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Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins

(oxidative damage/*Escherichia coli*/catalase)

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ABSTRACT **Hydrogen peroxide treatment induces the synthesis of 30 proteins in *Salmonella typhimurium*. Five of these proteins are also induced by heat shock, including the highly conserved DnaK protein. The induction of one of these five proteins by heat shock is dependent on *oxyR*, a positive regulator of hydrogen peroxide-inducible genes, while the induction of the other four by heat shock is *oxyR* independent. Five of the 30 hydrogen peroxide-inducible proteins have been identified, and their structural genes have been mapped. Other stresses such as nalidixic acid, ethanol, or cumene hydroperoxide treatment also induce subsets of the 30 hydrogen peroxide-inducible proteins as well as additional proteins. Hydrogen peroxide-inducible proteins are shown to be largely different from those proteins induced by aerobiosis. In addition, the expression of the *katG* (catalase) gene is shown to be regulated by *oxyR* at the level of mRNA.**

All aerobic organisms must cope with reactive oxygen species such as superoxide anion, hydrogen peroxide, and the hydroxyl radical, which are formed in respiring cells as incomplete reduction products of molecular oxygen, and with singlet oxygen, which is formed photochemically (1–3). These reactive oxygen species can oxidize membrane fatty acids, thus initiating lipid peroxidation (4), can oxidize proteins (5), and can damage DNA (6, 7). Enteric bacteria have several enzymes that may protect cells from oxidative damage including superoxide dismutase and catalase (1). In addition, DNA repair enzymes such as exonuclease III (8), DNA polymerase I (9), RecBC nuclease (10), and the RecA protein (11) appear to be important in repairing DNA lesions resulting from oxidative damage. The evolution of these defense mechanisms that prevent or repair oxidative damage was probably critical to the survival of aerobic life forms (1). We have described a global response in enteric bacteria that is induced in response to oxidative stress (12).

In this response, *Salmonella typhimurium* becomes resistant to killing by hydrogen peroxide when pretreated with a nonlethal level of hydrogen peroxide (12). This adaptation occurs under conditions that are similar to those first described for *Escherichia coli* (13). We have shown that the adaptation results in the transient accumulation of a distinct group of proteins that are visible on two-dimensional (2D) protein gels (12). The induction of nine of the proteins is under positive control by the *oxyR* gene product in both *Salmonella* and *E. coli* (12). Three of the nine *oxyR*-dependent proteins are also heat inducible (12).

This paper presents additional information regarding regulation of the adaptation to oxidative stress. Proteins induced by hydrogen peroxide are found to overlap significantly with those induced by other stresses such as heat shock, as well as ethanol and nalidixic acid treatments. The significance of the overlaps among the different stress responses is discussed.

MATERIALS AND METHODS

Bacterial Strains and Labeling of Proteins. *S. typhimurium* LT2 and *E. coli* K12 were the wild-type strains used. Overnight cultures in VBC salts (14) supplemented with 0.4% glucose (VBC/glucose) at 28°C or 37°C were diluted, incubated, and then labeled during exponential growth (OD_{650} of 0.2–0.4). The labeling reactions were as described (12): 0.4-ml aliquots of the cultures were grown in VBC/glucose containing L-[^{35}S]methionine at 200 μCi/ml (1 Ci = 37 GBq; Dupont-NEN Products) and unlabeled L-methionine such that the final L-methionine concentration was 10 μM. Cultures were labeled for the designated period of time, and the labeling reaction was terminated by the addition of 10 μl of 0.1 M L-methionine. Cells were harvested by centrifugation for 5 min in a Fisher microfuge and resuspended in 2D gel electrophoresis sample buffer (15).

Conditions for Labeling Proteins During Stresses. Adaptation to hydrogen peroxide and temperature shift experiments were done as described (12). For ethanol treatment, cells were labeled for 30 min immediately following exposure to 4% (vol/vol) ethanol at 28°C. For nalidixic acid treatment, cells were labeled for 12 min immediately following exposure to nalidixic acid (60 μg/ml) at 37°C. Cells were treated with 125 μM cumene hydroperoxide at 37°C for 60 min. To compare cellular proteins synthesized during anoxic and aerobic growth conditions, cultures were grown at 37°C in VBC/glucose medium in tubes that were constantly bubbled with either air (aerobic) or 95% N_2/5% CO_2 (anoxic).

2D Gel Electrophoresis. 2D gel electrophoresis was done as described by O'Farrell (15). Proteins were classified using an alphanumeric system similar to that used by Neidhardt and coworkers (16). The numerical portion of each designation indicates the approximate molecular weight of the protein, and the letter portion indicates the approximate isoelectric point, as follows: C, <5.10; D, 5.10–5.59; E, 5.59–5.74; F, 5.74–6.18; G, >6.18 (Figs. 1 and 2).

RNA Isolation and Dot-Blot Hybridization. Total RNA was isolated from 5-ml aliquots withdrawn from exponentially growing cultures of LT2 and TA4100 (*oxyR1*) in VBC/glucose and Luria broth (LB). The cells were disrupted by phenol extraction, and the RNA was isolated as described (17). The RNA pellets were resuspended in 10 μl of distilled

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Abbreviation: 2D, two-dimensional.

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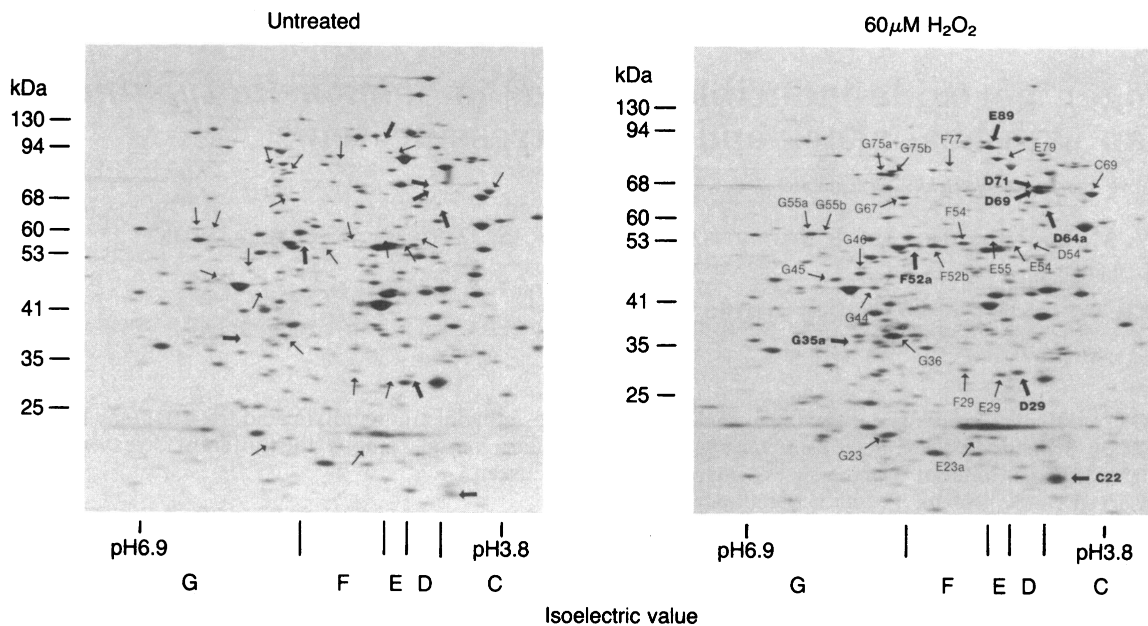

FIG. 1. Hydrogen peroxide-inducible proteins in *Salmonella*. Exponentially growing cells were labeled with L-[^{35}S]methionine for 60 min immediately following exposure to 60 μM hydrogen peroxide. Cells were then lysed and electrophoresed on an equilibrium isoelectric focusing gel followed by a 10% polyacrylamide/NaDodSO$_4$ gel as described (12). Only 29 proteins are visible in these gels. *oxyR*-regulated protein G18 is not visible on 10% polyacrylamide/NaDodSO$_4$ gels. Molecular sizes in kDa are indicated at the left. Values of isoelectric points are across the bottom. G, F, E, D, and C refer to the alphanumeric system used.

water. Aliquots (1 μl) of undiluted, 1:5, 1:10, and 1:50 diluted samples were blotted onto nitrocellulose filters preequilibrated in 20× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The filters were hybridized with 10^6 cpm of nick-translated (18) plasmid DNA containing the *katG* (catalase) gene on a 3.6-kilobase *Hin*dIII fragment (P. Loewen, personal communication), or with 10^6 cpm of a nick-translated 2.5-kilobase *Bgl* II/*Pst* I fragment containing the *hisJ* gene (19).

RESULTS

Hydrogen Peroxide-Inducible Proteins. Treatment of exponentially growing *Salmonella* cells with 60 μM hydrogen peroxide results in the transient induction of 30 proteins (Fig. 1). The nine proteins that are positively regulated by the *oxyR* gene (12) are marked with bold arrows, and the 21 *oxyR*-independent proteins are marked with light arrows in Fig. 1. Five of the 30 proteins have been identified (Table 1).

Proteins D69 and D71 are products of the *Salmonella katG* gene. In *E. coli katG* maps near *argH* at 89.2 minutes and represents the structural gene for the hydrogen peroxide-inducible catalase (20). We have obtained several deletions of the *katG* gene in *Salmonella* (e.g., TA4113) by selecting for excision of a *Tn10* in the *argH* gene. The deletions eliminate proteins D69 and D71 from 2D gels, and also eliminate the two hydrogen peroxide-inducible catalase bands (HPI and

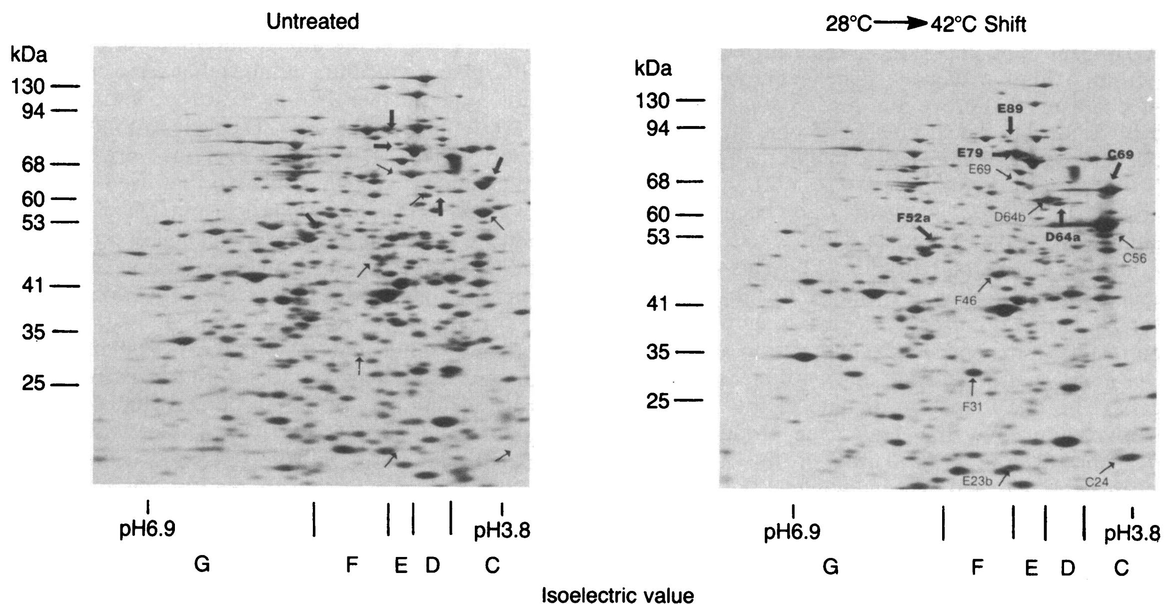

FIG. 2. Heat shock proteins in *Salmonella*. Exponentially growing cells were labeled with L-[^{35}S]methionine for 10 min immediately following a 28°C to 42°C shift. Cells were then lysed and run on an equilibrium isoelectric focusing gel followed by 10% polyacrylamide/NaDodSO$_4$ gel as described (12). Molecular sizes in kDa are indicated at the left. Values of isoelectric focusing points are across the bottom. G, F, E, D, and C refer to the alphanumeric system used.

Table 1. Hydrogen peroxide-inducible proteins that have been identified

Protein	Gene	Activity	Approximate map position	*oxyR* regulation	Mutant phenotype
D69	*katG*	Catalase	88 minutes	+	H_2O_2 hypersensitive
D71	*katG*	Catalase	88 minutes	+	H_2O_2 hypersensitive
F52a	*ahp*	Alkyl hydroperoxide reductase	13 minutes	+	Alkyl hydroperoxide hypersensitive
C22	*ahp*	Alkyl hydroperoxide reductase	13 minutes	+	Alkyl hydroperoxide hypersensitive
C69	*dnaK**	DNA biosynthesis	0 minutes	−	Death

+, uninducible in deletions of *oxyR*. −, inducible in deletions of *oxyR*.
*Mutants isolated only in *E. coli* (21).

HPII) (12) from native polyacrylamide gels stained for catalase activity (data not shown). Therefore, the *oxyR*-regulated *katG* gene also maps near *argH* (at 89 minutes) in *Salmonella*. The *Salmonella katG* deletions are hypersensitive to hydrogen peroxide killing, but otherwise grow normally, as is the case for *E. coli katG* mutants (20).

Proteins F52a and C22 are separable components of an alkyl hydroperoxide reductase activity in both *Salmonella* and *E. coli*. Each component has been purified to homogeneity from *Salmonella* strain TA4100 (*oxyR1*; unpublished results), in which both are constitutively overproduced (12). Several *Tn10*-mediated deletions have been obtained in the region near *entB* at 13 minutes in *Salmonella* and *E. coli* that both: (*i*) eliminate proteins F52a and C22 from 2D gels; and (*ii*) result in hypersensitivity to killing by alkyl hydroperoxides (Table 1). We have isolated a cosmid clone of this genetic locus (called *ahp*) from *E. coli* that appears to represent the structural genes for proteins F52a and C22 (unpublished results). The fifth hydrogen peroxide-inducible protein to be identified (C69) is the highly conserved heat shock protein DnaK (see below). In addition, glutathione reductase and Mn-containing superoxide dismutase activities are elevated in *oxyR1* and presumably account for more of the *oxyR*-regulated proteins (12).

Hydrogen Peroxide-Inducible Heat Shock Proteins. Five heat shock proteins in *Salmonella* can also be induced by hydrogen peroxide treatment. Fig. 2 shows a 2D gel of *Salmonella* cells pulse-labeled with L-[^{35}S]methionine for 10 min following a 28°C to 42°C temperature shift. There are 13 proteins induced by heat shock in *Salmonella* as determined by 2D gels. The five spots marked with bold arrows in Fig. 2 are also induced by 60 μM hydrogen peroxide (compare with Fig. 1).

One of the heat shock proteins induced by hydrogen peroxide, C69, has been identified as the highly conserved DnaK protein. Introduction of a runaway copy number plasmid that carries the *dnaK* gene from *E. coli* (21) results in the overproduction of a protein that comigrates exactly with *Salmonella* protein C69 on 2D protein gels (data not shown). In addition, C69 is one of the most abundant heat shock proteins in *Salmonella* as DnaK is in *E. coli*. Therefore, it is reasonable to conclude that C69 is the DnaK protein. E79 and C56 probably correspond to *E. coli* heat shock proteins F84.1 and GroEL, respectively, based on their degree of heat inducibility and their position on 2D gels. E79 is induced by hydrogen peroxide. In contrast, GroEL (C56) appears not to be induced by hydrogen peroxide based on visual inspection of 2D gels. However, immunoprecipitation of the GroEL protein before and after hydrogen peroxide treatment reveals a significant induction of this protein by hydrogen peroxide in *E. coli* (Lucy Shapiro, personal communication). As reported previously, three of the heat shock proteins that are also induced by hydrogen peroxide require *oxyR* for hydrogen peroxide induction (D64a, F52a, and E89) while two do not (C69, E79) (12).

Protein F52a, which is one component of the alkyl hydroperoxide reductase, is distinctive in that it requires *oxyR* for both heat shock induction and for hydrogen peroxide induction. In strains containing a deletion of *oxyR*, the F52a protein cannot be induced by heat shock (12). Deletions of the *ahp* locus, which eliminate protein F52a from 2D gels, also prevent F52a from being induced by either hydrogen peroxide treatment or heat shock (data not shown). This is strong evidence that the heat- and hydrogen peroxide-induced spots in the F52a region of the gel are, in fact, the same protein. F52a is also unusual in that it is not regulated in the same way in *E. coli* and *Salmonella*. In *E. coli* there is a spot analogous to the *Salmonella* F52a spot (unpublished results), which is also controlled by *oxyR* and forms one component of an alkyl hydroperoxide reductase activity. However, this protein is not heat inducible in *E. coli* (data not shown). Analysis of the cloned gene(s) (*ahp*) for the F52a protein in *Salmonella* and *E. coli* may reveal the reason for this difference.

Other Stresses Induce Some Heat Shock Proteins in *Salmonella*. In *E. coli* the DnaK and F84.1 (analogous to E79 in *Salmonella*) heat shock proteins can also be induced by ethanol (22), by UV irradiation and nalidixic acid (23), by bacteriophage λ infection (24), and by amino acid starvation (25). We have found that DnaK and E79 proteins in *Salmonella* can be induced by ethanol, by nalidixic acid, and by cumene hydroperoxide (data not shown) in addition to hydrogen peroxide. Each stress also induces distinct proteins. These data and data reported for *Salmonella* are shown schematically in Fig. 3.

Proteins Synthesized Anoxically Versus Aerobically. Catalase and Mn-containing superoxide dismutase are regulated by *oxyR* and have been reported to be induced by anaerobic to aerobic shifts (26, 27). Therefore, we have investigated whether other proteins that are more abundant during aerobic growth overlap with hydrogen peroxide-inducible proteins. The protein synthesis patterns for wild-type *Salmonella* cells grown in nonshaking tubes bubbled with either air (aerobic) or 95% N_2/5% CO_2 (anoxic) were compared. The steady-state pattern of protein synthesis as determined by 2D gels for nonshaking tubes bubbled with air was essentially identical to cells grown aerobically on a rotary shaker. Cells bubbled with air differed from cells bubbled with N_2/CO_2 in the abundance of 26 proteins (data not shown). There are 12 proteins that are more abundant under air and 14 proteins that are more abundant under N_2/CO_2 (Fig. 3). In *E. coli* 19 proteins are induced by aerobiosis, and 18 proteins are induced by anaerobiosis (28).

Seven of the 26 proteins whose levels are affected by the degree of aeration are hydrogen peroxide-inducible proteins. However, of these seven, four are more abundant during aerobic growth (G67, G45, G55a, and E89), and three are more abundant during anoxic (N_2/CO_2) growth (D69, D71, and F52a). Therefore, hydrogen peroxide-inducible proteins are not identical to proteins induced during aerobiosis. This is consistent with a report that has identified six oxygen-

	C22*	D69*	D71*	G35a	D29	G18	F52a*	E89	D64a	F77	G67	G55b	F52b	G75a	G75b	G23	E29	F29	G36	G44	E55	G55a	F54	E23a	G45	D54	E54	G46	C69*	E79	E23b	F31	E69	D64b
oxyR1	■	■	■	■	■	■	■	■	■																									
H_2O_2 - inducible	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■				
Heat-inducible							■	■	■																				■	■	■	■	■	■
Ethanol-inducible								■	■							■						■	■						■	■				■
Nalidixic acid -inducible																													■	■				
Anoxic		■	■				■																											
Aerobic								■			■											■			■									
Cumene hydroperoxide -inducible	■	■	■				■														■											■		

	C56*	C24	F46	C16	G87	F79a	G89	F71	D48	C45	G42	G28a	E52	F53	G77	G79a	G71	G73	F73	G55c	G48	G52	G68	G53	G27	F79b	C18	G28b	G64	G79b	G20	G35b	C23	F39
oxyR1																																		
H_2O_2 - inducible																																		
Heat-inducible	■	■	■	■																														
Ethanol-inducible	■				■	■	■	■	■	■	■	■	■																					
Nalidixic acid -inducible	■												■																					
Anoxic														■	■	■	■	■	■	■	■	■	■	■										
Aerobic																									■	■	■	■	■	■	■	■		
Cumene hydroperoxide -inducible																																	■	■

FIG. 3. Schematic diagram of the overlaps among stress responses in *Salmonella*. The dark boxes represent proteins that are induced by a given stress as determined by 2D-gel protein analysis. The top line of the figure shows the alphanumeric designation assigned to each protein. Proteins marked with an asterisk have been identified (C22, alkyl hydroperoxide reductase component; D69, catalase; D71, catalase; F52a, alkyl hydroperoxide reductase component; C69, DnaK protein; and C56, GroEL protein).

inducible Mu*d*-1 (Ap, *lac*) operon fusions in *Salmonella*, none of which are regulated by *oxyR* (29).

katG (catalase) mRNA Is Elevated in *oxyR1* Compared to Wild Type. The steady-state level of katG mRNA is elevated greater than 50-fold in *Salmonella oxyR1* mutants compared to an otherwise isogenic parent strain (Fig. 4). This is in agreement with the 50-fold increase in catalase activity observed in *oxyR1* mutants (12). The glucose repression described for catalase in wild type (30) and in *oxyR1* (12) also appears to involve a change in the steady-state level of katG mRNA (Fig. 4). The mechanism of this regulation is not yet understood. The hisJ probe was used as a control to demonstrate that roughly equal amounts of RNA were spotted on the filters from the *oxyR1* and wild-type strains.

DISCUSSION

Hydrogen Peroxide Adaptation, Heat Shock, and Other Stresses. Hydrogen peroxide adaptation results in the induction of 30 proteins in *Salmonella*. Nine of the most rapidly induced proteins are coordinately regulated by the *oxyR* gene product. Five of the 30 hydrogen peroxide-inducible proteins are also heat inducible. Of these five, three (D64a, E89, and F52a) are proteins regulated by *oxyR* (see Fig. 3). Two of these three proteins (D64a and E89) are heat inducible in an *oxyR*-independent manner.

The third heat-inducible and hydrogen peroxide-inducible protein F52a is of special interest because its heat and hydrogen peroxide induction both require *oxyR*. Therefore, a single regulatory element, *oxyR*, affects the induction of

FIG. 4. Steady-state level of katG mRNA in *oxyR1* versus wild type (wt). An aliquot of total cellular RNA isolated from either wild-type or *oxyR1* cells was spotted on nitrocellulose filters that were then probed with nick-translated DNA fragments from the *katG* or *hisJ* genes. RNA was diluted with water in the relative proportions indicated on the left side of each panel prior to being spotted on the nitrocellulose filter.

F52a in response to very different environmental conditions. Deletions of *ahp* which abolish the expression of the hydrogen peroxide-inducible F52a protein, also prevent the heat induction of a spot at the F52a position on 2D gels, providing very strong evidence that the heat- and hydrogen peroxide-induced spots are, in fact, the same protein. The F52a protein may also require the *htpR* gene product for heat induction, but this has not been tested.

The overlap among stress proteins induced by different agents in *Salmonella* is extensive (Fig. 3). However, each stress induces a characteristic group of proteins along with a smaller number of proteins that overlap with other stress proteins. This suggests that the overlaps do not result from the generation of a common intracellular signal, but rather that the genes coding for the proteins that can be induced by several stresses contain the cis-acting regulatory sequences required for several forms of regulation. We know this to be the case for proteins D64a and E89 that are induced by both heat shock and hydrogen peroxide. The induction of these two proteins by heat shock in deletions of *oxyR* is completely normal, whereas their hydrogen peroxide inducibility is abolished. In contrast, the induction of heat shock proteins in *E. coli* by ethanol (22) or by nalidixic acid (23) requires the *htpR* gene product. This could mean that these stresses generate the heat shock inducing signal. Alternatively, the *htpR* gene itself could be regulated via multiple cis-acting regulatory sequences that mediate its induction by different agents.

It has been shown that $CdCl_2$ treatment of *E. coli* also induces most of the heat shock proteins and most of the *oxyR*-regulated oxidative stress proteins (R. A. Van Bogelen, P. M. Kelley, and F. C. Neidhardt, personal communication). We have also observed this in *Salmonella* (D. Milligan and B.A., unpublished results). In *Salmonella*, $CdCl_2$ treatment may lead to the generation of the heat shock- and oxidative stress-inducing signals, since it strongly induces both responses. $CdCl_2$ is very effective at inducing adenylylated nucleotides in *Salmonella*, which have been postulated to be involved in both oxygen defense and heat shock regulation (31, 32).

Hydrogen Peroxide-Inducible Proteins Are not the Same as Aerobically Induced Proteins. Some of the enzymes that are regulated by *oxyR* such as catalase and Mn-containing superoxide dismutase are induced by aerobiosis (26, 27). However, most hydrogen peroxide-inducible proteins (25 out of 30) are not proteins induced by aerobiosis. This argues against a role for the *oxyR*-regulatory network in defending cells from a simple anaerobic to aerobic shift. It is surprising that spots D69 and D71 are more abundant during anoxic

growth compared to aerobic growth (see Fig. 3) since these catalases are known to be repressed during strictly anaerobic conditions (26, 30).

It has also been proposed that the *oxyR* network may be involved in pathogenesis (12). The *oxyR* locus and several other loci affecting sensitivity to killing by oxidizing agents have been shown to affect pathogenesis of virulent *Salmonella* strains, as well as their ability to survive an oxidative burst from macrophages *in vitro* (ref. 33; P. I. Fields and F. Heffron, personal communication).

Hydrogen Peroxide Induction and Transcription. The hybridization experiment shown in Fig. 4 shows that the observed increase in catalase activity in *oxyR1* mutants is coincident with higher steady-state levels of katG mRNA. Most likely this results from increased transcription of the *katG* gene in *oxyR1* mutants, although an increase in mRNA stability in *oxyR1* is also possible. The other major stress regulons in enteric bacteria are regulated at the level of transcription initiation (reviewed in ref. 34).

How Does Hydrogen Peroxide Induce Gene Expression? For the *oxyR*-dependent stress response, as for the *htpR*-, *recA/lexA*-, and *ada*-dependent stress responses, some of the key structural and regulatory elements have been identified. The work of Teo *et al.* (35) has led to the identification of the intracellular signal inducing the adaptive response to alkylating agents. The induction occurs via methylation of the regulatory protein itself (Ada) that converts it to an efficient activator of transcription of *ada*-dependent genes. The primary signal inducing gene expression for the other responses remains unknown, although there is some evidence that the heat shock signal in *E. coli* may be the presence of abnormally folded proteins (36).

Since oxidative stress and heat shock rapidly increase the levels of dinucleotides such as AppppA in *Salmonella* (31, 32), we have suggested that the primary signal in initiating these responses may be a dinucleotide. We have cloned and sequenced the *oxyR* gene from *E. coli* (unpublished results) as a first step toward understanding the induction of genes by oxidative stress at the molecular level.

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