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Mutational Analysis To Define an Activating Region on the Redox-Sensitive Transcriptional Regulator OxyR[▽]

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The OxyR transcription factor is a key regulator of the *Escherichia coli* response to oxidative stress. Previous studies showed that OxyR binding to a target promoter enhances RNA polymerase binding and vice versa, suggesting a direct interaction between OxyR and RNA polymerase. To identify the region of OxyR that might contact RNA polymerase, we carried out alanine scanning and random mutagenesis of *oxyR*. The combination of these approaches led to the identification of several mutants defective in the activation of an OxyR target gene. A subset of the mutations map to the DNA-binding domain, other mutations appear to affect dimerization of the regulatory domain, while another group is suggested to affect disulfide bond formation. The two mutations, D142A and R273H, giving the most dramatic phenotype are located in a patch on the surface of the oxidized OxyR protein and possibly define an activating region on OxyR.

The OxyR transcription factor was originally identified as a regulator of the *Salmonella enterica* serovar Typhimurium and *Escherichia coli* responses to hydrogen peroxide but has since been discovered in many bacterial species (reviewed in references 13 and 21). The protein is both the sensor and the transducer of peroxide stress. During normal growth, OxyR is reduced and acts as a repressor of a subset of genes, including negative autoregulation of its own expression. Upon exposure to elevated levels of hydrogen peroxide, OxyR is oxidized and activates the expression of a regulon of genes encoding defense activities. In previous studies, we showed that OxyR is activated by reversible disulfide bond formation between cysteine residues 199 and 208 (1, 9, 26) and that the reduced and oxidized forms of the tetrameric transcription factor make different contacts along the promoter DNA (25). The solutions of the crystal structure of the regulatory domain (residues 80 to 305) in the reduced and oxidized conformation showed that the two redox-active cysteines are approximately 17Å apart in the reduced structure and that disulfide bond formation in the oxidized form results in a significant structural change in the regulatory domain (3). The different orientations of the monomers relative to each other in the reduced and oxidized conformations can explain the different DNA-binding footprints observed for the two forms of OxyR.

OxyR is a member of the LysR family of transcriptional regulators. This family comprises the most abundant class of transcriptional regulators in bacterial cells (12). Most of the LysR-type regulators are between 30 and 35 kDa in molecular size and form homodimers or homotetramers. The proteins have a very conserved amino-terminal domain containing a helix-turn-helix DNA-binding motif. Unlike OxyR, most of the LysR family members are activated by binding to effector molecules. However, for all of the LysR-type transcriptional regulators, the carboxy-terminal part of the protein constitutes the inducer-binding or regulatory domain.

OxyR, like most of the LysR family members, binds overlapping or adjacent to the promoter to repress or activate transcription (reviewed in reference 16). Footprinting experiments showed oxidized OxyR increases RNA polymerase binding to the OxyR-dependent promoters, suggesting that OxyR activates transcription by recruiting RNA polymerase (8). This interaction may be due to direct contacts between the α subunit of RNA polymerase (encoded by *rpoA*) and OxyR, since strains expressing α mutants lacking the carboxy-terminal domain (α-CTD) are unresponsive to OxyR activation (22). A screen for *rpoA* mutations that resulted in decreased OxyR activation of the *katG* target gene led to the isolation of 11 α mutants with substitutions for amino acids 265, 268, 269, 293, 294, 298, 299, 300, and 307 in the α-CTD (23). However, it has not been established whether OxyR makes direct contacts with this domain of RNA polymerase. Since all of the mutations map to the α DNA-binding domain, the reduced activation observed may solely be due to reduced RNA polymerase binding to the *katG*, *ahpC*, and *oxyS* promoters rather than an OxyR contact site on the α-CTD. To gain insight into possible contacts between OxyR and RNA polymerase, we carried out alanine scanning and random mutagenesis of *oxyR* and screened for mutants unable to activate transcription.

MATERIALS AND METHODS

Plasmids and bacterial strains. Derivatives of pAQ5 (20) were used for all phenotypic assays. For the overexpression and purification of mutant OxyR

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TABLE 1. *E. coli* strains used for this study

Strain	Relevant genotype	Source or reference
DH5α		BRL
XL1-Blue		Stratagene
XL1-Red	*mutD5 mutS mutT* Tn*10*(tet)	Stratagene
TA4484	*oxyR*Δ*3*/pMC7	24
N9716	GC4468 Δ*oxyR*::Sp	W. Gillette
FÅ369	Δ*ahpCF*::Kan	1
GSO5	Δ*oxyR*::Kan Φ(*oxyS-galK*)	8
GSO130	MC4100 Φ(*oxyS-lacZ*)	25
GSO131 (WX16)	MC4100 Φ(*oxyS-lacZ*) Δ*oxyR*::Sp	This study
GSO132 (WX21)	MC4100 Φ(*oxyS-lacZ*) Δ*oxyR*::Sp Δ*ahpCF*::Kan	This study
GSO133	MC4100 Φ(*oxyR-lacZ*) Δ*oxyR*::Kan	25

proteins, the 0.8-kb RsrI/HindII *oxyR* fragment from the pAQ5 derivatives was used to replace the same fragment in pGSO69 (8). The bacterial strains used in the present study are listed in Table 1. DH5α and XL1-Blue were routinely used for plasmid preparation. GSO5 (8) was used for oxidant sensitivity test and for primer extension assays, TA4484 (24) was used for the overexpression and purification of mutant OxyR proteins, and GSO133 (25) was used for the *oxyR-lacZ* fusion assays. The Δ*oxyR*::Sp deletion was moved into GSO130 (25) by P1 transduction from N9716 (kindly provided by W. Gillette) to generate GSO131 (WX16). The Δ*ahpCF*::Kan deletion similarly was introduced into GSO131 by P1 transduction from FÅ369 (1) to construct GSO132 (WX21). P1 transductions were carried out as described previously (18).

Growth conditions. Strains were routinely grown at 37°C in Luria-Bertani (LB) medium (11). Ampicillin (50 to 100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (Kan; 25 μg/ml), streptomycin (Sp; 25 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 40 μg/ml) was added where appropriate. Mutants were identified on MacConkey or tetrazolium medium containing 1% lactose (11, 18).

Mutagenesis. The desired alanine substitutions were generated by using the QuikChange site-directed mutagenesis kit (Stratagene) and oligonucleotides carrying the appropriate codon substitutions. Two approaches were used to create the random mutations in the *oxyR* gene carried on pAQ5. First, pAQ5 was transformed into a mutator strain (XL1-Red). The plasmid isolated from about 500 transformants grown in LB medium with chloramphenicol for ~24 h was then used to transform GSO132. As a second approach, chemical mutagenesis of pAQ5 was carried out as described previously (8). Briefly, 20 μl of pAQ5 plasmid DNA (5 μg) was mixed with 80 μl of 0.5 M potassium phosphate buffer (pH 6.0) containing 5 mM EDTA and 100 μl of 1 M hydroxylamine. The mixture was incubated at 65°C for 60 min. The DNA was dialyzed extensively with Tris-Cl–EDTA buffer and then used to transform XL1-Blue. The XL1-Blue transformants were collected, and plasmid DNA was again isolated and used to transform GSO132. For each mutant, the sequence of the entire *oxyR* gene was confirmed by sequencing.

Zone-of-inhibition assays. Aliquots (0.1 ml) of overnight cultures were mixed with 2.5 ml of top agar and plated on LB medium containing the appropriate selection. Disks impregnated with 10 μl of either 10% H_2O_2 or 4% cumene hydroperoxide were placed on the plates. The zones of inhibition surrounding the disks were measured after overnight incubation.

Primer extension assays. Cultures were grown to an optical density at 600 nm of 0.3 to 0.5 and split; half was left untreated, and the other half was exposed to 0.2 mM hydrogen peroxide. The cultures were shaken for 5 min, and the cells were collected. The total RNA was extracted by using TRIzol reagent (BRL). Primer extension assays were carried out with 5 μg of total RNA and an oligonucleotide (5′-GCAAAAGTTCACGTTGG) complementary to the *oxyS* gene that was labeled with T4 polynucleotide kinase. The probe was annealed to the RNA, and a 60-min extension reaction was carried out at 42°C using reverse transcriptase (Life Sciences Inc.). The products were analyzed by using an 8% sequencing gel.

Protein expression and purification. The wild-type and mutant OxyR proteins were overexpressed and purified as described previously (8). Briefly, TA4484 carrying the pGSO69 derivatives was grown to an optical density at 600 nm of 0.5, and expression was induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. Cells were harvested and lysed by three passages through a French press. Cell debris was removed by centrifugation, and the OxyR protein in the supernatant was purified by passage over heparin-Sepharose and Mono-S columns (Pharmacia).

DNase I footprinting assays. DNase I footprinting assays were carried out as described previously (24). An end-labeled DNA fragment was incubated with 50 to 200 ng of purified protein in 25 μl of 0.5× TM buffer. The binding reaction mixtures were then treated with DNase I for 2 min, extracted with phenol-chloroform, and examined on 8% sequencing gels.

Immunoblotting. α-OxyR antiserum was generated by immunizing rabbits with purified OxyR protein (Covance). Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide and transferred to a nitrocellulose filter by electroblotting. The filter was probed with a 1:10,000 dilution of antiserum. Bound antibody was visualized by rabbit antiserum using the enhanced chemiluminescence Western blotting system from Amersham.

β-Galactosidase assays. β-Galactosidase assays were carried out according to the method of Miller (11).

RESULTS AND DISCUSSION

Alanine-scan screen for activation-defective mutants. The DNA-binding domain of LysR type factors is highly conserved and located at the amino termini of these proteins (reviewed in reference 16). Based on the assumption that regions important for the interaction with RNA polymerase also may be somewhat conserved among LysR family proteins, we aligned several *E. coli* members of this family of transcriptional regulators (Fig. 1). We noticed two patches of conservation in addition to the DNA-binding domain. One region spans residues 135 to 145, and another region spans residues 231 to 241, encompassing the position of the A233V mutation found for the *oxyR2* constitutive mutant (4). To determine whether any of these residues are important for transcriptional activation by OxyR, we substituted alanine for each of the amino acids. The mutant derivatives carried on the pACYC184 plasmid were then introduced into the *oxyR* deletion strain GSO5, and the strains were examined for their sensitivity to hydrogen peroxide and cumene hydroperoxide (Table 2). These assays revealed that two substitutions, D142A and T238A, resulted in significantly increased sensitivity to both oxidants. All other mutants showed wild-type or only slightly increased sensitivity to the peroxides. The expression of *oxyS*, an OxyR target gene, also was examined by primer extension assays in these mutant strains (Fig. 2). The results of the primer extension assay were consistent with those of the oxidant inhibition test. The D142A and T238A mutants showed significantly decreased induction of the OxyR target gene, indicating that the two mutants cannot activate transcription in response to hydrogen peroxide treatment. The L239A and V231A mutants also showed reduced induction of the OxyS RNA in the primer extension assay but were not hypersensitive to the oxidants. For the L239A mutant, the wild-type resistance most likely is due to the partially constitutive nature of this mutant; some OxyS expression was observed even in the absence of hydrogen peroxide treatment.

The T238A mutant is locked in a reduced conformation. Examination of the structure of the OxyR regulatory domain, solved during the course of these studies (3), showed that the T238 residue is buried in the core of the region containing the redox-active C199 and C208 residues involved in disulfide bond formation. This proximity of the T238 residue to the redox-active cysteines raised the possibility that the activation defect in the T238A mutant might be due to defective disulfide

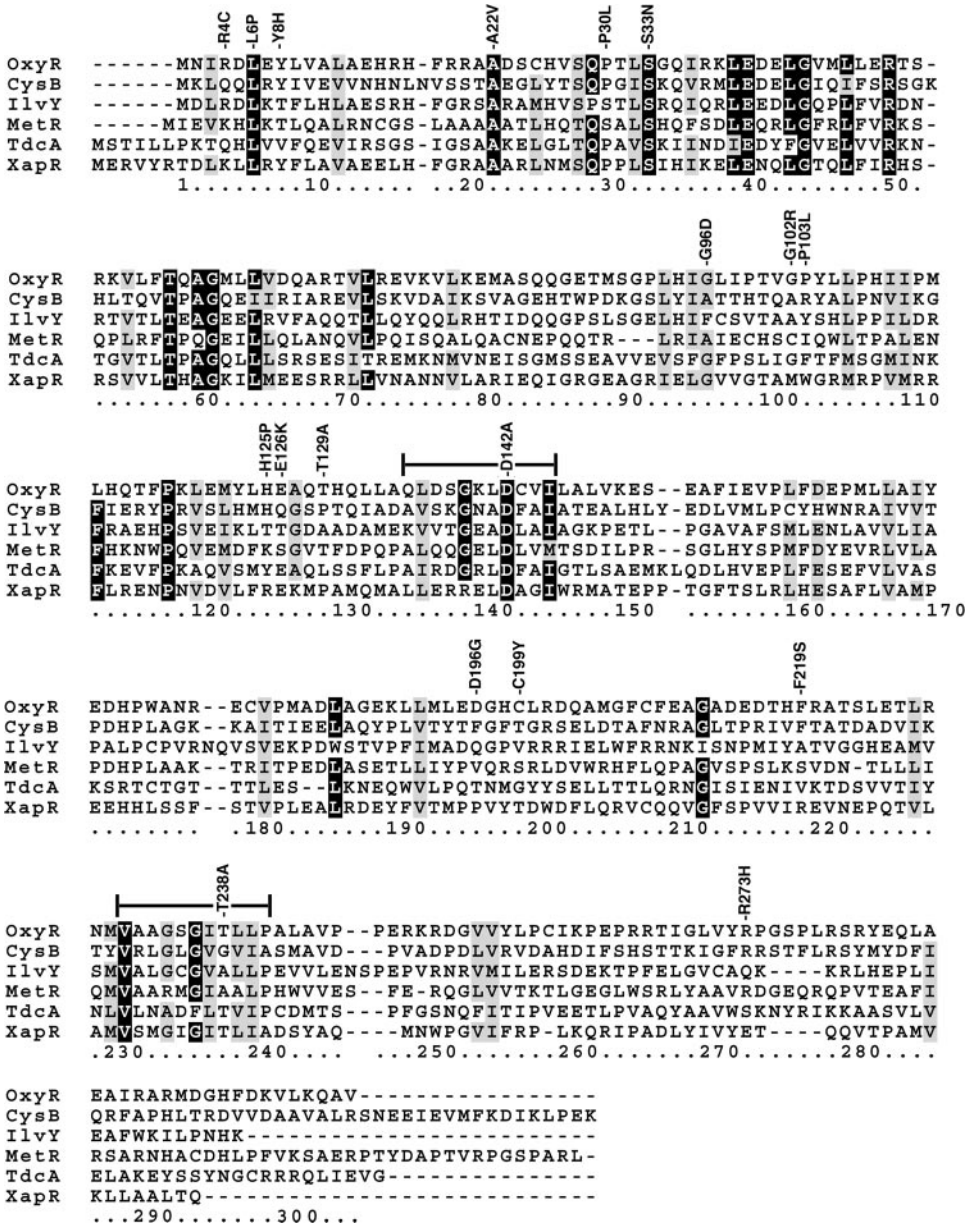

FIG. 1. Alignment of *E. coli* LysR family members. The amino acid sequences of the *E. coli* OxyR, CysB, IlvY, MetR, TdcA, and XapA LysR-type transcriptional regulators were aligned by using CLUSTAL W software. Numbering is based on the OxyR sequence. Amino acids identical in five of the six proteins are highlighted in black, and conserved residues are highlighted with gray. Brackets denote regions mutated in the alanine-scan mutagenesis. Positions of activation-defective mutations isolated in the random mutagenesis screen are indicated above the sequence.

bond formation. To examine this possibility, we carried out DNase I footprinting experiments to assay T238A protein binding to the site between the divergent *oxyR* and *oxyS* promoters. Wild-type OxyR binds to this site in both its reduced and oxidized confirmations but makes different DNA contacts in the two conformations (25). The assays with purified T238A showed that, under oxidizing conditions, this mutant has a reduced footprint highly similar to the C199S mutant, which is locked in the reduced conformation (Fig. 3). This result suggests that T238 influences C199-C208 disulfide bond formation and that the T238A mutant is less readily oxidized. In contrast, the purified D142A protein behaves more like the wild-type protein and shows a predominantly oxidized footprint under these conditions.

A negative charge of D142 is essential for gene activation by OxyR. D142 is a negatively charged residue. To test the importance of the charge of this residue, we examined the properties of mutants in which D142 was substituted by another negatively charged residue (E), neutral amino acids (N and Q), and a positively charged residue (K) (Table 3 and Fig. 4). The D142E mutant showed wild-type resistance to oxidants and wild-type induction of *oxyS* upon treatment with hydrogen per-

TABLE 2. Peroxide sensitivity of alanine-scan mutants

Plasmid	Codon exchange	Zone of inhibition (mm)[a] with:	
		10% H_2O_2	4% CHP
pACYC184		39	29
pAQ5		22	17
Q135A	CAA-GCA	21	17
L136A	CTG-GCG	26	20
D137A	GAC-GCC	22	18
S138A	AGC-GCC	22	17
G139A	GGC-GCC	25	18
K140A	AAA-GCA	23	18
L141A	CTC-GCC	22	17
D142A	GAT-GCT	34	25
V144A	GTG-GCG	23	18
I145A	ATC-GCC	23	17
pACYC184		38	29
pAQ5		22	19
V231A	GTG-GCG	22	19
G234A	GGT-GCT	23	18
S235A	AGC-GCC	23	17
G236A	GGG-GCG	25	20
I237A	ATC-GCC	23	16
T238A	ACT-GCT	31	26
L239A	TTA-GCA	22	15
L240A	CTG-GCG	23	16
P241A	CCA-GCA	24	20

[a] That is, the total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values correspond to the average of three independent assays.

oxide. In contrast, the D142Q and D142N mutants had increased sensitivity and showed reduced ability to activate transcription, and the D142K mutant behaved like the Δ*oxyR* mutant strain, indicating a negatively charged residue is important in this position. The levels of the D142K mutant protein were elevated compared to the wild-type protein, although not to the extent observed for mutants carrying substitutions in

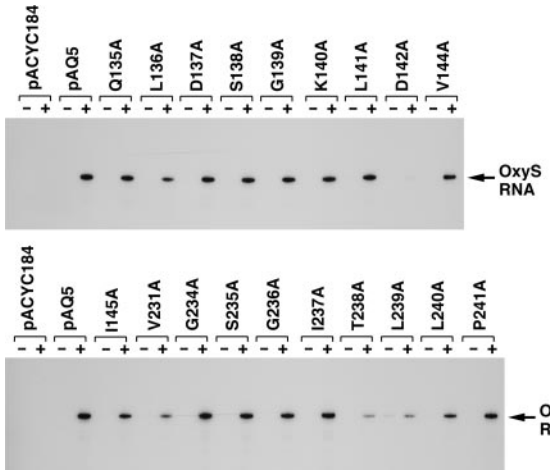

FIG. 2. Primer extension analysis of *oxyS* induction in alanine-scan mutants. Total RNA was isolated from the corresponding *E. coli* strains grown to mid-exponential phase and either left untreated (−) or exposed to 200 μM hydrogen peroxide (+) for 5 min. A labeled oligonucleotide capable of hybridizing to the OxyS RNA was incubated with 5 μg of each RNA sample and extended with reverse transcriptase.

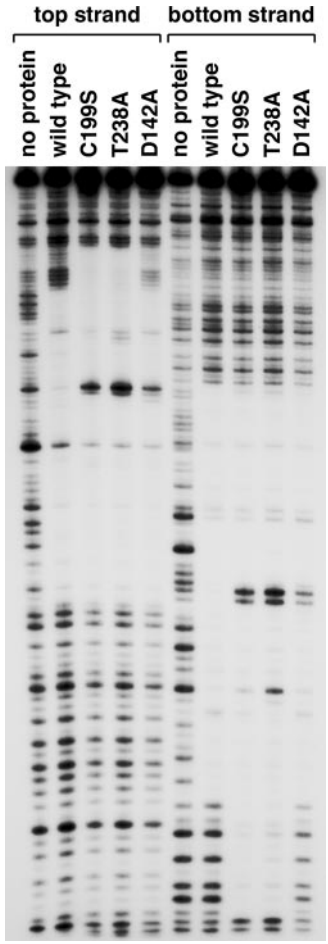

FIG. 3. DNase I footprint analysis of purified D142A and T238A OxyR mutant proteins binding to an *oxyS-oxyR* promoter fragment. The 100-bp EcoRI-HindIII fragment of pGSO40 (25) labeled at the HindIII site (top strand relative to the *oxyS* promoter) or EcoRI site (bottom strand relative to the *oxyS* promoter) was incubated with 50, 160, 170, and 150 ng of purified wild-type, C199S, T238A, and D142A OxyR, respectively. Footprinting assays were carried out in the absence of dithiothreitol; a short oxidized footprint is observed for wild-type OxyR under these conditions.

the DNA-binding domain (see below). Thus, the D142K mutant may be somewhat defective in autorepression and DNA binding, and some of the lack of activation may be attributable to decreased promoter binding. However, the levels of the

TABLE 3. Peroxide sensitivity of D142 mutants

Plasmid	Codon exchange	Zone of inhibition (mm)[a] with:	
		10% H_2O_2	4% CHP
pACYC184		38	27
pAQ5		23	18
D142A	GAT-GCT	32	24
D142E	GAT-GAA	22	19
D142N	GAT-AAT	33	26
D142Q	GAT-CAG	31	24
D142K	GAT-AAA	37	28

[a] That is, the total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values correspond to the average of three independent assays.

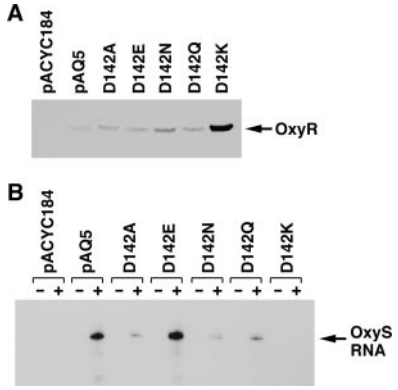

FIG. 4. (A) Immunoblot of D142 mutant protein levels. The total protein corresponding to equal numbers of cells was separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with α-OxyR antiserum. (B) Primer extension analysis of *oxyS* induction in D142 mutants. The total RNA was isolated from the corresponding *E. coli* strains grown to mid-exponential phase and either left untreated (−) or exposed to 200 μM hydrogen peroxide (+) for 5 min. A labeled oligonucleotide capable of hybridizing to the OxyS RNA was incubated with 5 μg of each RNA sample and extended with reverse transcriptase.

D142A, D142N, and D142Q mutants were similar to the wild-type levels, indicating that these activation-defective mutants are fully able to bind DNA.

Random mutagenesis screen for activation-defective mutants. In previous studies, we designed genetic screens for constitutive and for nonbinding OxyR mutants (7, 8). To screen for an activation-defective OxyR mutant, we used an *ahpCF* deletion strain, which lacks the alkylhydroperoxide reductase and thus has elevated endogenous levels of hydrogen peroxide (17). In this background the wild-type OxyR protein is constitutively in its oxidized (activated) conformation (15). The strain also was engineered to carry a prophage with a *lacZ* fusion to the OxyR target *oxyS*, as well as a deletion of the chromosomal copy of *oxyR*. On MacConkey plates, the Φ(*oxyS-lacZ*) Δ*oxyR*::Sp Δ*ahpCF*::Kan strain (GSO132) carrying the pACYC184 control vector is white, whereas this strain carrying the pAQ5 plasmid encoding wild-type *oxyR* is red. On tetrazolium indicator plates, the GSO132/pACYC184 strain is red, and the GSO132/pAQ5 strain is white. pAQ5 was mutagenized by passage through a mutator strain or by treatment with hydroxylamine and transformed into GSO132. Plasmids were extracted from all white colonies identified on MacConkey plates and all red colonies identified on tetrazolium plates. The phenotypes were confirmed by reintroducing the plasmids into the same background. All of the mutants showing decreased *oxyS-lacZ* expression were tested for hydrogen peroxide sensitivity (Table 4). Immunoblots were also carried out to eliminate all mutants expressing truncated versions of OxyR (Fig. 5A and data not shown). The plasmids associated with hypersensitivity to hydrogen peroxide and expressing full-length OxyR were then sequenced.

Mutants are impaired in DNA binding, oligomerization, disulfide bond formation, and transcriptional activation. At least four categories of activation-defective mutants were expected: (i) mutants defective in DNA binding and therefore also defective in *oxyR* autoregulation, (ii) mutants altered in oligomerization, (iii) mutants in which oxidation and disulfide bond formation are affected, and (iv) mutants defective in the interaction with RNA polymerase. To distinguish between these possibilities, we carried out a number of assays (Table 4 and Fig. 5) and mapped the positions of the mutations on the crystal structures of the OxyR regulatory domain (Fig. 6).

Six mutations—R4C, Y8C, Y8H, A22V, P30L, and S33N—

TABLE 4. Phenotype of uninducible mutants

Plasmid	Codon exchange	Zone of inhibition (mm)[a] with:		OxyR protein[b]	*oxyR-lacZ*[c]	Interpretation
		10% H_2O_2	4% CHP			
pACYC184		38	28	No expression	380	
pAQ5		22	19	Wild type	91	
R4C	CGT-TGT	39	29	Overexpressed (+++)	350	DNA binding
Y8C	TAC-TGC	36	28	Overexpressed (+++)	340	DNA binding
Y8H	TAC-CAC	29	23	Overexpressed (+++)	350	DNA binding
A22V	GCA-GTA	39	28	Overexpressed (+++)	360	DNA binding
P30L	CGA-TGA	37	27	Overexpressed (+++)	350	DNA binding
S33N	AGC-AAC	38	29	Overexpressed (+++)	350	DNA binding
G96D	GGT-GAT	38	28	Overexpressed (++)	220	Oligomerization
G102R	GGA-AGA	37	28	Overexpressed (++)	240	Oligomerization
P103L	CCG-CTG	31	27	Wild type	33	Oligomerization
H125P	CAT-CCT	32	27	Wild type	29	Oligomerization
E126K	GAA-AAA	36	26	Overexpressed (++)	220	Oligomerization
T129A	ACC-GCC	36	27	Wild type	22	Oxidation
D196G	GAT-GGT	28	24	Wild type	24	Oxidation
C199Y	TGT-TAT	30	18	Wild type	65	Oxidation
F219S	TTC-TCC	37	27	Overexpressed (+)	47	Oligomerization
T238A	ACT-GCT	30	25	Wild type	24	Oxidation
R273H	CGT-CAT	45	34	Overexpressed (++)	80	Positive control

[a] That is, the total diameter of the growth inhibition zone caused by the addition of hydrogen peroxide (H_2O_2) or cumene hydroperoxide (CHP). The values correspond to the average of three independent assays.
[b] Approximate amounts of OxyR proteins in Fig. 5.
[c] Miller units of β-galactosidase activity. The values correspond to the average of two experiments.

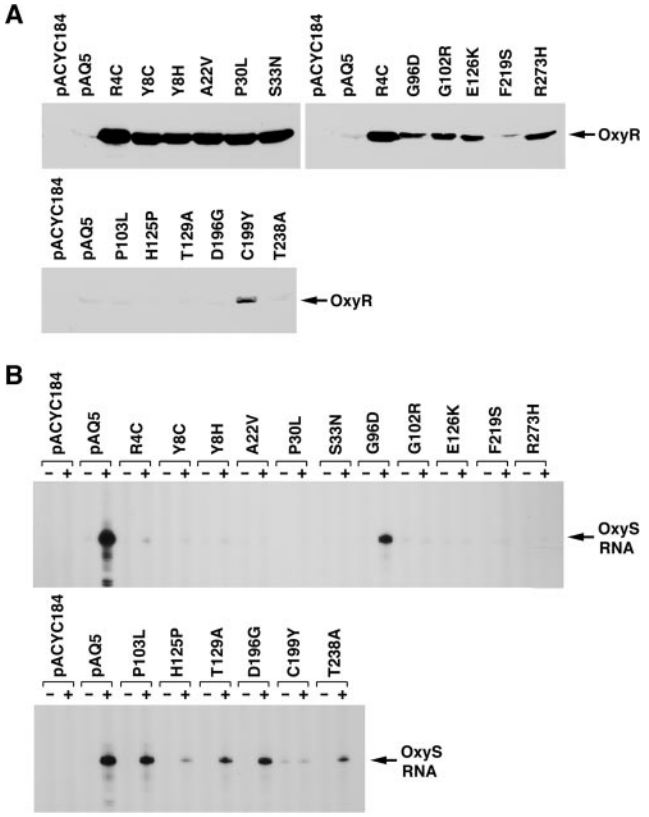

FIG. 5. (A) Immunoblot of OxyR protein levels in activation-defective mutants. Total protein corresponding to equal numbers of cells was separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with α-OxyR antiserum. (B) Primer extension analysis of *oxyS* induction in activation-defective mutants. Total RNA was isolated from the corresponding *E. coli* strains grown to mid-exponential phase and either left untreated (−) or exposed to 200 μM hydrogen peroxide (+) for 5 min. A labeled oligonucleotide capable of hybridizing to the OxyS RNA was incubated with 5 μg of each RNA sample and extended with reverse transcriptase.

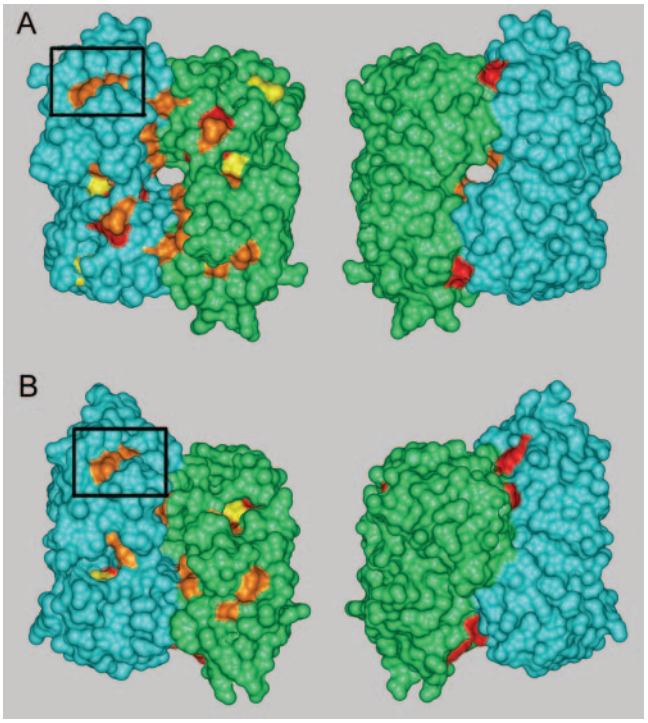

FIG. 6. Positions of constitutive and activation-defective mutations on the OxyR structure. Space-filling model of the OxyR regulatory domain in the reduced (A) and oxidized (B) conformations was generated by using InsightII software (Accelrys Software, Inc.). The front view is given on the left, and the back view is on the right. One monomer is shown in aqua, and the second monomer is shown in green. The positions of the C199 and C208 residues are indicated in yellow. The residues affected by the constitutive mutations (T100, H114, H198, R201, and A233) isolated in a previous screen for elevated expression of an *oxyS-galK* fusion (8) are indicated in red. The residues found to be mutated in the activation-defective mutants (G96, G102, P103, H125, E126, T129, D142, F219, T238, and R273) described here are indicated in orange. The black box surrounds residues D142 and R273.

mapped to the helix-turn-helix DNA-binding domain not present in the crystal structure. Immunoblots of all of the corresponding mutants showed highly elevated OxyR expression (Fig. 5A). These mutants also were unable to repress the *oxyR-lacZ* fusion (Table 4). Thus, we suggest that the R4C, Y8C, Y8H, A22V, P30L, and S33N mutants are defective in DNA binding. Two of these mutants, R4C and S33N, were previously isolated in the screen for nonbinding mutants (7).

An additional six mutations—G96D, G102R, P103L, H125P, E126K, and F219S—mapped to the interface between the two monomer subunits. Two of the corresponding mutants, P103L and G96D, had only minor defects in OxyS induction, and a third, H125P, was only partially defective (Fig. 5B). In contrast, no induction was detected for the G102R, E126K, and F219S mutants. Two of these stronger mutants, G102R and E126K, also had elevated OxyR levels (Fig. 5A) and increased *oxyR-lacZ* expression (Table 4), indicating that possible defects in oligomerization might be associated with defects in DNA binding. Interestingly, the G96D mutant had similar elevated levels of the OxyR protein and *oxyR-lacZ* expression but was far less defective in OxyS induction, raising the possibility that the G96D substitution actually enhances activation. Given the locations of the mutations, we suggest all six affect activation by altering the interaction between the monomers.

Four substitutions—T129A, D196G, C199Y, and T238A—mapped at or near C199 and C208, the two redox-active cysteine residues. The C199Y mutation affects one of the two redox-active cysteines. The T129 residue touches C199, and the D196 residue is on the same loop as C199; it is likely that both of the corresponding mutations affect the reactivity of C199. The T238A mutation is equivalent to the mutation generated by the alanine-scan mutagenesis and is also thought to affect the reactivity of the redox-active cysteines. The T129, C199Y, and T238A mutant proteins had reduced footprints under oxidizing conditions (Fig. 2 and data not shown), a finding consistent with the suggestion that these mutants are impaired in disulfide bond formation. The T129A and T238A mutants are both hypersensitive to oxidants but still show some OxyS RNA induction. In contrast, the C199Y mutant is partially constitutively active, similar to what has been observed for a C208S mutant (9). The footprint of the D196G mutant protein was similar to the wild-type protein under oxidizing

conditions. However, the mutation had minor effects; the D196G mutant was only partially sensitive to oxidants and only showed slightly reduced OxyS induction.

The remaining mutation, R273H, mapped to a surface-exposed residue and had a dramatic effect. Absolutely no OxyS induction was observed in this strain, indicating that the mutant is completely impaired in transcriptional activation. The R273H protein levels were somewhat elevated, though the expression of the *oxyR-lacZ* fusion was similar to the wild-type OxyR strain. We do not know the reason for the discrepancy between these two assays of autorepression, but the increased R273H levels may reflect a partial defect in DNA binding or altered sensitivity to degradation. Intriguingly, the R273H mutant was even more sensitive to both hydrogen peroxide and cumene hydroperoxide than the pACYC184 vector control strain, suggesting that expression of the mutant protein is somewhat detrimental to the cell.

D142 and R273 define a possible activating region. One mutant, D142A, isolated in our alanine-scan approach and another isolated in our random screen, R273H, show strong defects in OxyR activation of the *oxyS* target gene but only partial defects in DNA binding. Intriguingly, the two residues affected by the mutations are adjacent in the OxyR regulatory domain (Fig. 6). There are several possible explanations for the observed defect in transcription activation. D142A and R273H map to an interaction surface between the regulatory and DNA-binding domains of the structure of the full-length *Ralstonia eutropha* CbnR protein solved in the absence of inducer (14). Thus, the substitutions may be affecting transcription activation through some effects on DNA binding. The structure of a full-length LysR-type regulator in an inducer-bound, activated conformation is not yet available. However, given that R273 maps onto the surface of the CbnR structure and both D142 and R273 have the potential to be exposed in the activated conformation, the two residues may also correspond to a point of contact between OxyR and RNA polymerase and thus define an activating region on the OxyR protein. Alternatively, the D142A and R273H may be altering the OxyR conformation such that the actual contact residues can no longer interact with RNA polymerase.

Activating regions are well defined for a number of transcriptional regulators that are not members of the LysR family (reviewed in references 2 and 5). For example, the E34 and D38 residues of the λ*c*I activator have been shown to contact the R588 and R596 residues of the σ^{70} subunit of RNA polymerase. Similarly, the D241 residue of the *E. coli* RhaS protein, a member of the AraC/XylS family of transcriptional regulators, interacts with R599 of σ^{70}. Multiple activating regions that interact with both the α and σ^{70} subunits of RNA polymerase also have been defined for the *E. coli* CRP activator; ARI (T158), ARII (H19, H21, D21, and K101) and ARIII (D53, E54, E55, and E58). It is notable that many of the residues shown to be involved in contacting RNA polymerase are charged, as is the case for D142 and R273.

Less is known about possible activating regions for LysR-type transcriptional regulators. Two studies propose that residues in the DNA-binding domain of these transcription factors contact the α subunit of RNA polymerase. L30A, F31A, and F32L substitutions in the *E. coli* GcvA regulator result in reduced activation of the *gcvT* promoter but do not reduce DNA binding or autorepression (6). Similarly, derivatives of the *E. coli* CysB protein with substitutions of the Y27, T28, and S29 residues are defective for activation of the *cysP* promoter but not for inducer or DNA binding (10). It is possible that LysR family members have multiple activating regions, as is the case for CRP. The GcvA protein has in fact been shown to interact with both α and σ^{70} subunits of RNA polymerase (19).

The strongest support for the model that the D142 and R273 residues act as a contact point for RNA polymerase would come from the isolation of suppressor mutations in subunits of RNA polymerase, as has been the case for some of the non-LysR regulators described above. All genetic screens to isolate such suppressors for the putative OxyR positive control mutants thus far have been unsuccessful (data not shown), and no such suppressors have been reported for other LysR family members. Thus, additional biochemical and structural studies of LysR-type transcriptional regulators will need to be carried out before the precise contacts between this family of transcription factors and RNA polymerase can be defined.

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