14 are Ahp subunits (13). Polypeptides 11, 12, and 13 may also be Ahp subunits, since mutants that overproduce Ahp contain these same polypeptides (13; data not shown). Polypeptide 57 is endonuclease IV, as confirmed by using purified endonuclease IV (26) added to a labeled extract and by the specific overproduction of this same species in bacteria containing the *nfo* gene on a multicopy plasmid (8).

The inducible G6PD activity was the product of the *zwf* gene because strains containing a *zwf*::*lacZ* transcriptional fusion (kindly supplied by R. E. Wolf, Jr.) exhibited PQ-inducible β-galactosidase activity (data not shown). The position of G6PD has not been determined on the two-dimensional gels used in this study.

Inducible resistance to MD. The levels of most of the oxidants used in this work did not cause any decrease in cell growth or in the survival of CFUs, with the exception of NEM and the higher level of H_2O_2 (Fig. 3). Thus, the protein inductions observed here occurred largely under conditions compatible with cell viability, which was consistent with their interpretation as adaptive responses against continued or still higher levels of stress. Indeed, the pretreatment of bacteria with 1.45 mM MD conferred substantial resistance to a transient challenge with up to 100 mM MD in the presence of 50 μM $CuCl_2$ (Fig. 4). An adaptive effect has also been reported for the redox-cycling agents plumbagin (11) and PQ (25).

DISCUSSION

The responses to oxidative stress in *E. coli* are complex and probably involve several intracellular inducing signals. The induction of the *oxyR* regulon by MD and PQ is likely an indirect result of superoxide radical formation. The O_2^- formed by redox cycling can react with transition metals or superoxide dismutase to form H_2O_2 (and subsequently · OH) by Fenton (and Haber-Weiss) chemistry (12, 18). It is not known whether H_2O_2 itself, rather than a product of its action, is the direct activator of the OxyR regulatory protein.

PQ and MD are known to produce O_2^- efficiently in *E. coli* (15, 16). These agents induced a set of activities including SodA, endonuclease IV, and G6PD that were not elevated by H_2O_2 treatment of wild-type *E. coli*. The signal for the

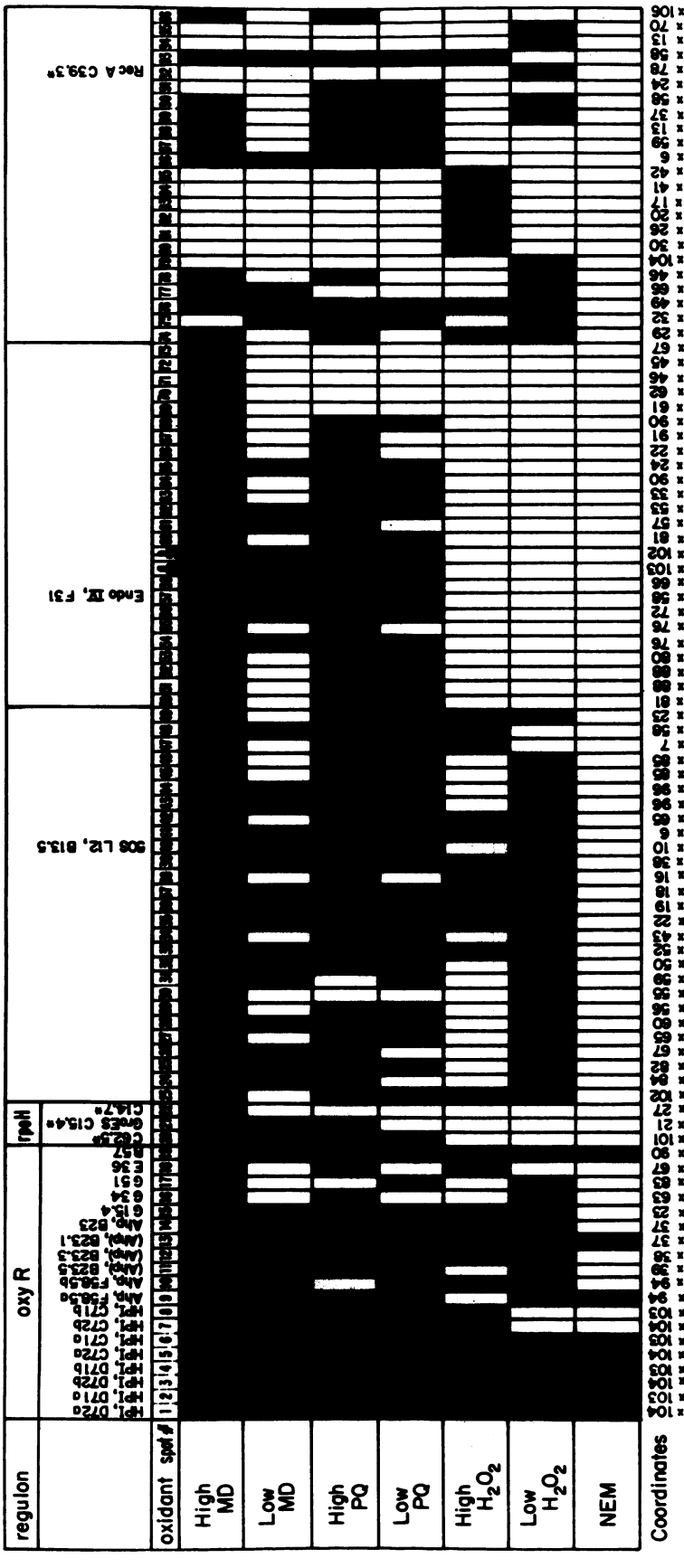

FIG. 2. Summary of induction of polypeptides by different agents. ■, Induction. Cells were pulse-labeled for 0 to 20 min or 20 to 40 min after treatment with the indicated oxidant, except for the NEM and the 2 mM H_2O_2 exposures, which were for only 0 to 20 min. Concentrations of the oxidants used were as follows: high MD, 1.45 mM; low MD, 0.145 mM; high PQ, 1.3 mM; low PQ, 0.13 mM; high H_2O_2, 2 mM; low H_2O_2, 0.06 mM; NEM, 0.04 mM. Induction experiments for H_2O_2, MD, and PQ were done with strain JTG100 (Fig. 1), as well as with at least two of the following strains: AB1157 (10), JTG102 (13), and GC4468 (4). The induction experiment for NEM was done only with strain JTG100. Induced proteins were estimated visually to be increased at least twofold. Proteins identified on a reference gel supplied by Neidhardt and co-workers (31) are indicated by an asterisk. Other designations are solely for cross-referencing with Fig. 1 and are not necessarily generally applicable. Spots 11 to 13 were tentatively identified as Ahp proteins (see legend to Fig. 1). Endo IV, DNA endonuclease IV; 50S L12, 50S ribosomal subunit protein L12. Spots 1 to 73 are indicated in Fig. 1; spots 74 to 96 were found to be elevated in other experiments. Spots 1 to 19 are proteins in the *oxyR* regulon. Spots 20 to 22 are proteins in the *rpoH* regulon. Spots 23 to 49 are proteins that were induced by both redox-cycling agents and H_2O_2, along with other spots not marked in Fig. 1 (spots 74 to 78, 88 to 90, and 93). Spots 50 to 73 were induced only by redox-cycling agents, along with several spots not marked in Fig. 1 (spots 86 to 88, 91, and 96).

TABLE 1. Enzyme levels in extracts of JTG100 cells treated with oxidants

Agent[a]	Enzyme activities (U/mg)[b] of:	
	G6PD	Catalase
None	0.12 ± 0.04	3.0 ± 1.5
H_2O_2	0.14 ± 0.05	6.8 ± 1.3
MD	0.39 ± 0.09	25 ± 7.4
PQ	0.22 ± 0.07	7.4 ± 3.4

[a] The treatments were with 60 μM H_2O_2, 1.45 mM MD, or 1.30 mM PQ.
[b] Each number represents the average of three experiments plus or minus standard deviation.

induction of these proteins and probably others may depend on the formation of intracellular superoxide rather than H_2O_2. Thus, the inductions of SodA, endonuclease IV, and G6PD by PQ and MD depend on O_2 (15; unpublished observations), as do the PQ-inducible *soi*::*lacZ* gene fusions (24). The SodA protein and the *soi* genes are also moderately inducible by increased oxygen tensions in *sod*$^+$ *E. coli* (14, 24). In addition, bacteria lacking all superoxide dismutase activity (*sodA sodB*) have elevated levels of a number of the oxidative stress proteins only when cultured aerobically (unpublished observations). These same superoxide dismutase-deficient mutants also exhibit oxygen-inducible endonuclease IV activity (6). Taken together, these observations point to a group of proteins whose synthesis is stimulated in response to elevations of the intracellular O_2^- concentration.

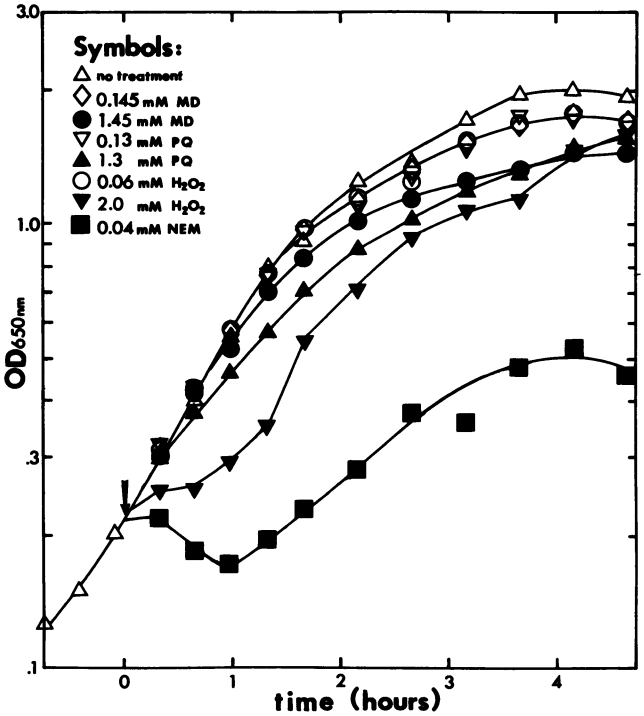

FIG. 3. Growth of cells in the presence of different oxidants. Exponentially growing bacteria were cultured at 37°C with shaking at 200 rpm in supplemented M9 medium, and at time zero (arrow) different oxidants were added. Cell growth was monitored by measuring the optical densities of the cultures. Essentially the same results were obtained by plating samples for colony-forming ability (data not shown).

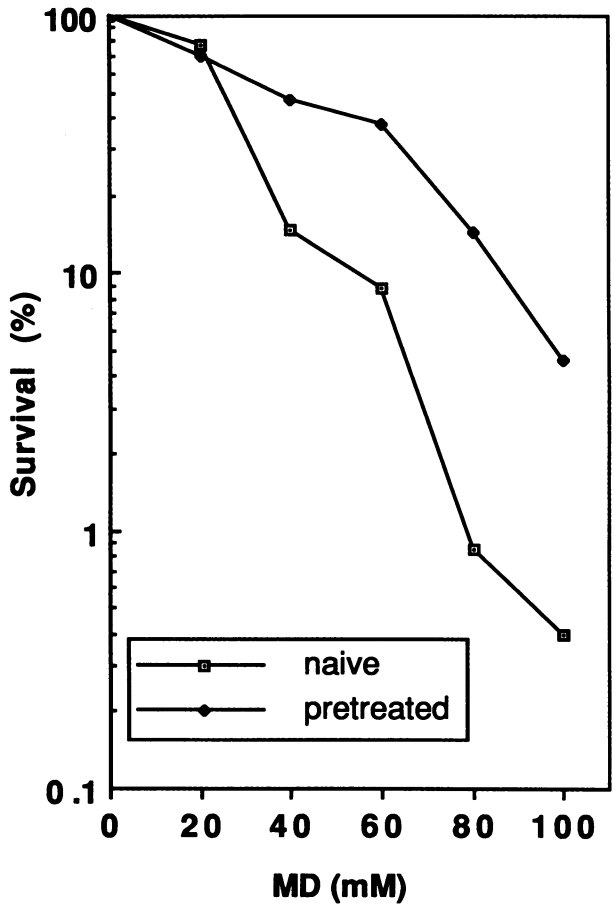

FIG. 4. Induction of bacterial resistance to MD. Bacteria, either naive or pretreated with 1.45 mM MD, were challenged as described in Materials and Methods.

The level of SodA is sensitive to oxygen tension (14, 39) and the addition to anaerobic cells of metal-chelating agents (28, 34), NO_3^- or NO_3^- plus PQ (33), as well as a variety of other compounds that change the cellular redox potential (36). At present, it is not clear whether all these inductions are controlled by the same regulatory mechanism. Other proteins induced by redox-cycling agents such as endonuclease IV (6) may not be as sensitive to these treatments as SodA. For example, the levels of catalases, endonuclease IV, and G6PD were unaffected by NO_3^- treatment of anaerobic cells (unpublished observations). Furthermore, the pattern of proteins induced by NO_3^- under anaerobic conditions (35) is different from the pattern induced by MD or PQ (Fig. 2). Once genes that affect the regulation of *sodA* and other oxidative stress proteins are identified, the regulatory mechanisms can be more fully explored.

Conditional resistances to oxidative stresses in *E. coli* have been described previously, one elicited by H_2O_2 (10) and another activated by superoxide-generating agents (11, 15, 25). It is now clear that these responses are not entirely separate; a partial model for how they are connected is shown in Fig. 5. Both H_2O_2 and redox-cycling agents (by leading to H_2O_2 formation) can activate the *oxyR* regulon to provide cellular protection against both types of oxidants (13). Both types of agent induce many of the same *oxyR*-independent proteins. However, redox-cycling drugs induce an additional global response in *E. coli* that is detectable as

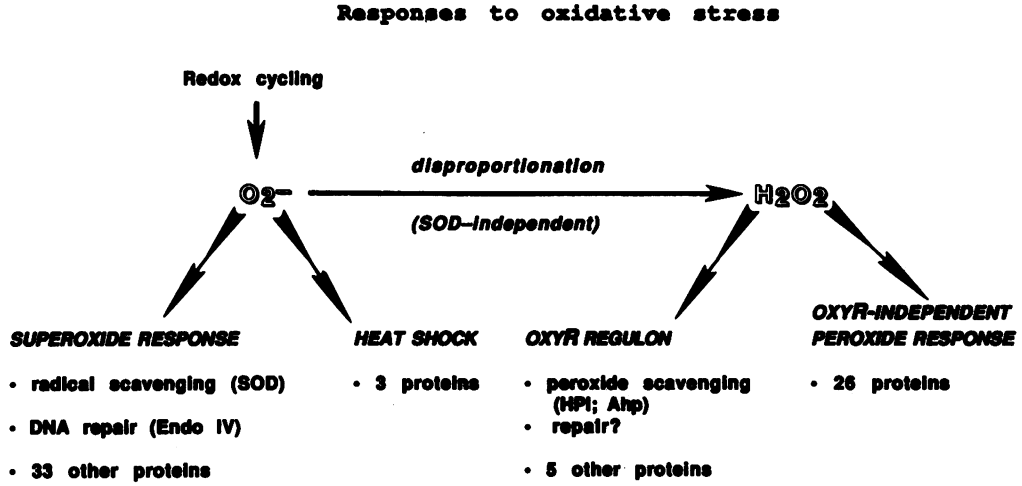

FIG. 5. Model for the cellular responses to oxidative stress. Superoxide produced by redox cycling can be converted to H_2O_2, inducing the *oxyR* regulon and many *oxyR*-independent proteins that are also elevated by H_2O_2 directly. Superoxide generation triggers the synthesis of a variety of additional proteins, including those that confer increased cellular capacity to scavenge radicals (superoxide dismutase [SOD]) and repair oxidized DNA (endonuclease IV [Endo IV]). The arrows do not necessarily imply that O_2^- or H_2O_2 are the direct signals for the induction of all the proteins. Some of the proteins induced by redox cycling could be triggered by effects such as NADPH depletion, but such separate groups are not distinguished here. These global patterns of induction and the limited genetic studies performed thus far indicate the existence of multiple regulators that respond to low-level oxidative stress to provide adaptive protection. Not all proteins observed in Fig. 2 are accounted for in this scheme. Only proteins that were induced at least threefold were included in the different categories. In a few cases where proteins were seen to be induced weakly by H_2O_2 but strongly by O_2^- (e.g., oxidant spots 39, and 43 to 46), those proteins are considered to be in the superoxide-induced category.

major changes in the production of many proteins. At least some of these proteins are increased in response to intracellular O_2^-, as discussed above. Since the reduction of PQ and MD may occur at the expense of cellular reducing equivalents such as NADPH (17, 22), the induction of still others of these proteins might be triggered, for example, by the depletion of the NADPH pool.

The elevated production of the redox-inducible proteins aids cells in overcoming the lethal effects of high superoxide challenges, independent of the H_2O_2 response. For example, the *sodA*-encoded superoxide dismutase, which is inducible by MD and PQ but not by H_2O_2, accounts for much of the cellular resistance to MD (unpublished observations) or PQ (4). This is also true for G6PD, since mutants lacking this enzyme are hypersensitive to MD (unpublished observation). G6PD is probably responsible for providing NADPH for enzymes such as Ahp (19) and glutathione reductase. We have recently isolated *E. coli* mutants that express elevated levels of SodA, endonuclease IV, and G6PD, along with several other proteins that usually require redox-cycling agents for their induction (J. T. Greenberg, P. Monach, and B. Demple, manuscript in preparation). These mutants have an increased resistance to MD and other agents that is only partly dependent on the *sodA* and *nfo* genes. Characterization of these new mutants will aid our dissection of the complex of oxidation stress-inducible regulons of *E. coli* and the different intracellular signals that activate them.

Inducible responses to oxidative stress may be general protective mechanisms that are not restricted to bacteria. Mn-superoxide dismutase is inducible in plants by a variety of stresses (3). Mn-superoxide dismutase mRNA is inducible by tumor necrosis factor alpha in human cells; thus, Mn-superoxide dismutase may be responsible for resistance to radiation induced by tumor necrosis factor alpha (42). Human skin fibroblasts induce heme oxygenase in response to challenges with near-UV light, MD, or H_2O_2 (23). This induction could serve to protect cells against high levels of oxidative stress by increasing the cellular levels of the antioxidant metabolite of heme, bilirubin (23). Human lymphocytes possess an adaptive response to [^{3}H]thymidine or X rays which involves the induction of specific proteins (S. Wolff, J. K. Wiencke, V. Afzal, J. Youngblom, and F. Cortes, *Proceedings of the 14th L. H. Gray Conference on Low Dose Radiation Risk Assessment*, in press). Most of the proteins induced by these latter treatments have not yet been identified. Whether these eucaryotic oxidation stress responses include proteins with functions analogous to those seen in *E. coli* awaits further work.

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