DNA Microarray-Mediated Transcriptional Profiling of the *Escherichia coli* Response to Hydrogen Peroxide

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The genome-wide transcription profile of *Escherichia coli* cells treated with hydrogen peroxide was examined with a DNA microarray composed of 4,169 *E. coli* open reading frames. By measuring gene expression in isogenic wild-type and *oxyR* deletion strains, we confirmed that the peroxide response regulator OxyR activates most of the highly hydrogen peroxide-inducible genes. The DNA microarray measurements allowed the identification of several new OxyR-activated genes, including the *hemH* heme biosynthetic gene; the six-gene *suf* operon, which may participate in Fe-S cluster assembly or repair; and four genes of unknown function. We also identified several genes, including *uxuA*, encoding mannonate hydrolase, whose expression might be repressed by OxyR, since their expression was elevated in the ΔoxyR mutant strain. In addition, the induction of some genes was found to be OxyR independent, indicating the existence of other peroxide sensors and regulators in *E. coli*. For example, the *isc* operon, which specifies Fe-S cluster formation and repair activities, was induced by hydrogen peroxide in strains lacking either OxyR or the superoxide response regulators SoxRS. These results expand our understanding of the oxidative stress response and raise interesting questions regarding the nature of other regulators that modulate gene expression in response to hydrogen peroxide.

The *Salmonella enterica* serovar Typhimurium and *Escherichia coli* responses to hydrogen peroxide initially were analyzed 15 years ago using two-dimensional gel separation of proteins (10, 20, 33). These studies showed that the expression of approximately 30 proteins is induced by hydrogen peroxide treatment: 12 proteins are maximally induced within 10 min and 18 proteins are maximally induced between 10 and 30 min after the addition of hydrogen peroxide (10). Mutational studies led to discovery of the OxyR regulatory protein, which was shown to regulate the expression of 9 of the 12 rapidly induced proteins (10). A variety of approaches have led to the identification of some of the OxyR-activated genes, including *katG* (encoding hydroperoxidase I), *ahpCF* (encoding an alkyl hydroperoxide reductase), *oxyS* (encoding a small regulatory RNA), *dps* (encoding a nonspecific DNA binding protein), *gorA* (encoding glutathione reductase), *grxA* (encoding glutaredoxin 1), *trxC* (encoding thioredoxin 2), *fur* (encoding the Fur repressor of ferric ion uptake), and *dsbG* (encoding a disulfide chaperone-isomerase) (41; also reviewed in reference 30). OxyR also has been shown to be a repressor of its own expression as well as that of *fhuF* (encoding a ferric ion reductase) and *flu* (encoding the antigen 43 outer membrane protein). Nevertheless, the identity of many of the hydrogen peroxide-inducible proteins has remained unknown.

The recently developed microarray technology has allowed the parallel study of the expression of every gene in an organism. This approach has already been successfully used in studying *E. coli* gene expression under a number of different growth conditions (13, 26, 31, 35, 36). Here, we report a survey of gene expression in response to hydrogen peroxide. In addition to confirming the hydrogen peroxide induction of most known OxyR-regulated genes, we have identified several new members of the OxyR regulon. We also have found that many genes are induced by hydrogen peroxide in an OxyR-independent fashion, revealing complex regulation of the cellular response to oxidative stress.

MATERIALS AND METHODS

Plasmids and strains. The DNA sequences and coordinates throughout the study are for *E. coli* from GenBank accession no. U00096 (5). The plasmids used in the study were constructed using fragments PCR amplified from chromosomal DNA. The sequences of all oligonucleotides are listed in Table 1. To generate the *hemH* promoter plasmids (pGSO131 and pGSO132), a 280-bp fragment produced using primers 819 and 821 was cloned into pCR2.1 (Invitrogen) in both orientations. To generate a *sufA* promoter plasmid (pGSO133), a 378-bp fragment produced using primers 820 and 825 was cloned into pCR2.1. To generate the *isc* promoter plasmid (pGSO135), a 500-bp fragment produced using primers 699 and 700 was cloned into the *Eco*RI and *Bam*HI sites of pUC18. The ΔoxyR::kan (GSO9 [32]) mutant allele was moved into MG1655 (2) by P1 transduction (27) to generate GSO77. MC4100 (wild type), GSO47 (MC4100 ΔoxyR::kan), GSO71 (MC4100 ΔsoxRS), and GSO72 (MC4100 Δfur::kan) were described previously (40).

RNA isolation. Cultures were grown under aeration at 37°C in Luria-Bertani (LB) rich medium (27). Exponential-phase cultures (optical density at 600 nm = 0.2 to 0.5) were split into aliquots; one aliquot was left untreated, and the other aliquots were treated with the indicated amounts of hydrogen peroxide or paraquat. After 10 min, the cells from 5, 10, or 25 ml of culture were harvested and resuspended in 1 ml of Trizol equilibrated at 4°C (Gibco BRL). All subsequent purification steps were carried out according to the Trizol reagent manual (based on reference 9).

DNA microarray experiments. Fabrication of the *E. coli* DNA microarray and procedures for cDNA labeling, hybridization, and array quantification were described previously (28, 35).

Primer extension assays. Total RNA samples were subjected to primer extension assays as described previously (37), using primer 819 specific to *hemH*,

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TABLE 1. Sequences of oligonucleotides used in the study

No.	Sequence[a]
188	5′-GCAAAAGTTCACGTTGG
686	5′-GCATTGCGGTCACGGCATAG
699	5′-GCCAAC<u>GGATCC</u>GGGCCCGCTTCA
700	5′-GATGCGACGCG<u>GAATTC</u>GTCTTAT
819	5′-TCGGGCGTACCCAGGTTTG
820	5′-TCAGCGTTAAGCCTTGCCA
821	5′-AAGCGCGCACGGACAGTC
823	5′-TGAATAATTTTCTGATGGGACAT
825	5′-AGCCTGTGTCGCACAGAC
828	5′-CTGATGTGTGGGTTAAC

[a] Restriction sites are underlined.

primer 820 specific to *sufA*, primer 188 specific to *oxyS*, primer 823 specific to *soxS*, and primer 686 specific to the *yfhP* gene in the *isc* operon.

DNase I footprinting. DNase I footprinting assays of purified OxyR binding to the *hemH* and *sufA* promoters were carried out as described previously (32).

RESULTS

DNA microarray measurements. Wild-type (MG1655) cells and isogenic Δ*oxyR* (GSO77) mutant cells were grown to exponential phase in LB rich medium. The cultures were split, and half of each culture was treated with 1 mM hydrogen peroxide. After 10 min, total RNA was isolated from the untreated and treated cultures. To check whether the RNA samples showed a well-characterized peroxide stress response, we examined the expression of the *oxyS, ahpC, katG,* and *fhuF* genes using primer extension assays. As observed previously, *oxyS, ahpC,* and *katG* showed OxyR-dependent induction by hydrogen peroxide, and *fhuF* showed repression in the wild-type strain and slight induction in the *oxyR* deletion strain (data not shown).

Each of the RNA samples was used as a template for cDNA synthesis with attendant incorporation of either of two fluorescent dyes, Cy3 and Cy5. Pairs of differentially labeled untreated and treated cDNA samples from each strain then were hybridized to a glass slide on which two sets of the 4,169 *E. coli* open reading frames (ORFs) were printed. For each strain, two slides were used for hybridization: for one slide, the untreated sample was labeled with Cy3 and the treated sample was labeled with Cy5, and for the second slide, the dye-sample pairings were reversed. Thus, the expression for each gene was measured four times. The average of the four data points is reported here. Overall, the mRNA levels of 140 genes in the wild-type strain showed >4-fold induction after treatment with hydrogen peroxide, and the mRNA levels of 167 genes in the Δ*oxyR* strain showed >4-fold induction. The 30 genes whose expression was induced most strongly in the wild-type strain are listed in Table 2, and the 30 genes whose expression was induced most strongly in the Δ*oxyR* strain are listed in Table 3. All of these genes have induction ratios of >10-fold.

OxyR-dependent response. A hallmark of the *E. coli* response to hydrogen peroxide is the rapid and strong induction of a set of OxyR-regulated genes, including *dps, katG, grxA, ahpCF,* and *trxC.* The observed >20-fold induction of all of these genes in the wild-type strain (Table 2) but not the Δ*oxyR* strain (Table 3) provided an internal validation of the microarray experiment. Of the other known OxyR-activated genes, *fur* and *gorA* were slightly induced and *dsbG* was unchanged in the

TABLE 2. The 30 most strongly hydrogen peroxide-induced genes in the wild-type strain

Gene	b no.	Induction ratio[a]	Function[b]
dps	b0812	180	Stress response DNA binding protein
yaiA	b0389	56	Function unknown
katG	b3942	44	Catalase hydrogen peroxidase I
grxA	b0849	37	Glutaredoxin I
yfiA	b2597	36	Function unknown
ibpA	b3687	29	Chaperone, heat-inducible protein of HSP20 family
yjiD	b4326	29	Function unknown
ycfR	b1112	26	Function unknown
ahpF	b0606	22	Alkyl hydroperoxide reductase large subunit
trxC	b2582	21	Thioredoxin 2
sufA	b1684	21	Homology with IscA
ymgB	b1166	20	Function unknown
ahpC	b0605	20	Alkyl hydroperoxide reductase small subunit
ibpB	b3686	18	Chaperone, heat-inducible protein of HSP20 family
yaaA	b0006	18	Function unknown
tnaA	b3708	18	Tryptophanase
fpr	b3924	17	Ferredoxin NADP$^+$ reductase
cysK	b2414	16	Cysteine synthase
sufB	b1683	16	Function unknown
dsdX	b2365	15	Homology with gluconate permease
ybjM	b0848	15	Function unknown
yeeD	b2012	14	Function unknown
dsdA	b2366	13	D-Serine deaminase
soxS	b4062	13	Regulatory protein of *soxRS* regulon
sbp	b3917	12	Periplasmic sulfate binding protein
sufC	b1682	12	Putative ABC transporter
phoH	b1020	12	Member of *pho* regulon
hemH	b0475	11	Ferrochelatase
yljA	b0881	11	Function unknown
ycgZ	b1164	11	Function unknown

[a] Ratio of transcript levels for hydrogen peroxide-treated wild-type (MG1655) strain to transcript levels for untreated wild-type strain.
[b] Function descriptions are taken from http://genolist.pasteur.fr/Colibri/.

wild-type strain treated with hydrogen peroxide. Of the known OxyR-repressed genes, *flu* was unchanged and *fhuF* was slightly repressed. A comparison of the induction ratios between the wild-type strain and the Δ*oxyR* strain indicated that, among the 30 most highly induced genes, 8 additional genes (*hemH, sufABC, yaiA, yaaA, yljA,* and *ybjM* [Table 4]) might be regulated by OxyR. The remaining 16 most highly induced genes showed approximately equal levels of hydrogen peroxide induction in the wild-type strain and the Δ*oxyR* strain.

(i) hemH. One gene, *hemH* (b0475), whose expression was induced 11-fold by hydrogen peroxide in the wild-type strain and <2-fold in the Δ*oxyR* strain, encodes a ferrochelatase that catalyzes the incorporation of ferrous ion into protoporphyrin IX in the final step in protoheme biosynthesis (3). The *hemH* locus also was named *visA* since some ferrochelatase mutants of *E. coli* were identified by virtue of having a photosensitive phenotype (19). It was determined previously that the photosensitivity was caused by the increased levels of protoporphyrin IX which accumulate in the mutants lacking ferrochelatase (21). To confirm OxyR regulation of *hemH,* we carried out primer extension assays. As shown in Fig. 1B, *hemH* induction by hydrogen peroxide in vivo was clearly dependent on OxyR. The start of the transcript corresponds to an A residue at

TABLE 3. The 30 most strongly hydrogen peroxide-induced genes in the ΔoxyR strain

Gene	b no.	Induction ratio[a]	Function[b]
yfiA	b2597	109	Function unknown
ibpB	b3686	54	Chaperone, heat-inducible protein of HSP20 family
ibpA	b3687	35	Chaperone, heat-inducible protein of HSP20 family
tnaA	b3708	30	Tryptophanase
yjiD	b4326	29	Function unknown
cysK	b2414	25	Cysteine synthase
uxuA	b4322	23	Mannonate hydrolase
dsdX	b2365	22	Homology with gluconate permease
ytfK	b4217	20	Function unknown
recN	b2616	20	Recombination and repair
soxS	b4062	19	Regulatory protein of soxRS regulon
dsdA	b2366	18	D-Serine deaminase
fpr	b3924	18	Ferredoxin NADP⁺ reductase
ymgB	b1166	17	Function unknown
yeeD	b2012	17	Function unknown
ygaQ	b2654	17	Function unknown
yaiA	b0389	16	Function unknown
yceP	b1060	16	Function unknown
glgS	b3049	15	Glycogen synthesis protein
ydcH	b1426	15	Function unknown
tnaL	b3707	15	Regulatory leader peptide for tna operon
phoH	b1020	14	Member of pho regulon
ymgA	b1165	14	Function unknown
ydeN	b1498	14	Function unknown
sbp	b3917	12	Periplasmic sulfate binding protein
ynaF	b1376	12	Function unknown
cysP	b2425	12	Periplasmic sulfate binding protein
yaeH	b0163	12	Function unknown
manX	b1817	11	Mannose phosphotransferase system
ycgK	b1178	10	Function unknown

[a] Ratio of transcript levels for hydrogen peroxide-treated ΔoxyR (GSO77) strain to transcript levels for untreated ΔoxyR strain.
[b] Function descriptions are taken from http://genolist.pasteur.fr/Colibri/.

TABLE 4. OxyR regulon

Gene	b no.	Basal level (ppm)[a]	Induction ratio in strain:	
			Wild type	ΔoxyR
dps	b0812	330	180	2.0
katG	b3942	270	44	2.1
grxA	b0849	54	37	1.2
ahpF	b0606	350	22	1.2
trxC	b2582	73	21	2.1
ahpC	b0605	2,000	20	2.2
fur	b0683	390	2.9	1.1
gor	b3500	160	2.1	0.9
dsbG	b0604	93	0.7	0.7
flu	b2000	110	1.0	1.9
fhuF	b4367	200	0.4	5.7
hemH	b0475	39	11	1.1
sufA	b1684	270	21	3.6
sufB	b1683	90	16	4.0
sufC	b1682	120	12	3.4
sufD	b1681	160	8.3	3.0
sufS	b1680	220	3.5	1.5
sufE	b1679	140	8.2	3.5
yaaA	b0006	110	18	4.2
yaiA	b0389	130	56	16
ybjM	b0848	51	15.0	1.1
yljA	b0881	250	11.0	4.6

[a] Levels in cells during exponential growth in LB medium. The data are from unpublished data of Y. Wei and R. A. LaRossa.

position 497255, 24 bp upstream of the hemH start codon. An in vitro DNase I footprinting experiment showed that oxidized OxyR binds to the hemH promoter centered at 497211, overlapping and just upstream of the −35 region (Fig. 1C).

(ii) suf operon. Three genes, sufA/ydiC (b1684), sufB/ynhE (b1683), and sufC/ynhD (b1682), whose expression was strongly induced in the wild-type strain but not the ΔoxyR strain, are members of a six-gene cluster (b1679 to b1684). These genes are transcribed in the same direction and show potential for translational coupling. Patzer and Hantke (24) suggested that the gene cluster forms an operon and, because of possible involvement in sulfur mobilization, named the genes sufA, sufB, sufC, sufD, sufS, and sufE. Of the six ORFs, the sufS gene and its product are the best characterized. This gene encodes one of three NifS homologs in E. coli and also has been named csdB (18). All three E. coli NifS homologs, IscS, CSD, and SufS/CsdB, catalyze the elimination of sulfur from L-cysteine and selenium from L-selenocysteine (18). However, the SufS/CsdB protein was shown to be 290 times more active on L-selenocysteine than on L-cysteine and was thus considered the E. coli counterpart of the mammalian selenocysteine lyase (18). The gene products of the remaining five suf genes are less well studied, but some show interesting homologies to other characterized proteins. sufA encodes a homolog of IscA which, together with the products of the iscS and iscU genes, is involved in Fe-S cluster formation and repair. The sufB, sufC, and sufD genes encode components of an ATP binding cassette (ABC) transporter. No biochemical data can be found with regard to these three genes. However, genetic studies have shown that E. coli sufC mutants have delayed soxR-dependent induction of a soxS-lacZ gene fusion (22), and the stability of the [2Fe-2S] ferric ion reductase protein encoded by fhuF is decreased in sufD mutants (24). The final gene in the operon, sufE, encodes a conserved oxidoreductase, a homolog of which is present downstream of the csd gene.

Although only sufA, sufB, and sufC are among the most highly induced genes, sufD (b1681), sufS (b1680), and sufE (b1679) also are induced with higher ratios in the wild-type strain than in the ΔoxyR strain (Table 4 and Fig. 2A). To confirm OxyR regulation of this operon and to map the start of the suf transcript, we carried out primer extension assays (Fig. 2B). Consistent with the microarray data, we detected OxyR-dependent induction of sufA. A strong primer extension product ending in a T residue at position 1762442, 32 bp upstream of the sufA start codon at position 1762410, was observed when RNA isolated from the wild-type strain was used as the template. We also carried out DNase I footprinting experiments to test for OxyR binding to the sufA promoter. In a computational scan of the E. coli genome, we predicted a putative OxyR site centered at 1762663, 253 bp upstream of the sufA start codon (41). The DNase I footprinting carried out using a 378-bp fragment from the sufA-ydiH intragenic region showed that oxidized OxyR exclusively bound to the predicted site (Fig. 2C). However, this single OxyR binding site was far up-
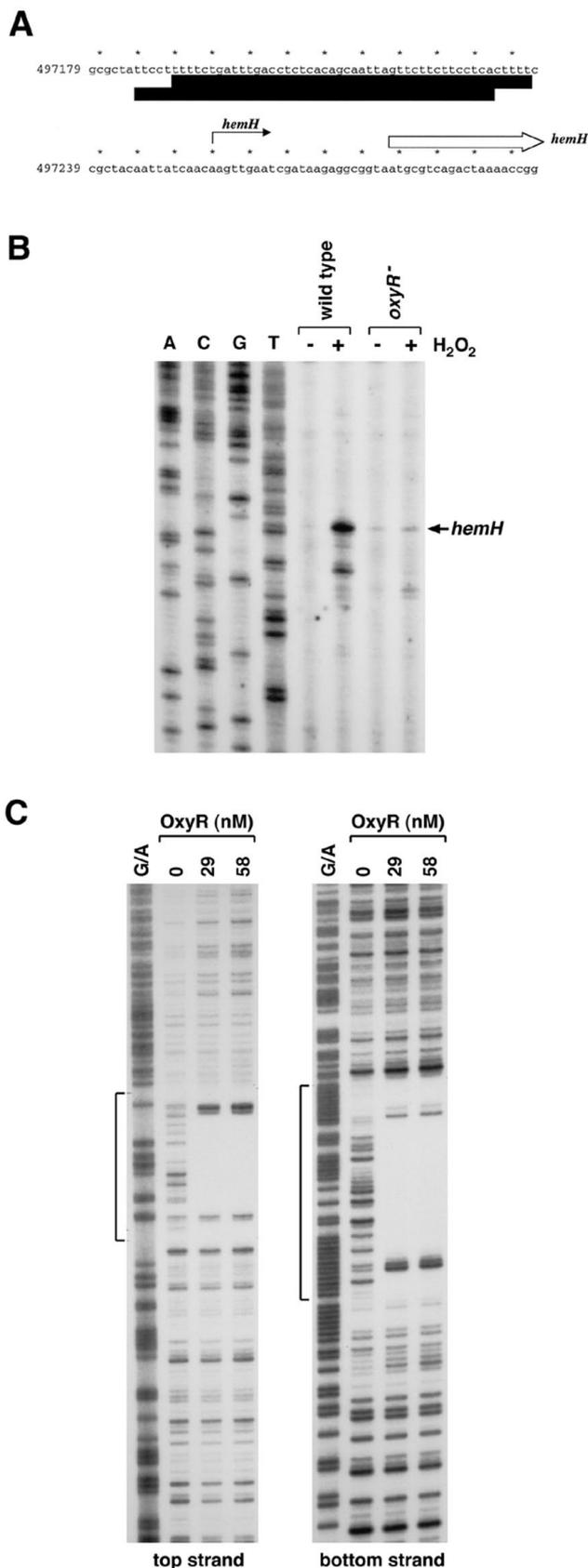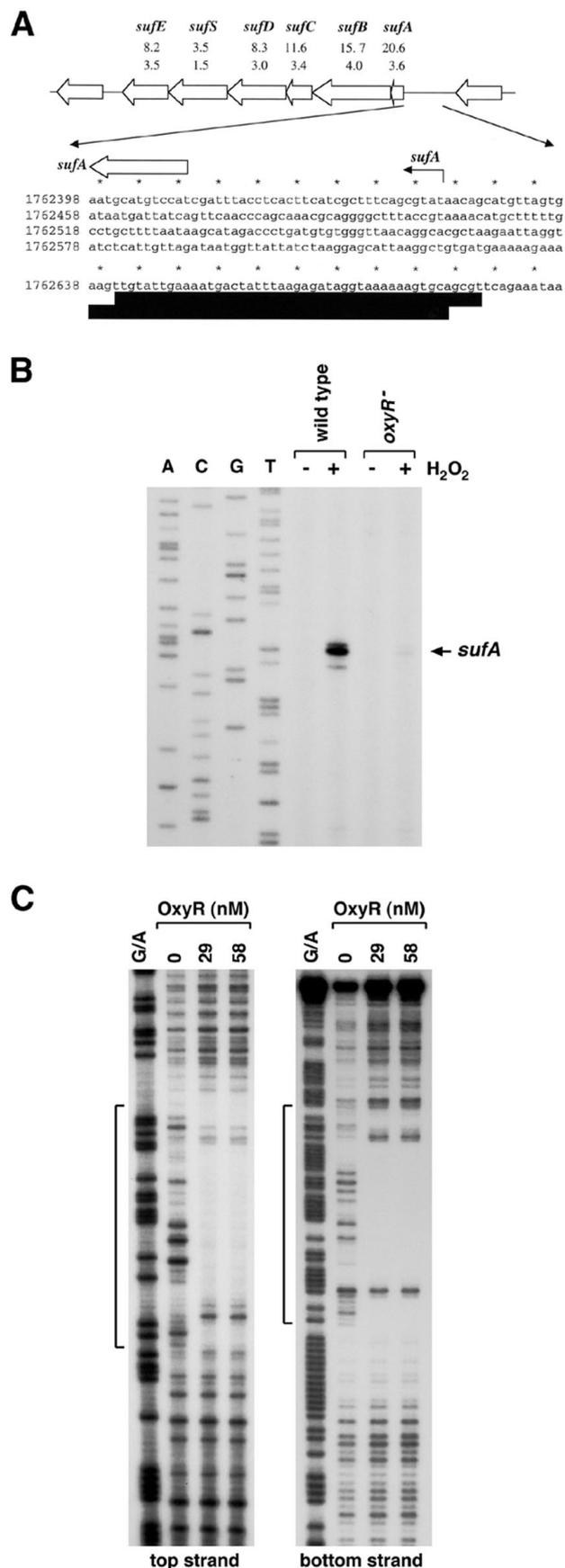
stream from the end of the primer extension product, and at all other known OxyR-activated promoters, the transcription factor binds at a position overlapping or directly upstream of the -35 sequence of the promoter. Possibly, the initial *sufA* transcript is longer and is either processed or folded into a complex secondary structure impervious to reverse transcriptase. Given the unusually high conservation of the *sufA-ydiH* intragenic region (34), it is likely that the regulation of *sufA* expression is complex. We did not observe strong Fur regulation of *sufA* expression under conditions used in our experiments (data not shown). However, two putative Fur binding sites, centered at positions 1762460 and 1762466, have been predicted in the *suf* promoter (K. Lewis, B. Doan, M. Zheng, G. Storz, and T. D. Schneider, unpublished data), and Fur-dependent expression of *sufD-lacZ* and *sufS-lacZ* fusions has been observed previously (24). More experiments are needed to fully understand the regulation of the *suf* operon.

(iii) Genes of unknown function. Four ORFs of unknown function, *yaiA*, *yaaA*, *yljA*, and *ybjM*, showed much stronger peroxide induction in the wild-type strain than in the *oxyR* deletion strain. *yaaA* and *yljA* are conserved ORFs, but experimental data about the function of the corresponding proteins cannot be found in the literature. Primer extension assays confirmed that transcripts initiating directly upstream of the predicted first codon of the *yaiA*, *yaaA*, and *yljA* ORFs are induced by hydrogen peroxide in an OxyR-dependent manner (data not shown). The computational search for OxyR binding sites (41) predicted a putative OxyR binding site centered at position 922026 in the *yljA* promoter. Interestingly, we were not able to detect a transcript that would encode the predicted 125-amino-acid (aa) YbjM ORF. Instead, Northern blots and primer extension analysis showed that the OxyR-regulated *grxA* transcript is approximately 600 nucleotides in length and extends into the strand opposite *ybjM*. Inspection of the *ybjM* antisense sequence revealed that this opposite strand also encodes an 81-aa ORF. The arrays used in our experiments do not distinguish between strands. Thus, we suggest that the signal detected for *ybjM* actually corresponds to OxyR activation of a gene which is encoded on the opposite strand and is likely to be in an operon with *grxA*.

(iv) Possible OxyR-repressed genes. In comparing genes induced by hydrogen peroxide in the wild type (Table 2) and the Δ*oxyR* mutant (Table 3), we noted a number of genes, *uxuA*,

arrow, and the start of the corresponding ORF is denoted by the white arrow. The DNase I footprints for OxyR binding are indicated by the dark boxes. (B) Primer extension assays of *hemH* expression in wild-type (MC4100) and Δ*oxyR* (GSO47) strains grown in LB medium. Exponential-phase cultures were split into two aliquots: one aliquot was left untreated, and the other was treated with 1 mM hydrogen peroxide. The cells were harvested after 10 min, total RNA was isolated, and primer extension assays were carried out with primer 819 specific to *hemH*. The neighboring sequencing reactions were carried out with the same primer. (C) DNase I footprinting assays of oxidized OxyR binding to the top and bottom strands relative to the *hemH* promoter. The regions protected by OxyR on both strands are indicated by the brackets. The plasmids carrying the *hemH* promoter fragment in both orientations were digested with *Not*I, labeled with ^{32}P, and then digested with *Bam*HI to give the labeled top and bottom strands. The samples were run in parallel with Maxam-Gilbert G/A sequencing ladders.

FIG. 1. OxyR-dependent induction of *hemH*. (A) Sequence of the *hemH* promoter. The *hemH* transcription start is marked by the black

ygaQ, *ytfK*, *ydcH*, *ydeN,* and *yaeH*, that were induced more strongly in the Δ*oxyR* background. OxyR is both an activator and a repressor. Thus, it is possible that OxyR represses these genes in response to oxidative stress. Our computational search predicted an OxyR binding site at position 4549044 between *uxuAB* (encoding mannonate hydrolase and mannonate oxidoreductase) and the divergent *gntP* gene (encoding a possible gluconate permease) (Fig. 3). Since *uxuB* and *gntP* also showed higher induction ratios in the absence of OxyR, it is intriguing to speculate that the two divergent promoters are repressed by oxidized OxyR. Similarly, there are two predicted OxyR binding sites at positions 2784053 and 2784276 between *ygaQ* and a putative divergent gene designated b2653, and both *ygaQ* and b2653 showed a higher induction ratio in the Δ*oxyR* mutant strain. In this context, it also is noteworthy that three genes, *ybjC*, *nfsA*/*mdaA* (encoding nitrofuran reductase I activity B), and *rimK* (encoding a ribosomal modification protein), divergent to *grxA* were induced in the Δ*oxyR* mutant but not the wild-type strain. No OxyR binding sites were predicted upstream of *ytfK*, *ydcH*, *ydeN,* and *yaeH,* so these genes may or may not be repressed directly by OxyR. OxyR binding to these promoters and the predicted sites will need to be tested experimentally.

OxyR-independent responses. Below, we describe the expression of genes that are induced similarly by hydrogen peroxide in the wild-type strain and the Δ*oxyR* mutant strain. It is interesting that among genes induced moderately in both the wild-type strain and the Δ*oxyR* deletion strain were a number of heat shock genes (*groEL*, *groES*, *grpE*, *dnaK*, and *htpG*) including those encoding proteolytic activities (*clpA, clpB, clpX,* and *clpP*). SOS genes (*recA*, *recN*, *lexA*, and *dinD*); sulfate and cysteine metabolism genes (*cysKAUPNDHJ* and *sbp*); genes specifying tricarboxylic acid cycle enzymes (*acnA* and *fumA*); the *nrdHIEF* operon, which directs synthesis of a second ribonucleotide reductase system; and the universal stress gene *uspA* also were induced to some extent in both the wild-type strain and the mutant strain. In contrast, the expression of

FIG. 2. OxyR-dependent induction of the *suf* operon. (A) Structure of the *suf* operon and sequence of the *sufA* promoter. The induction ratios observed for the wild-type and Δ*oxyR* mutant strains in the microarray experiment are given below each gene. The *sufA* transcription start is marked by the black arrow, and the start of the corresponding ORF is denoted by a white arrow. The DNase I footprints for OxyR binding are indicated by the dark boxes. Our computational search (41) predicted an OxyR binding site of 9.6 bits centered at position 922026. (B) Primer extension assays of *sufA* expression in wild-type (MC4100) and Δ*oxyR* (GSO47) strains grown in LB medium. Exponential-phase cultures were split into two aliquots: one aliquot was left untreated, and the other was treated with 1 mM hydrogen peroxide. The cells were harvested after 10 min, total RNA was isolated, and primer extension assays were carried out with primer 820 specific to *sufA*. The neighboring sequencing reactions were carried out with the same primer. (C) DNase I footprinting assays of oxidized OxyR binding to the top and bottom strands relative to the *sufA* promoter. The regions protected by OxyR on both strands are indicated by the brackets. For OxyR binding to the top strand, the ^{32}P-labeled primer 828 and unlabeled primer 825 were used to PCR amplify a 187-bp fragment. For OxyR binding to the bottom strand, the 378-bp *Not*I-*Bam*HI fragment of pGSO133 was labeled with ^{32}P at the *Bam*HI site. The samples were run in parallel with Maxam-Gilbert G/A sequencing ladders.
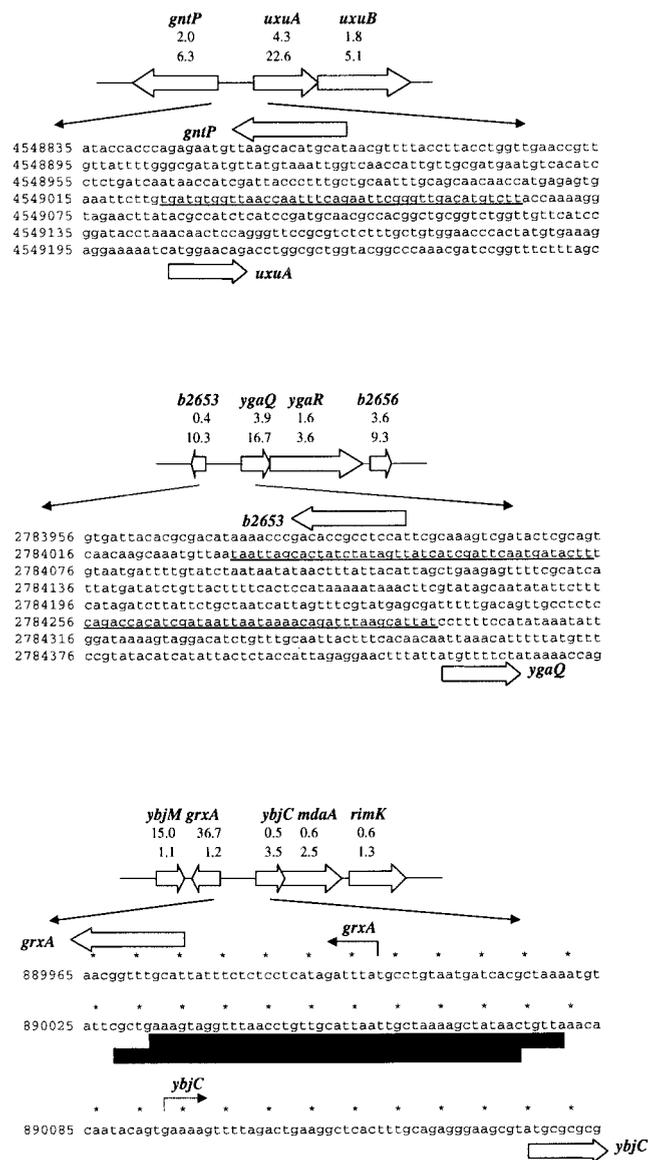
FIG. 3. Possible OxyR-dependent repression of *gntP* and *uxuAB*; b2653, *ygaQ*, *ygaR*, and b2656; and *ybjC*, *nfsA*/*mdaA*, and *rimK*. The gene organization of the corresponding operons is shown. Predicted OxyR binding sites (41) of 7.0 bits centered at position 4549044, 8.8 bits centered at position 2784053, and 5.4 bits centered at position 2784276 are underlined. The confirmed OxyR binding site upstream of the *grxA* promoter (39) is indicated by the black boxes. The transcription starts documented for *grxA* and predicted for *ybjC* are denoted by solid and dotted arrows, respectively. The induction ratios observed for the wild-type and Δ*oxyR* mutant strains in the microarray experiment are given below each gene.
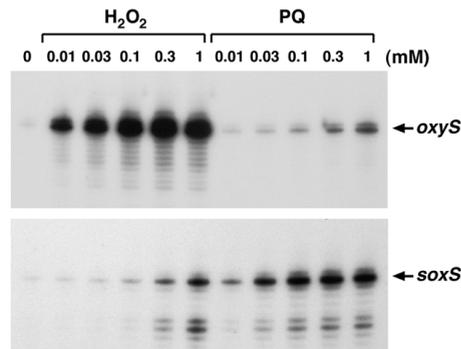

FIG. 4. *oxyS* and *soxS* induction by hydrogen peroxide and paraquat. The figure shows the results of primer extension assays of *oxyS* and *soxS* transcript levels in wild-type (MC4100) cells grown in LB medium. An exponential-phase culture was split into aliquots: one aliquot was left untreated, and the other aliquots were exposed to the indicated concentrations of hydrogen peroxide and paraquat. The cells were harvested after 5 min, total RNA was isolated, and primer extension assays were carried out with primer 188 specific to *oxyS* and primer 823 specific to *soxS*.

many ribosomal protein genes, cold shock genes, ATP synthase genes, and transporter genes was repressed.

(i) SoxRS regulon. The SoxRS regulon was reported previously to be induced primarily by superoxide-generating compounds and not by hydrogen peroxide (23). Thus, we were surprised to find that several members of this regulon such as *fpr* (encoding ferredoxin-flavodoxin reductase) and *sodA* (with an induction ratio of 8 and encoding manganese superoxide dismutase), as well as *soxS* itself, were among the genes most

strongly induced by 1 mM hydrogen peroxide in both the wild-type background and the Δ*oxyR* mutant strain background. To directly compare the induction of OxyR and SoxR target genes by both hydrogen peroxide and superoxide-generating compounds, we treated wild-type cells with either 0, 0.01, 0.03, 0.1, 0.3, and 1 mM hydrogen peroxide or the same concentrations of paraquat (methyl viologen), a standard inducer of the *soxRS* regulon. We then carried out primer extension assays to examine the expression of *oxyS*, a primary OxyR target, and *soxS*, the only known SoxR target (Fig. 4). As expected, *oxyS* was strongly induced by all concentrations of hydrogen peroxide and *soxS* was induced by all concentrations of paraquat, consistent with the notion that OxyR primarily senses hydrogen peroxide while SoxR primarily responds to superoxide-generating compounds. However, we also observed that *soxS* was partially induced by high concentrations of hydrogen peroxide and that *oxyS* was slightly induced by high concentrations of paraquat.

(ii) *isc* operon. The *isc* gene cluster, *yfhP* (b2531), *iscS*/*yfhO* (b2530), *iscU*/*yfhN* (b2529), *iscA*/*yfhF* (b2528), *hscB* (b2527), *hscA* (b2526), *fdx* (b2525), and *yfhJ* (b2524), has received attention due to its role in Fe-S cluster formation and cysteine-related metabolism (12, 25, 38). The fact that Fe-S cluster damage is a major consequence of oxidative stress prompted us to examine the regulation of this cluster. The expression profiles indicated that the first four genes (*yfhP*, *iscS*, *iscU*, and *iscA*) in the cluster were modestly induced in both the wild-type strain and the *oxyR* deletion strain (Fig. 5A). The induction ratios for the last four genes (*hscB*, *hscA*, *fdx*, and *yfhJ*) were within the error of the experiment, suggesting that these four genes were not induced and that they are regulated differently. This result is consistent with a report that the *hscBA* genes are transcribed independently of the *isc* genes (15). Primer extension assays showed that *isc* operon induction by hydrogen peroxide (Fig. 5B) and paraquat (data not shown) was independent of both OxyR and SoxRS. These assays also allowed the transcription start to be mapped to a G residue (on

A

iscA iscU iscS yfhP
3.2   3.3   4.2   5.8
4.6   4.1   5.3   8.5

yfhP

2660149 catgtcttacttcacctcaaactcgcccctgcccgggggtttttttattgtaaaagtgggggg

yfhP

2660209 tattgcatagcagggtcaagtctgacattccccgagtaaattggtcaactatttacttgac
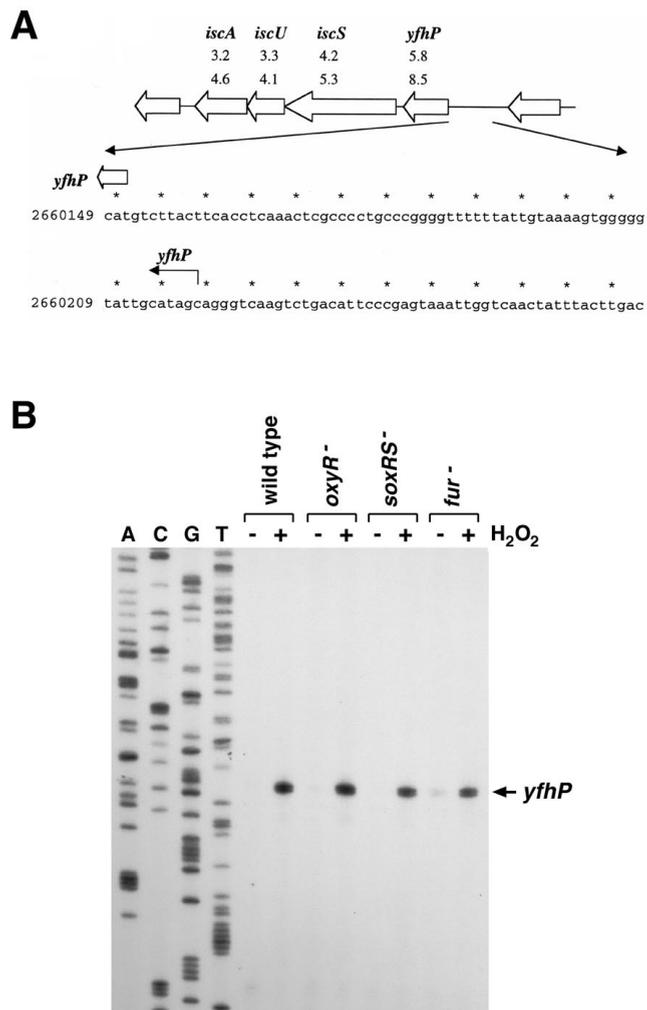
B

← yfhP

FIG. 5. OxyR- and SoxRS-independent induction of the *isc* operon. (A) Structure of the *isc* operon and sequence of the *yfhP* promoter. The induction ratios observed for the wild-type and $\Delta oxyR$ mutant strains in the microarray experiment are given below each gene. The *yfhP* transcription start is marked by the black arrow, and the start of the corresponding ORF is denoted by a white arrow. (B) Primer extension assays of *yfhP* expression in wild-type (MC4100), $\Delta oxyR$ (GSO47), $\Delta soxRS$ (GSO71), and Δfur (GSO72) strains grown in LB medium. Exponential-phase cultures were split into two aliquots: one aliquot was left untreated, and the other was treated with 1 mM hydrogen peroxide. The cells were harvested after 10 min, total RNA was isolated, and primer extension assays were carried out with primer 686 specific to *yfhP*. The neighboring sequencing reactions were carried out with the same primer.

the strand opposite the one shown in Fig. 5A) at position 2660219, 68 bp upstream of the *yfhP* start codon.

(iii) Induction of other genes. Among the other genes whose expression was strongly induced in an OxyR-independent manner were *cysK*, encoding cysteine synthase, which catalyzes the last step in cysteine synthesis; *sbp* and *cysP*, encoding periplasmic sulfate binding proteins; and *dsdA,* encoding a D-serine deaminase. We also observed modest induction of other genes in the cysteine biosynthesis pathway. The induction of these genes suggested a concerted effort to accumulate more cysteine in response to hydrogen peroxide treatment. Two other

genes, *tnaA* and *tnaL*, that were highly induced in an OxyR-independent manner are involved in amino acid catabolism. The *ibpA* and *ibpB* genes encoding heat shock proteins also were strongly induced in both the wild-type and $\Delta oxyR$ strains (Tables 1 and 2). The observed induction of the genes encoding the HSP20 chaperones in response to oxidative stress was consistent with a recent report that *ibpA*-, *ibpB*-, and *ibpAB*-overexpressing strains are resistant not only to heat but also to paraquat treatment (14).

There are 10 genes of unknown function among the most highly induced genes whose expression is independent of OxyR. *yfiA* was strongly induced (36-fold) in the wild-type strain and became the most strongly induced (109-fold) gene in the $\Delta oxyR$ mutant strain. The function of YfiA is not clear. Recent reports have shown that the YfiA protein is associated with 70S ribosomes and stabilizes ribosomes against dissociation (1, 16). Nine other strongly induced genes encode relatively small ORFs: *yjiD* (133 aa), *ymgB* (88 aa), *yeeD* (75 aa), *ycfR* (85 aa, induced 9-fold in the $\Delta oxyR$ strain), *ycgZ* (78 aa, induced 9-fold in the $\Delta oxyR$ strain), *ymgA* (90 aa, induced 3-fold in the wild type), *yceP* (84 aa, induced 10-fold in the wild type), *ynaF* (144 aa, induced 8-fold in the wild type), and *ycgK* (133 aa, induced 9-fold in the wild type). Homology searches suggest that *ynaF* encodes a filament protein. YeeD and YcfR show homology to other unknown ORFs, but all of the other genes encoding the small ORFs at best have very low scores in BLAST searches. We note that the small size of many of these predicted proteins may have precluded them from being detected on the two-dimensional gels initially used to characterize the response to hydrogen peroxide.

DISCUSSION

Use of DNA microarrays to characterize the oxidative stress response. DNA microarray technology already has been shown to be a useful tool in studying global expression patterns in response to a number of different growth conditions; here, we examine the *E. coli* response to oxidative stress. In the experiment that we have presented, we were able to confirm the induction of many oxidative stress genes that were laboriously identified over a period of almost 20 years. In addition, we were able to identify several new hydrogen peroxide-inducible genes: some new members of the OxyR regulon and others induced by an OxyR-independent mechanism. We do note a few limitations of our experiment. First, since the glass slides that we used carry only DNA corresponding to ORFs, we did not detect expression of the strongly induced OxyS RNA. Second, for reasons that are not understood, the induction of some of the OxyR-regulated ORFs such as *dsbG* was not detected. Thus, it has been an advantage to simultaneously carry out a computational search for additional OxyR binding sites (41). A third limitation is that the arrays are not strand specific. From the array data, we assumed that the annotated *ybjM* gene was induced by OxyR; however, primer extension and Northern experiments showed that hydrogen peroxide treatment actually leads to the induction of a transcript on the opposite strand. Despite the limitations listed above, future microarray experiments to examine the global gene response to hydrogen peroxide and other oxidants over a range of concentrations and times should give even further insight into the *E. coli* response

to oxidative stress. Experiments to examine the gene expression profiles in specific mutants also should help to further delineate the roles of specific regulators. For example, by examining the hydrogen peroxide response in strains lacking the OxyS small RNA regulator, we may be able to differentiate those genes regulated directly by OxyR and those regulated indirectly through OxyS RNA.

Identification of OxyR-regulated genes. We have identified several new OxyR-regulated genes. One example is *hemH* encoding the ferrochelatase that catalyzes the conversion of protoporphyrin IX to protoheme, the final step of protoheme biosynthesis. Heme is an essential cofactor for both the *katG*- and the *katE*-encoded hydroperoxidase enzymes, and it has long been known that *katG* transcription and hydroperoxidase I activity are strongly induced in response to oxidative stress. If ferrochelatase is the limiting step in protoheme biosynthesis, the induction of *hemH* may be needed to satisfy the need for increased heme levels associated with increased hydroperoxidase production. Protoporphyrin IX can generate reactive oxygen species in the presence of light (21). Thus, the induction of *hemH* may be important to reduce the concentration of the potentially toxic protoporphyrin IX intermediate. Since hydrogen peroxide oxidizes the pool of intracellular ferrous iron, increased ferrochelatase production also may be required to allow for the enzyme to compete for lowered levels of iron. Additional experiments are needed to distinguish between these possible explanations for the OxyR-dependent induction of *hemH*. It is interesting that, in *Bacillus subtilis*, an operon (*hemAXCDBL*) encoding enzymes for the early steps of heme biosynthesis is induced by hydrogen peroxide (6, 8), although our DNA microarray experiments did not provide evidence for the induction of the corresponding genes in *E. coli*.

We also discovered that the *sufA, sufB, sufC, sufD, sufS,* and *sufE* genes are part of the OxyR regulon. The observation that all of the genes were induced by hydrogen peroxide in an OxyR-dependent way is consistent with a previous proposal that these genes form an operon (24). Although the exact in vivo function of the *suf* operon is not clear, limited biochemical evidence suggests that *suf*-encoded proteins are involved in Fe-S cluster and/or S and/or Se metabolism. Since Fe-S clusters are one of the primary cellular targets of oxidative stress (reviewed in reference 29), there is a clear need to induce proteins that help to assemble or repair these clusters. Patzer and Hantke (24) isolated *sufD* and *sufS* mutants based on their requirement for the *fhuF*-encoded ferric ion reductase activity. Given this observation, it seems contradictory that the *suf* operon is induced by oxidized OxyR while *fhuF* is repressed by oxidized OxyR (41). We suggest that this opposing regulation indicates that the *suf*-encoded proteins are required for cellular functions in addition to FhuF. In general, since *hemH* and the *suf* operon, as well as some of the OxyR-regulated genes of unknown function, are highly conserved among prokaryotic species, future genetic and biochemical studies of these genes and their gene products will increase our understanding of cellular defenses against oxidative stress.

Overlap with other regulatory pathways. We observed *soxS* induction by 1 mM hydrogen peroxide in our DNA microarray measurements and, by primer extension assays, confirmed that *soxS* could be induced by 0.3 and 1 mM hydrogen peroxide. Recent assays of a SoxRS-regulated *micF::luxCDABE* fusion

(4) and measures of *soxS* transcript levels by multiplex reverse transcription-PCR (17) also suggest that SoxR is activated by high concentrations of hydrogen peroxide. These results expand the overlap between the superoxide and peroxide responses in *E. coli*. The observed *soxS* induction may be due to some SoxR oxidation by high peroxide concentrations. Alternatively, hydrogen peroxide might lead to the generation of another signal that activates SoxR or to inefficient SoxR reduction, by as yet uncharacterized mechanisms. Two known members of the SoxRS regulon, *fpr* and *sodA*, were among the most highly hydrogen peroxide-induced genes in both the wild-type and the ΔoxyR mutant cells. It is possible that the expression of other transcripts that are induced independently of OxyR is under the control of the SoxRS regulators.

Our DNA microarray results also show that there is overlap between the oxidative stress and heat shock and SOS responses. This overlap was presaged by the initial two-dimensional gel experiments (20, 33) as well as by assays of small panels of stress-responsive promoters fused to *luxCDABE* (4). A systematic identification of all the genes regulated by particular transcription factors should help to map out the complex genetic regulatory network among the different stress responses. Two recent studies have examined the whole-genome expression pattern in *Saccharomyces cerevisiae* cells exposed to a variety of stress conditions including oxidative stress and heat shock (7, 11). One difference between the bacterial and yeast responses to hydrogen peroxide is noteworthy; while there is a predominant, clearly defined OxyR-regulated response in *E. coli*, the *S. cerevisiae* response involves a large set of general stress proteins. In the ΔoxyR mutant strain, the induction of other general stress transcripts becomes more pronounced, making the peroxide response in the ΔoxyR strain more akin to the *S. cerevisiae* response. Possible parallels can be drawn between the response to oxidative stress and that to amino acid starvation. While wild-type *E. coli* cells induce specific operons in response to deprivation of specific amino acids (for example, Khodursky et al. [13] describe the specific response to tryptophan starvation), wild-type *S. cerevisiae* cells induce a generalized response upon encountering a limited supply of any one amino acid. Inactivation of an *E. coli* response to a particular amino acid-mediated regulatory circuit results in an emphasis upon the second, more generalized stringent response.

Our study points out that the activities of transcription factors, in addition to OxyR and SoxRS, may be modulated by oxidative stress. An example of such regulation is the *isc* operon. Both microarray and primer extension measurements showed that *isc* expression is induced by peroxide. Given the role of the *isc* gene products in Fe-S assembly, this induction is not surprising. However, it was unexpected to find that the induction is independent of both OxyR and SoxR, indicating the existence of an unidentified redox regulatory pathway. We also were intrigued by the strong OxyR-independent induction of the *yfiA* gene. Identification of these alternate pathways of hydrogen peroxide-dependent gene induction and the characterization of the redox sensing mechanisms involved are important directions for future studies.

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