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OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins

(oxidative stress/autoregulation/LysR/NodD)

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ABSTRACT **The *oxyR* gene is required for the induction of a regulon of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*. The *E. coli oxyR* gene has been cloned and sequenced, revealing an open reading frame (305 amino acids) that encodes a 34.4-kDa protein, which is produced in maxicells carrying the *oxyR* clone. The OxyR protein shows homology to a family of positive regulatory proteins including LysR in *E. coli* and NodD in *Rhizobium*. Like them, *oxyR* appears to be negatively autoregulated: an *oxyR*::*lacZ* gene fusion produced 5-fold higher levels of β-galactosidase activity in *oxyR* null mutants compared to *oxyR*$^+$ controls, and extracts from an OxyR-overproducing strain were able to protect regions (−27 to +21) of the *oxyR* promoter from DNase I digestion. DNA sequence analysis of the *oxyR2* mutation, which causes overexpression of *oxyR*-regulated proteins in the absence of oxidative stress, showed that the *oxyR2* phenotype is due to a missense mutation (C·G to T·A transition) that changes alanine to valine at amino acid position 234 of OxyR.**

Cloning and DNA sequence analysis of the genes encoding bacterial and phage transcriptional regulatory proteins has facilitated the understanding of the molecular mechanisms used to control gene expression by directing attention to a few evolutionarily conserved domains in these regulatory proteins (1–4). Numerous transcriptional regulatory proteins contain a conserved "helix-turn-helix" domain by which many bacterial (and some eukaryotic) proteins recognize and bind to specific DNA sequences (reviewed in refs. 1 and 5). Several bacterial proteins have since been found to be alternative sigma factors for RNA polymerase (reviewed in ref. 2). Recently, it has also been noted that for many bacterial regulons, two proteins are involved in regulating gene expression and that these proteins share homology (reviewed in ref. 3). In these systems, one protein is thought to function as the sensor of an environmental change and the other component is a transcriptional activator. Through extensive sequence comparisons, Kofoid and Parkinson (4) have extended these classes and denoted transmitter and receiver domains in many bacterial regulatory proteins.

We have previously described a regulator, *oxyR*, of hydrogen peroxide-inducible genes in *Salmonella typhimurium* and *Escherichia coli* (6). Treatment of bacterial cells with low doses of hydrogen peroxide induces resistance to subsequent lethal doses of hydrogen peroxide and the synthesis of 30 proteins. Nine of these proteins, including catalase, glutathione reductase, and an alkyl hydroperoxide reductase, require the *oxyR* gene for hydrogen peroxide induction. Strains with deletions of *oxyR* are unable to induce this regulon and are hypersensitive to hydrogen peroxide. Strains carrying a dominant mutation, *oxyR1* in *S. typhimurium* and *oxyR2* in *E. coli*, are resistant to hydrogen peroxide killing and constitutively overproduce the *oxyR*-regulated proteins. As a first step toward understanding the molecular mechanism of OxyR action, we have sequenced the *oxyR*$^+$ and *oxyR2* genes from *E. coli* and investigated the regulation of *oxyR*. Here we report that OxyR is a member of a family of bacterial regulator proteins.§

MATERIALS AND METHODS

Plasmids. The inserts carried by pAQ16-pAQ21 are illustrated in Figs. 1 and 2*A*. pAQ16 and pAQ17 are derived from pUC12 (9). pAQ19 and pAQ21 carry inserts cloned into pUC18, and pAQ18 and pAQ20 carry inserts in pUC19. pAQ22 carries a 2.3-kilobase (kb) fragment isolated from TA4110 (*oxyR2*) chromosomal DNA digested with *Bam*HI and *Eco*RI and cloned into pUC18. pAQ23 carries a 0.2-kb *Hin*cII/*Ssp* I fragment of pAQ17 cloned into the unique *Sma* I site of pRS415 (10).

DNA Manipulations. DNA isolation, analysis, and cloning were generally carried out as described by Maniatis *et al.* (11). DNA sequencing was carried out by the dideoxynucleotide chain-termination method (12) with Klenow enzyme (Boehringer Mannheim) or Sequenase (United States Biochemical) enzyme. The C·G to T·A mutation at amino acid position 234 was generated by using an oligonucleotide-directed *in vitro* mutagenesis system from Amersham.

Maxicells. Plasmid-encoded proteins were labeled with [^{35}S]methionine in maxicells as described by Sancar *et al.* (13). CSR603 (13) cells carrying the desired clones were grown to midlogarithmic phase (A_{600} = 0.41–0.52) in M9 medium containing 1% Casamino acids and thiamine (0.1 μg/ml) and then irradiated 15 sec with a GE germicidal lamp at a 29.5-cm distance. After a 1-hr recovery, cells were treated with cycloserine (100 μg/ml) and incubated in the dark for 8–12 more hr. The cells were then collected, resuspended in Hershey medium, and incubated for another hour, after which 5 μl of [^{35}S]methionine (50 μCi; 1 Ci = 37 GBq) was added for 1 hr. Cells were collected, resuspended in Laemmli (14) buffer, and electrophoresed on a 12% polyacrylamide gel.

Primer Extension. Total cellular RNA was isolated from K12 and TA4112 by phenol extraction as described (15). A 30-nucleotide (CTCAAGATCACGAATATTCATTATCCATCC) single-stranded end-labeled synthetic oligonucleotide was added to 25 μg of RNA, was allowed to anneal, and then extended with reverse transcriptase (Life Sciences,

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04553).

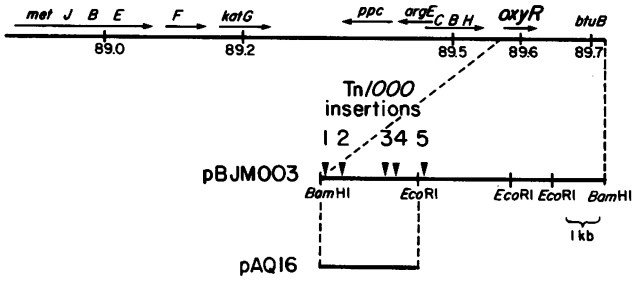

FIG. 1. Map position of *oxyR* in *E. coli*. The map position of *oxyR* relative to *metJBLF*, *katG*, *ppc*, *argECBH*, and *btuB* is based on the phenotype of Tn*10*-mediated deletions in *E. coli* and *S. typhimurium*. (The gene orientations were taken from refs. 7 and 8.) The lines below the chromosomal map denote the DNA carried by pBJM003 and the pAQ16 subclone.

Saint Petersburg, FL) as described (16). The extended products were analyzed by electrophoresis on 8% denaturing polyacrylamide gels. Dideoxynucleotide sequencing reactions primed from the end-labeled oligonucleotide were electrophoresed in neighboring lanes.

β-Galactosidase Assays. β-Galactosidase assays were performed as described by Miller (17) after cells were lysed as described (18).

Footprinting. *oxyRΔ3* strains carrying pMC7 (carrying *lacI*^{q}; ref. 19) and pKK177-3 (carrying the *tac* promoter, derived from pKK223-3; ref. 20) or pAQ25 (*oxyR* with the *des* Shine–Dalgarno sequence cloned behind the *tac* promoter in pKK177-3; G.S., L. A. Tartaglia, and B.N.A., unpublished data) were treated with 1 mM isopropyl β-D-thiogalactopyranoside for 2 hr. Samples (5 ml) of the treated cells were then centrifuged and the pellets were resuspended in 800 μl of 10 mM Tris·HCl (pH 7.5). The cells were lysed by two 10-sec sonication steps, centrifuged 5 min at 3000 × *g* to remove unlysed cells, followed by a second centrifugation at 13,000 × *g* to remove the membrane fraction. The footprinting reactions were carried out as described (21). Aliquots of the soluble fraction (final protein concentration, 5 or 50 μg/ml) were incubated with a 200-base-pair (bp) *Bam*HI/*Tth*111-I *oxyR* fragment (5 fM, labeled at the *Bam*HI site with [γ-^{32}P]ATP) and poly(dI-dC) competitor DNA (0.1 μg) for 10 min at room temperature, after which DNase I (2 μl; 1.25 μg/ml; Cooper Biomedical) was added for 1 min. The cleaved products were analyzed on an 8% denaturing polyacrylamide gel alongside a G/A sequencing ladder.

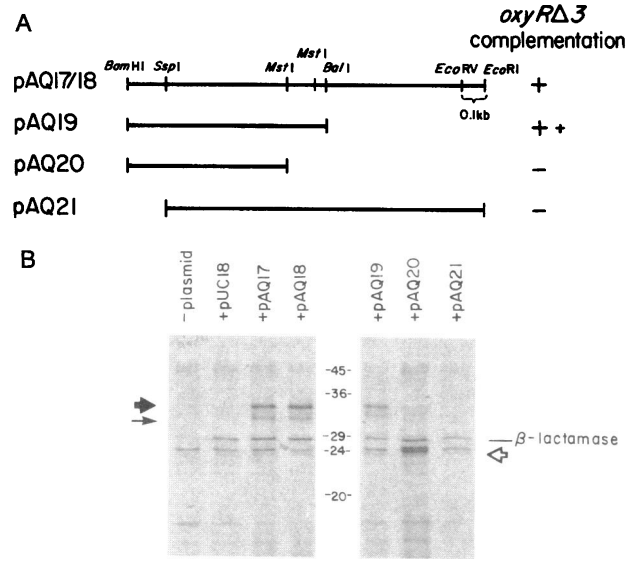

FIG. 2. Proteins encoded by *oxyR* subclones. (*A*) DNA carried by the *oxyR* subclones. pAQ17, pAQ18, and pAQ19, but not pAQ20 and pAQ21, are able to complement the hydrogen peroxide sensitivity of *oxyRΔ3*. (pAQ17 and pAQ18 carry the same 1.5-kb fragment cloned in opposite orientations relative to the *lacZ* promoter in pUC12 and pUC19.) (*B*) Autoradiograph corresponding to the proteins produced by maxicells carrying various *oxyR* subclones. The large solid arrow points to the prominent 34-kDa protein encoded by pAQ17, pAQ18, and pAQ19. The open arrow denotes a prominent species <34 kDa encoded by pAQ20. (The small arrow indicates a minor 32-kDa species, possibly a modified form of OxyR, encoded by pAQ17, pAQ18, and pAQ19.)

RESULTS

Cloning *oxyR* from *E. coli*. We previously isolated deletions of *oxyR* in *S. typhimurium* (*oxyRΔ2*, TA4108) and *E. coli* (*oxyRΔ3*, TA4112) by selecting for the loss of Tn*10* insertions in the nearby *argH* (89.5 min) and *btuB* genes (89.7 min) (6). Since Tn*10*-mediated deletions extend in only one direction from the original insertion, *oxyR* had to be located in the region between *argH* and *btuB* as shown in Fig. 1. A 15.4-kb clone (pBJM003) carrying the *E. coli btuB* gene and a series pBJM003 derivatives carrying Tn*1000* (γ-δ) insertions were described by Heller *et al.* (22). pBJM003 and five insertion derivatives were transformed into the *E. coli oxyR* deletion strain, and transformants were then tested for sensitivity to hydrogen peroxide and cumene hydroperoxide. The hypersensitivity to peroxides exhibited by *oxyRΔ3* is corrected by pBJM003 and all of the Tn*1000* derivatives except pBJM003-Tn*1000-2* (data not shown). Therefore, the *oxyR* gene is located at or near the site of the Tn*1000-2* insertion, about 800 bp from the *Bam*HI site in pBJM003.

A 2.3-kb *Eco*RI/*Bam*HI fragment of pBJM003, which carries the site of Tn*1000-2* insertion, was isolated and cloned into the *Eco*RI and *Bam*HI sites of pUC12 (pAQ16). This 2.3-kb insert complemented the peroxide hypersensitivity of *oxyRΔ3* (Table 1). To further define the sequences required for complementation of the chromosomal deletion of *oxyR*, a 1.0-kb internal *Eco*RV fragment was removed from the 2.3-kb clone to give pAQ17 and pAQ18. pAQ17 and pAQ18 were still able to complement the *oxyR* deletion strain (Table 1). The restriction map of pAQ17 is given in Fig. 2*A*, and the DNA carried by further subclones is indicated. The ability of the subclones to complement the *oxyRΔ3* sensitivity to hydrogen peroxide and cumene hydroperoxide is reflected by the zones of inhibition given in Table 1. pAQ19 still complements the *oxyRΔ3* sensitivity and even confers slightly greater resistance than any of the larger clones. pAQ20 and pAQ21 no longer complement *oxyRΔ3*, indicating that essential portions of the *oxyR* gene have been deleted. pAQ16 and pAQ17 were also found to restore the inducibility of *oxyR*-regulated proteins by hydrogen peroxide as determined by two-dimensional protein gels in *oxyR* deletion strains (data not shown).

Table 1. Sensitivity to killing of strains carrying *oxyR* clones

		Zones of inhibition, mm	
		Hydrogen peroxide (10%)	Cumene hydroperoxide (5%)
TA4467	*oxyRΔ3*/pUC18	35	26
TA4470	*oxyRΔ3*/pAQ16	25	20
TA4471	*oxyRΔ3*/pAQ17	25	20
TA4472	*oxyRΔ3*/pAQ18	23	19
TA4473	*oxyRΔ3*/pAQ19	20	18
TA4474	*oxyRΔ3*/pAQ20	34	25
TA4475	*oxyRΔ3*/pAQ21	35	26

The zones of inhibition were determined as described (6) except that the strains were grown in and plated on Luria broth (LB) containing ampicillin. The zone sizes in mm are the means of three determinations.

OxyR Is a 34-kDa Protein. The proteins encoded by the various *oxyR* subclones were examined by labeling the plasmid-encoded proteins in maxicells. The vector control (pUC18) and pAQ17, -18, -19, -20, and -21 were transformed into CSR603, a *recA*$^-$, *uvrB*$^-$ strain. When this strain is irradiated with UV light, most of the chromosomal DNA is damaged, but, because of the large number of plasmids, some of these escape UV damage. The cells are unable to repair the UV damage and degrade all the damaged DNA. If [^{35}S]methionine is added several hours later, only proteins encoded by the undamaged plasmid DNA are synthesized and labeled. As shown in Fig. 2*B*, the clones capable of complementing the *oxyR*Δ*3* strain (pAQ17, -18, -19) encode a prominent 34-kDa protein and a minor 32-kDa protein that are not present in the pUC18 vector control lane. The cells carrying pAQ20 encode a protein that is smaller than 34 kDa, possibly a truncated form of the major 34-kDa species, suggesting that the *Mst* I end of the clone corresponds to the C-terminal end of the OxyR protein. pAQ21 does not encode any plasmid-specific proteins other than the β-lactamase, suggesting that the *Bam*HI to *Ssp* I section of the *oxyR* clone may carry the *oxyR* promoter region.

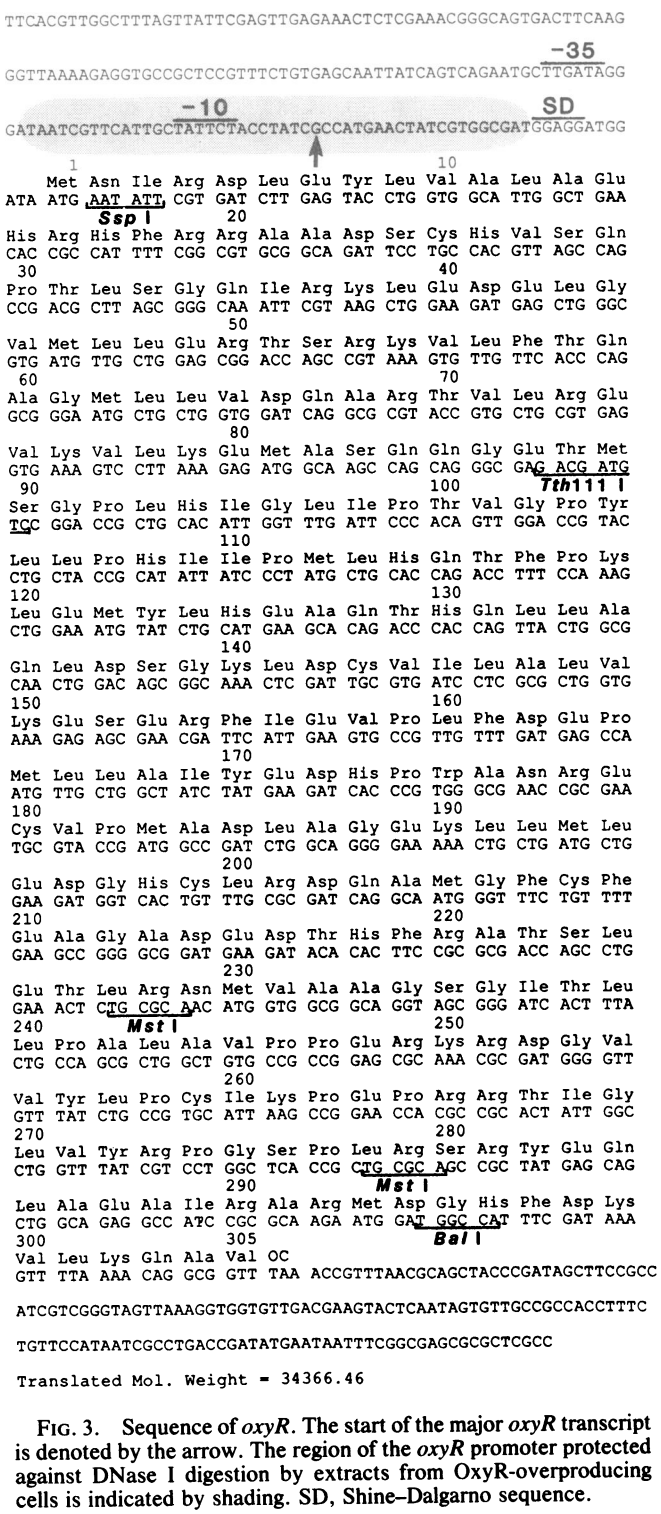

FIG. 3. Sequence of *oxyR*. The start of the major *oxyR* transcript is denoted by the arrow. The region of the *oxyR* promoter protected against DNase I digestion by extracts from OxyR-overproducing cells is indicated by shading. SD, Shine–Dalgarno sequence.

The Sequence of *oxyR* Reveals Homology to a Family of Transcriptional Activator Proteins in Bacteria. The DNA sequence of a 1200-bp region extending from the *Bam*HI site toward the *Eco*RI site was determined. The one long open reading frame encoded by the DNA sequence corresponds to a 34.4-kDa protein extending from the *Bam*HI site toward the *Eco*RI end (Fig. 3). The size and orientation of the protein agree with the maxicell data. The predicted charge of the OxyR protein (pI 6.18–6.9) agrees with the position of the protein on two-dimensional gels probed with antibodies directed against a β-galactosidase–OxyR fusion protein (G.S. and B.N.A., unpublished results), and the codon usage is typical of *E. coli* (23). To confirm that the reading frame was correct, we created a frameshift mutation by filling-in the unique *Tth*111-I site within *oxyR* (a net two-nucleotide insertion). The frameshift mutant was unable to complement the hydrogen peroxide sensitivity of an *oxyR* deletion (data not shown).

Comparing the amino acid sequence of the OxyR protein with sequences in the Dayhoff data bank, we found that OxyR shows extensive homology to the *E. coli* protein LysR (Fig. 4). LysR is a positive regulator of *lysA*, which encodes the enzyme responsible for the last step in lysine biosynthesis (24, 27). OxyR also shares significant homology with the *Rhizobium meliloti 1021* protein NodD (25) (Fig. 4) and other *Rhizobium* NodD proteins (28). NodD regulates several genes involved in the formation of nodules in the symbiotic relationship between *Rhizobium* and legumes (25, 28). Henikoff *et al.* (29) have recently recognized that these proteins and others [*E. coli* IlvY, regulator of *ilvC* and other genes in the isoleucine–valine biosynthetic pathway (30); *E. coli* and *S. typhimurium* CysB, positive regulator of the cysteine regulon (31); *S. typhimurium* MetR, regulator of the *metE* and *metH* genes (32); and the *Enterobacter cloacae ampR* regulator of the *ampC* gene, which confers resistance to cephalosporin (33)] comprise a family of bacterial regulatory

FIG. 4. Homology among OxyR, LysR, and NodD. Shading between residues indicates identity or conservative leucine–valine–isoleucine substitutions between OxyR and LysR (24) and NodD (25). Inducer-independent missense mutations in OxyR and NodD (26) are indicated with arrows. Amino acids are designated by the single-letter code.

proteins. The sequence of *Pseudomonas aeruginosa trpI*, the positive regulator of the *trpAB* operon, has shown that TrpI is yet another member of the family (34). All of the bacterial regulatory proteins described above are between 30 and 35 kDa in size and act as transcriptional activators. The most extensive homology is in the N-terminal ends of the proteins, although there is homology throughout the amino acid sequences.

Start of the *oxyR* Transcript. Total cellular RNA was isolated from wild-type and *oxyR2* mutant cells. The start point of the *oxyR* transcript was determined by extension of a primer hybridizing to the 5′ region of the *oxyR* mRNA (Fig. 5). The major transcript starts with a G residue. Corresponding −10 and −35 sequences that contain five of the six residues found in the canonical sigma70 promoters are indicated in Fig. 3. The similar levels of the *oxyR* transcript in both the wild-type and *oxyR2* mutant strains indicated by the approximately equal intensity of the primer-extension products suggests that the constitutive phenotype of the *oxyR2* mutant is not due to vast overproduction of the *oxyR* message and protein.

The Constitutive Phenotype of the *oxyR2* Mutant Is Due to an Alanine to Valine Missense Mutation. An *oxyR2* mutant strain constitutively overexpresses *oxyR*-regulated catalase and alkyl hydroperoxide reductase activities (6). To learn more about the mechanism of *oxyR* activation, the mutant *oxyR2* gene was cloned. Chromosomal DNA was isolated from the *oxyR2* mutant and digested with *Eco*RI and *Bam*HI. Fragments between 2.0 and 2.3 kb were cloned into the *Eco*RI and *Bam*HI sites of pUC18. Colonies obtained after transforming *oxyRΔ3* were screened for hybridization to a fragment of the wild-type *oxyR* clone. Three *oxyR2* clones were isolated in this fashion, of which pAQ22 is one representative. Although *oxyRΔ3* carrying pAQ22 (TA4476) was very slow growing and pAQ22 was unstable, the strain had increased levels of the *oxyR*-regulated proteins and increased resistance to hydrogen peroxide (data not shown).

We sequenced the entire *oxyR2* coding region and found that the only difference between *oxyR2* and the wild-type sequence was a C·G to T·A missense mutation causing a valine to be substituted for an alanine at amino acid position 234. To confirm that the C·G to T·A missense mutation was in fact the sole mutation to cause the *oxyR2* constitutive phenotype, this transition mutation was also generated by site-specific mutagenesis. The site-specific mutant contained on pUC18 conferred the same phenotypes as pAQ22, showing that the valine for alanine substitution at position 234 in OxyR is sufficient to cause constitutive induction of hydrogen peroxide-inducible genes.

***oxyR* Is Negatively Autoregulated by the Binding of OxyR to Its Own Promoter.** To study the expression from the *oxyR* promoter, the *oxyR* upstream and promoter region sequences were fused to the *lacZ* gene on a multicopy vector (pRS415) to give pAQ23. This construct was moved into *oxyR*$^+$ (TA4477) and *oxyRΔ3* (TA4478) backgrounds, and the level of β-galactosidase activity was determined during exponential growth. Interestingly, the *oxyR*::*lacZ* fusion gave 5-fold higher levels of activity in the *oxyR* deletion background (19,000 units; A_{600} = 0.593) compared to the *oxyR*$^+$ background (3800 units; A_{600} = 0.613). This finding suggests that OxyR is a negative regulator of its own expression.

To learn more about the nature of the repression we investigated the ability of the OxyR protein to bind its own promoter. Extracts made from cells that greatly overproduce the OxyR protein were added to *oxyR* promoter fragments and then tested for the ability to protect against DNase I digestion. A distinct region of protection was seen extending from −27 to +21 relative to the start of the *oxyR* transcript (Fig. 6). This protection is not seen with extracts made from strains that do not overproduce OxyR and suggests that the mechanism by which OxyR negatively autoregulates its expression is by binding to, and therefore blocking, the DNA sequences that are required for its own transcription.

DISCUSSION

The cloning and DNA sequence of the *E. coli oxyR* gene, a positive regulator of a regulon for defenses against oxidative stress, revealed that OxyR is a 34-kDa protein that shows significant homology to a family of bacterial regulatory proteins. All of these regulators are between 30 and 35 kDa in size, all act as positive regulators, and many are known to

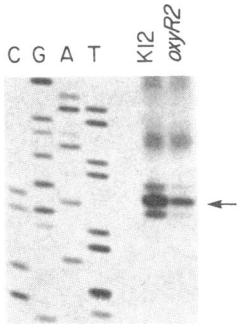

FIG. 5. Start of the *oxyR* transcript. The start of the major *oxyR* transcript in wild-type and *oxyR2* mutant cells was determined by primer-extension studies. Occasional double bands in the adjacent sequencing ladder are due to hybridization between the 30-base primer and M13.

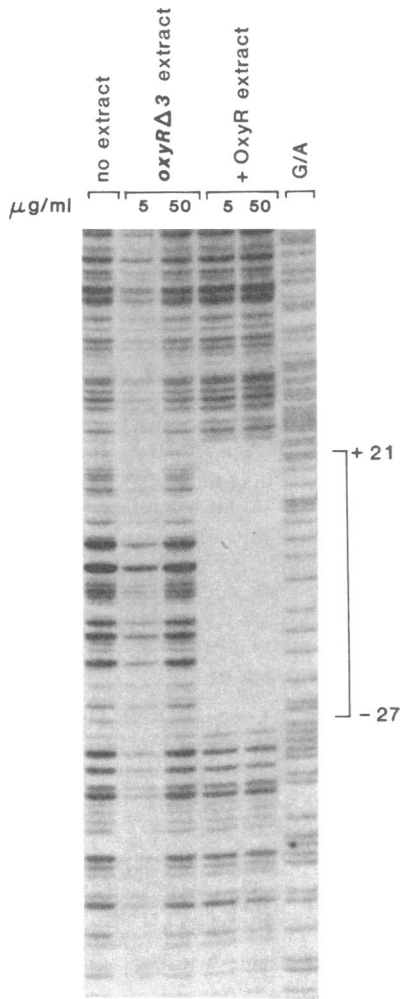

FIG. 6. Footprint of OxyR on the *oxyR* promoter. A 5′-end-labeled *oxyR* promoter fragment was incubated with extracts from an *oxyR* deletion strain or an OxyR-overproducing strain (final concentration, 5 and 50 μg of protein per ml) and then digested with DNase I as described. The protection is shown for the noncoding strand.

negatively regulate their own expression (29). The role of *oxyR* as a positive regulator has been described (6). The increased expression from a heterologous *oxyR::lacZ* gene fusion in an *oxyR* deletion strain compared to an $oxyR^+$ strain suggests that OxyR is also a negative regulator of its own expression. Interestingly, while OxyR, LysR, NodD, IlvY, and MetR all negatively regulate their own genes, they positively regulate a transcript from an overlapping promoter on the complementary strand (29, 36). The *oxyR* system differs from the other systems in that the transcript made opposite the *oxyR* structural gene is very small (only 100 nucleotides) and probably does not encode a protein (G.S. and B.N.A., unpublished data).

The footprinting experiments described here suggest that the OxyR protein binds to DNA. Purified OxyR can also protect specific regions of other *oxyR*-regulated promoters from DNase I digestion (G.S., L. A. Tartaglia, and B.N.A., unpublished data). Based on sequence analysis, Henikoff *et al.* (29) suggest that the LysR class of bacterial regulators have helix-turn-helix motifs toward the N-terminal domains of the proteins, but proof that these helices are interacting with the DNA will require structural studies. Another member of the LysR class, NodD, has been shown to bind a defined DNA sequence (NOD box; ref. 35), and studies on chimeric NodD proteins with C-terminal domains from different strains of *Rhizobium* do suggest that the N-terminal portion of the protein is involved in DNA recognition (28).

The inducer-responsive domain may be near the C terminus. Our finding that the mutation causing the constitutive phenotype maps within this region supports this claim. An inducer-independent *nodD* mutation was also found to map in the C-terminal region of NodD (ref. 26; also indicated in Fig. 4). It was surprising that the constitutive *oxyR2* phenotype was solely due to an alanine to valine missense mutation, but it has been found that an inducer-independent *crp* mutation can also be caused by an alanine to valine change (Sankar Adhya, personal communication). Possibly, the slightly larger size of the valine compared to the alanine causes a significant conformational change.

The molecular mechanism by which the OxyR protein is activated by oxidative stress is of interest. The fact that the constitutive phenotype of the *oxyR2* mutant is due to a missense mutation in OxyR and not due to overproduction of *oxyR* mRNA suggests that the OxyR protein itself may be modified to induce the regulon under stress conditions. It may be significant that none of the six cysteine residues found in the OxyR protein is present in LysR or NodD since cysteines might easily be oxidized. We are using the cloned *oxyR* gene to overproduce OxyR and to allow purification of the protein to determine the exact manner of OxyR activation by oxidative stress.

Note Added in Proof. We have recently learned that OxyR corresponds to the *mom* gene repressor (MomR), the methylation-dependent regulator of the *mom* gene of phage Mu (M. Bölker and R. Kahmann, personal communication).

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