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The Major Catalase Gene (*katA*) of *Pseudomonas aeruginosa* PA14 Is under both Positive and Negative Control of the Global Transactivator OxyR in Response to Hydrogen Peroxide▽§

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The adaptive response to hydrogen peroxide (H_2O_2) in *Pseudomonas aeruginosa* involves the major catalase, KatA, and OxyR. However, neither the molecular basis nor the relationship between the aforementioned proteins has been established. Here, we demonstrate that the transcriptional activation of the *katA* promoter (*katAp*) in response to H_2O_2 was abrogated in the *P. aeruginosa* PA14 *oxyR* null mutant. Promoter deletion analyses revealed that H_2O_2-mediated induction was dependent on a region of DNA −76 to −36 upstream of the H_2O_2-responsive transcriptional start site. This region harbored the potential operator sites (OxyR-responsive element [ORE]) of the *Escherichia coli* OxyR binding consensus. Deletion of the entire ORE not only abolished H_2O_2-mediated induction but also elevated the basal transcription, suggesting the involvement of OxyR and the ORE in both transcriptional activation and repression. OxyR bound to the ORE both *in vivo* and *in vitro*, demonstrating that OxyR directly regulates the *katAp*. Three distinct mobility species of oxidized OxyR were observed in response to 1 mM H_2O_2, as assessed by free thiol trapping using 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid. These oxidized species were not observed for the double mutants with mutations in the conserved cysteine (Cys) residues (C199 and C208). The uninduced transcription of *katAp* was elevated in an *oxyR* mutant with a mutation of Cys to serine at 199 (C199S) and even higher in the *oxyR* mutant with a mutation of Cys to alanine at 199 (C199A) but not in *oxyR* mutants with mutations in C208 (C208S and C208A). In both the C199S and the C208S mutant, however, *katAp* transcription was still induced by H_2O_2 treatment, unlike in the *oxyR* null mutant and the C199A mutant. The double mutants with mutations in both Cys residues (C199S C208S and C199A C208S) did not differ from the C199A mutant. Taken together, our results suggest that *P. aeruginosa* OxyR is a bona fide transcriptional regulator of the *katA* gene, sensing H_2O_2 based on the conserved Cys residues, involving more than one oxidation as well as activation state *in vivo*.

The vast majority of metabolic energy is generated primarily through oxidative phosphorylation in aerobic bacteria. This process, involving the reduction of molecular oxygen (O_2) to water, can potentially be dangerous to the cell. Such dangers surface when aberrant electron flow from the electron transport chain or cellular redox enzymes directly reduces O_2, which can lead to the successive production of reactive oxygen species (ROS) (17), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$), within cells (27). Besides the inevitable endogenous generation of ROS through normal aerobic metabolism, pathogenic bacteria can be exposed to exogenously generated ROS by human phagocytes during the infection process, which mount dramatic ROS-dependent antimicrobial responses (22). Detoxification of ROS is provided by iron sequestration, free-radical-scavenging agents, DNA-binding proteins, DNA repair enzymes, and most importantly antioxidant enzymes, such as superoxide dismutases (SODs), catalases, and peroxidases (20, 27). These elaborate detoxification systems often require specific regulators for proper gene expression, constituting multiple regulons important in the adaptive response to multiple oxidative stresses.

Key regulators modulating the adaptive response to oxidative stresses have been well characterized for model bacteria such as *Escherichia coli* and *Bacillus subtilis* (40, 54). Among them, the OxyR protein of *E. coli* is one of the best-characterized transcriptional regulators, and homologues are found in most proteobacterial and some Gram-positive genomes. OxyR is a 34-kDa LysR-type transcriptional regulator that controls a majority of the genes involved in the defense against H_2O_2 in *E. coli* and *Salmonella enterica* serovar Typhimurium (1, 13). OxyR senses H_2O_2 and can switch rapidly between reduced and oxidized states, but only the oxidized form acts as a transcriptional activator for target genes under its control. In the presence of H_2O_2, OxyR forms an intramolecular disulfide bond (between peroxidatic and resolving cysteines, i.e., Cys 199 and Cys 208) which can be deactivated by enzymatic reduction upon relief of the oxidative stress (61). Both oxidized and reduced forms of the *E. coli* OxyR protein possess DNA bind-

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ing activity, recognizing a motif comprised of four ATAG elements spaced at 10-bp intervals (57, 58). OxyR also acts as a repressor of its own transcription, as do other LysR-type regulators, independent of its redox state (49). Recent studies have revealed that there are OxyR homologs in other bacterial species whose properties differ from those of *E. coli* OxyR with regard to the mode(s) of peroxide sensing and transcriptional regulation. OxyR acts as a repressor for its primary target genes in various bacteria, such as *Neisseria gonorrhoeae*, *Legionella pneumophila*, *Xanthomonas campestris*, and *Pseudomonas putida* (24, 34, 38, 59). *Deinococcus radiodurans* OxyR lacks the conserved cysteine residue corresponding to the peroxidatic cysteine in *E. coli* OxyR (5). These findings indicate that the regulatory mechanisms governed by OxyR are considerably diversified among bacterial species.

Pseudomonas aeruginosa is an opportunistic human pathogen that can cause sepsis and even death in immunocompromised individuals, such as patients suffering from severe burns or other traumatic skin damage or from cystic fibrosis. It possesses OxyR, which is known as an H_2O_2-sensing transcriptional regulator (43). It is also known that typical antioxidant enzymes for the defense against H_2O_2 challenges include three catalases (KatA, KatB, and KatE) (2, 39) and three alkyl hydroperoxide-reducing proteins (AhpB, AhpC, and Ohr) (20, 42). Among them, the *katB*, *ahpB*, and *ahpC* genes are positively regulated by OxyR in response to H_2O_2 and menadione or paraquat (PQ) treatments, whereas the PQ-induced expression of the *katA* gene is not affected at all in the *oxyR* mutant (20).

The *katA* gene encodes the major catalase of *P. aeruginosa*, and its expression is constitutively high, in part, as a means to cope with the micromolar range of H_2O_2 that is generated under normal growth conditions in *P. aeruginosa*, as in *E. coli* (18, 20). Its expression is further increased when cells enter the stationary growth phase (15), like other major bacterial catalases, such as KatG from *E. coli*, KatA from *B. subtilis*, and CatA from *Streptomyces coelicolor* (3, 6, 9, 41). We previously reported the role of KatA in H_2O_2 resistance and osmoprotection (36). KatA is critical for the adaptive response to H_2O_2 and full virulence in mouse and *Drosophila melanogaster* infection models as well (36). Interestingly, KatA is detectable in stationary-phase culture supernatants, which restored the osmosensitivity of the *katA* mutant as well as the serial dilution defect of the *oxyR* mutant (20, 36). KatA is highly stable and can be found in the extracellular milieu, which ensures the survival of *P. aeruginosa* cells in its biofilm state and presumably during chronic infections, when organisms are continuously facing oxidative stress from phagocytic cells (15, 51). Although much has been unraveled about the physiological roles of KatA as the major H_2O_2-scavenging enzyme in H_2O_2 resistance and virulence of *P. aeruginosa*, relatively little is known about the regulatory mechanisms governing *katA* gene expression.

In the present study, we analyzed the transcription of the *katA* gene in response to H_2O_2 stress in *P. aeruginosa* strain PA14. We identified the *katA* promoter, whose transcription was induced by H_2O_2 exposure. Transcriptional activation was found to require the H_2O_2-sensing transcriptional regulator OxyR. The OxyR binding region within the *katA* promoter upstream region contains four ATAG-like elements located at a stretch of DNA located −72 and −33 upstream of the transcriptional start site. Based on the mutation analyses of the OxyR binding sites as well as the OxyR proteins for conserved cysteine (Cys) residues C199 and C208, we suggest that the OxyR protein directly regulates the *katA* gene by means of derepression and activation that involve more than one oxidation state, requiring both Cys residues in response to H_2O_2 stress.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* strains were grown at 37°C in Luria-Bertani (LB) medium (broth culture) or on 1.5% Bacto-agar solidified LB plates. Overnight, stationary-phase suspensions were inoculated (1.6×10^7 CFU/ml) into fresh LB broth; grown at 37°C to mid-logarithmic phase (optical density at 600 nm [OD_{600}] = 0.3), to late logarithmic phase (OD_{600} = 0.7), or to stationary phase (OD_{600} = 3.0); and then used for the experiments described herein. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml, and gentamicin (Gm), 15 μg/ml, for *E. coli*, and carbenicillin, 200 μg/ml, and Gm, 30 μg/ml, for *P. aeruginosa*.

DNA oligonucleotide primers. The DNA oligonucleotide primers used for gene deletion, gene expression, and gene detection in this study are listed in Table S1 in the supplemental material.

RNA isolation and S1 nuclease protection analysis. *P. aeruginosa* was grown aerobically until the mid-logarithmic (OD_{600} = 0.3), the late logarithmic (OD_{600} = 0.7), or the stationary (OD_{600} = 3.0) growth phase in LB medium, and then half of the culture was left untreated and the other half was treated with 1, 2, or 10 mM H_2O_2. The H_2O_2-treated culture samples were shaken before being collected at 10-min time intervals. Total RNA was isolated from approximately 1×10^9 cells by use of a Qiagen RNeasy kit (Qiagen). The oligonucleotide primer pairs used for the S1 nuclease protection were as follows: katA-N10 (5′ end at −133) and katA-S1C1 (5′ end at +264) for *katA*; katB-N5 (5′ end at −152) and katB-S1C1 (5′ end at +241) for *katB*; and rpoA-N1 and rpoA-C1 for the *rpoA* internal region, with a 30-bp unrelated sequence contained in rpoA-N1. PCR-generated probes were labeled with [γ-^{32}P]ATP by use of T4 polynucleotide kinase. S1 nuclease protection analysis was carried out using 50 μg of RNA samples as described elsewhere (8). For high-resolution S1 mapping, the unlabeled katA-S1C1 primer was used to generate the nucleotide sequence ladder, using a Sequenase version 2.0 DNA sequencing kit (USB) with [α-^{32}P]dATP and pQF-N10 as the template.

Construction of *lacZ* fusion plasmids and β-galactosidase assay. All of the deletion derivatives of the *katA* promoter in this study were created by PCR using the downstream primer katA-C1 (5′ end at +164) and one of the upstream primers katA-N10 (5′ end at −133), katA-N21 (5′ end at −76), katA-N22 (5′ end at −56), or katA-N23 (5′ end at −35). The amplified fragments were cloned into pQF50. LacZ (β-galactosidase) activity was determined using the mid-logarithmic-growth-phase cultures as described elsewhere (28). The results are presented as means with standard deviations and were analyzed by Student's *t* test using SPSS 5.0 statistical software. A *P* value of less than 0.005 was considered statistically significant.

Expression and purification of OxyR in *E. coli*. The coding region of the *oxyR* gene was prepared by PCR using primers oxyR-His-N0 (for tagging histidine at its N terminus) and oxyR-C0. The PCR products were cloned into pET15b using NcoI and BamHI, resulting in pET15H-oxyR, which was introduced into *E. coli* BL21(DE3)/pLysS. *E. coli* cells were grown for 5 h and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 30°C. The harvested cells were resuspended in TGED buffer (10 mM Tris-HCl [pH 7.8], 20% glycerol, 1 mM EDTA, and 0.1 mM dithiothreitol [DTT]) and disrupted by sonication. The lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was loaded onto a Ni-nitrilotriacetic acid (NTA) agarose column according to the manufacturer's recommendation (Qiagen). The proteins were eluted using TGED buffer containing 250 mM imidazole. The purified His-tagged OxyR protein was verified by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as having more than 90% homogeneity.

Antibody preparation and Western blot analysis. Anti-KatA and anti-RpoA antibodies were used as described previously (51). OxyR protein recovered from the insoluble fraction was used to raise the anti-OxyR antiserum as described elsewhere (8). An emulsion of OxyR protein (100 μg) in phosphate-buffered saline (PBS) (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.0) was injected into five mice (ICR females) at intervals of 2

TABLE 1. Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics or purpose[a]	Reference or source
P. aeruginosa strains		
PA14	Wild-type laboratory strain; Rif^r	47
lasR rhlR mutant	PA14 with in-frame deletion of *lasR rhlR*; Rif^r	46
mvfR mutant	PA14 with Tn*phoA* insertion at *mvfR*; Rif^r Km^r	4
rpoS mutant	PA14 with in-frame deletion of *rpoS*; Rif^r	46
oxyR mutant	PA14 with in-frame deletion of *oxyR*; Rif^r	12
phoB mutant	PA14 with in-frame deletion of *phoB*; Rif^r	This study
S33N mutant	*oxyR* with the chromosomally integrated mTn7-oxyR(S33N); Rif^r Gm^r	This study
C25S mutant	*oxyR* with the chromosomally integrated mTn7-oxyR(C25S); Rif^r Gm^r	This study
C199A mutant	*oxyR* with the chromosomally integrated mTn7-oxyR(C199A); Rif^r Gm^r	This study
C199S mutant	*oxyR* with the chromosomally integrated mTn7-oxyR(C199S); Rif^r Gm^r	This study
C208S mutant	*oxyR* with the chromosomally integrated mTn7-oxyR(C208S); Rif^r Gm^r	This study
C199A C208S mutant	*oxyR* with the chromosomally integrated mTn7-oxyR(C199A C208S); Rif^r Gm^r	This study
C199S C208S mutant	*oxyR* with the chromosomally integrated mTn7-oxyR(C199S C208S); Rif^r Gm^r	This study
E. coli strains		
DH5α	Multipurpose cloning	48
S17-1	Conjugal transfer of mobilizable plasmids; Tp^r Sm^r	53
BL21(DE3)/pLysS	High-stringency T7 promoter-based gene expression	Novagen
Plasmids		
pQF50	*lacZ* transcriptional fusion; Cb^r	16
pQF-N10	pQF50 with the *katA* promoter N10 (−133 to +164); Cb^r	This study
pQF-N21	pQF50 with the *katA* promoter N21 (−76 to +164); Cb^r	This study
pQF-N22	pQF50 with the *katA* promoter N22 (−56 to +164); Cb^r	This study
pQF-N23	pQF50 with the *katA* promoter N23 (−35 to +164); Cb^r	This study
pUCP18	General-purpose cloning; Cb^r	50
pUCP18-oxyR-FLAG	pUCP18 with the wild-type *oxyR* gene, with C-terminal FLAG tagging[c]; Cb^r	This study
pUCP18-oxyR(C199S)-FLAG	pUCP18 with the *oxyR* C199S mutant gene, with C-terminal FLAG tagging[c]; Cb^r	This study
pUCP18-oxyR(C208S)-FLAG	pUCP18 with the *oxyR* C208S mutant gene, with C-terminal FLAG tagging[c]; Cb^r	This study
pUCP18-oxyR(C199S C208S)-FLAG	pUCP18 with the *oxyR* C199S C208S mutant genes, with C-terminal FLAG tagging[c]; Cb^r	This study
pUC18T-mini-Tn*7*-Gm	mini-Tn*7*-Gm for single-copy complementation mobilizable from S17-1; Cb^r Gm^r	10
pTNS2	Transposase for mini-Tn*7*; Cb^r	10
mTn7-oxyR	pUC18T-mini-Tn7-Gm with the wild-type *oxyR* gene; Cb^r Gm^r	This study
mTn7-oxyR(S33N)	pUC18T-mini-Tn7-Gm with the *oxyR* S33N mutant gene; Cb^r Gm^r	This study
mTn7-oxyR(C25S)	pUC18T-mini-Tn7-Gm with the *oxyR* C25S mutant gene; Cb^r Gm^r	This study
mTn7-oxyR(C199A)	pUC18T-mini-Tn7-Gm with the *oxyR* C199A mutant gene; Cb^r Gm^r	This study
mTn7-oxyR(C199S)	pUC18T-mini-Tn7-Gm with the *oxyR* C199S mutant gene; Cb^r Gm^r	This study
mTn7-oxyR(C208S)	pUC18T-mini-Tn7-Gm with the *oxyR* C208S mutant gene; Cb^r Gm^r	This study
mTn7-oxyR(C199AC208S)	pUC18T-mini-Tn7-Gm with the *oxyR* C199A and C208S mutant genes; Cb^r Gm^r	This study
mTn7-oxyR(C199SC208S)	pUC18T-mini-Tn7-Gm with the *oxyR* C199S and C208S mutant genes; Cb^r Gm^r	This study
pET15b	T7 promoter-based expression vector; Cb^r	Novagen
pET15H-oxyR	pET15b with the *oxyR* gene, with direct His tagging[b]; Cb^r	This study

[a] Rif^r, rifampin resistant; Gm^r, gentamicin resistant; Cb^r, carbenicillin and ampicillin resistant; Tp^r, trimethoprim resistant; Sm^r, streptomycin resistant.
[b] Hexa-His tagging directly to the initiation codon, which is derived from the oligonucleotide primer (see Table S1 in the supplemental material).
[c] FLAG tagging directly to the last codon of the OxyR protein, which is derived from the oligonucleotide primer (see Table S1 in the supplemental material).

weeks. Antisera were obtained from the mice 1 week after the third injection. Western blot analysis was carried out using 50 μg of total protein more than three times as described previously (51).

Thiol trapping by 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). OxyR proteins were tagged with a FLAG epitope as described previously (37), using PCR primers oxyR-N1 and oxyR-FLAG-C0. The *oxyR* null mutant cells containing either FLAG-tagged OxyR (wild type [WT]) or one of the FLAG-tagged OxyR muteins with mutations in conserved Cys residues (C199S, C208S, and C199S C208S) were grown to logarithmic growth phase in LB broth at 37°C. After 1 mM H_2O_2 treatment, an aliquot of the culture (1 ml) was mixed with 110 μl ice-cold 100% (wt/vol) trichloroacetic acid (TCA) at various time points and harvested. The cell pellets were resuspended in 400 μl of 10% TCA and disrupted by sonication. After centrifugation, the pellets were mixed with 20 μl of AMS buffer (0.5 M Tris-HCl [pH 8.0], 10 mM AMS, 2% SDS, 100 mM NaCl, 1 mM EDTA, and 5% glycerol) and incubated in the dark for 1 h prior to cell extract preparation, followed by Western blot analysis using anti-FLAG M2 antibody (Sigma).

Gel mobility shift assay. A gel mobility shift assay was performed as described previously (7). A DNA fragment of the *katA* promoter region was generated by PCR with primer pair katA-N21 and katA-S1C1 or primer pair katA-N23 and katA-C1. The *ahpC* and the *pelA* promoter fragments were included as the positive and the negative control, respectively, and were generated using PCR primer pairs ahpC-N2 and ahpC-C1 and pelA-N3 and pelA-S1C1, respectively. The ORE fragment was prepared by annealing two 50-nucleotide (nt) complementary oligonucleotides encompassing the OxyR binding consensus (see O_1 to O_4 in Fig. 1B). The DNA fragments were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Ten femtomoles of the labeled probe was incubated with purified His-tagged OxyR in 20 μl of binding buffer (2 mM Tris-HCl [pH 7.8], 0.1 mM EDTA, 0.2 mM DTT, 4 mM KCl, 0.5 mM $MgCl_2$, 10 ng/ml bovine serum albumin [BSA], and 10% glycerol) containing 1 μg of poly(dI-dC) for 10 min at 25°C. The DNA-protein mixture was resolved on a 5% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer (48) and then analyzed with a phosphorimager analyzer (Fuji).

DNase I footprinting. The probe DNA (339 bp) was prepared by PCR with primer pair katA-N9 and katA-C2. Prior to PCR, the katA-C2 primer was 5′ end labeled for detection of the bottom strand. The binding reactions were performed as described for the gel mobility shift assay in a 50-μl reaction volume.

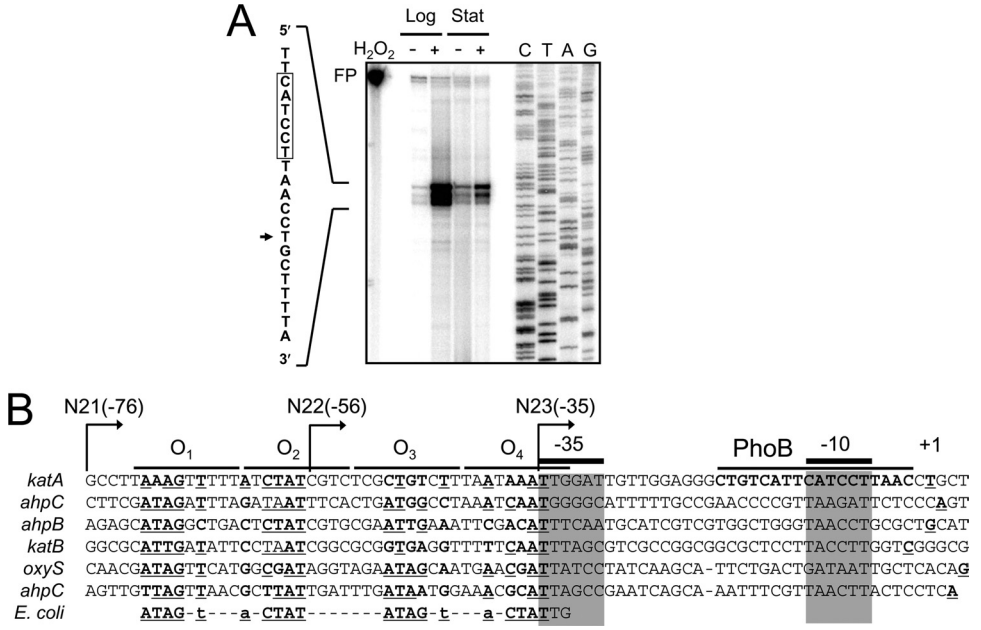

FIG. 1. Promoter region of the *katA* gene. (A) The transcriptional start site of the *katA* gene was determined by high-resolution S1 nuclease mapping. Total RNA (50 μg) was prepared from cells grown to the mid-logarithmic (Log; OD_{600} = 0.3) and the stationary (Stat; OD_{600} = 3.0) growth phases, with or without 1 mM H_2O_2 treatment for 10 min. The sequencing ladder (C, T, A, and G) was generated as described in Materials and Methods. The transcriptional start site is indicated by the arrow, and the putative −10 box is designated. (B) The alignment of the *katA* promoter region with the *P. aeruginosa* OxyR-regulated promoters (*ahpB*, *ahpC*, and *katB*) and the *E. coli* OxyR-regulated promoters (*ahpC* and *oxyS*) is shown, with the OxyR binding consensus indicated (O_1 to O_4; ATAG-t-N_5-a-CTAT-N_7-ATAG-t-N_5-a-CTAT). The strongly conserved nucleotides are designated by underlining, and the weakly conserved nucleotides are indicated by lowercase letters within the consensus. The −35 and −10 boxes and the experimentally determined +1 site are indicated. The heavy line above the *katAp* sequence indicates the PhoB binding site (60). N21(−76), N22(−56), and N23(−35) are the truncated promoter series, with the 5′ ends (see Fig. 2) indicated by the bent arrows. The numbers represent the nucleotide positions relative to the transcriptional start site.

The DNA-protein mixture was treated with DNase I as described elsewhere (52). Briefly, 50 μl of a $CaCl_2$-$MgCl_2$ solution (50 mM $CaCl_2$ and 10 mM $MgCl_2$) was added, and the mixture was incubated for 1 min and then treated with 0.015 U of RQI RNase-free DNase I (Promega) for 1 min. The digesting reaction was stopped with 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml tRNA), and following that the DNA was extracted and precipitated. The digested DNA was resolved on a 6% urea-polyacrylamide gel with sequencing ladders and analyzed with a phosphorimager analyzer (Fuji).

Chromatin immunoprecipitation assay. For chromatin immunoprecipitation (ChIP) experiments, a previously described protocol was adopted (32, 55), with some modifications. Briefly, the cultures (100 ml) of the logarithmic growth phase were washed with 40 ml PBS. The cultures were treated with 1.2% formaldehyde for 20 min. Cross-linking was stopped by adding glycine (125 mM), and then cells were sedimented by centrifugation at 8,000 rpm for 5 min at 4°C, washed twice with ice-cold PBS, and resuspended in 1 ml lysis buffer A (1 mM phenylmethylsulfonyl fluoride, 50 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], and 1% SDS). The resuspended pellet was disrupted by sonication and sedimented at 14,000 rpm for 15 min at 4°C. The supernatant was transferred into a new tube. An aliquot (30 μl) was used for the inputs, and the remaining amount, corresponding to 50 μg of total proteins, was used for the immunoprecipitations, which were performed by adding 470 μl lysis buffer containing 5 μl antiserum (either anti-OxyR antiserum or preimmune serum). The samples were incubated for 12 h at 4°C. Then, 20 μl of protein A Sepharose (100 mg/ml), 2 μg of sonicated salmon sperm DNA, and 10 μg of BSA were added to the samples, followed by incubation for 2 h at 4°C to reduce the nonspecific associations. The Sepharose beads were pelleted by centrifugation at 12,000 rpm for 5 min and washed once with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], and 150 mM NaCl), once with high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], and 500 mM NaCl), once with LiCl wash buffer (250 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 10 mM Tris-HCl [pH 8.0], and 1 mM EDTA [pH 8.0]), and twice with Tris-EDTA (TE) (48). The protein A bound complexes were eluted with 250 μl elution buffer (100 mM $NaHCO_3$ [pH 7.5] and 1% SDS) at 65°C and then centrifuged for 5 min at 12,000 rpm at 4°C. Two hundred microliters of TE containing 10 μg of proteinase K was added to 200 μl of input and precipitation samples, followed by incubation at 65°C for 5 h. The DNA was extracted twice with phenol-chloroform and once with chloroform and was ethanol precipitated. The pellets were dissolved and used for PCR detection using the primer pairs described in Table S1 in the supplemental material.

Creation of the *phoB* deletion and *oxyR* point mutants. The *phoB* deletion mutant was created based on the in-frame deletion by SalI digestion (456 bp) of the PCR fragment (1,037 bp) amplified using primers phoB-N1 and phoB-C1. The *oxyR* point mutant alleles for the DNA-binding domain (the 33rd serine residue to asparagine by a TCG-to-AAC mutation) (S33N) (30) and for the conserved cysteines (the 25th, 199th, and 208th cysteine residues to serine by a TGC-to-TCC mutation) (C25S, C199S, and C208S) were generated by gene splicing by overlap extension (SOE) (25) using 4 oligonucleotide primers (see Table S1 in the supplemental material). The PCR products (1,209 bp) were cloned into pUC18T-mini-Tn*7*-Gm at the KpnI and BamHI sites (Table 1). These *oxyR* point mutant alleles were introduced into the *oxyR* null mutant chromosome at the *att*Tn*7* site, which allows the *oxyR* gene expression from its own promoter, since no other potential promoter elements are provided (11, 12).

RESULTS

Identification of the H_2O_2-responsive *katA* promoter region in *P. aeruginosa*. The transcriptional start site of the *P. aeruginosa katA* gene was previously identified under phosphate-limiting conditions as 132 nucleotides (nt) upstream (G-132) of the *katA* translation initiation codon (60). To identify the transcriptional start site in response to H_2O_2 exposure, we performed high-resolution S1 nuclease mapping and identified the transcriptional start site as 155 nt upstream (T-155) of the initiation codon (Fig. 1A). A protected band corresponding to a G residue at position −132 was observed, but it was a minor tran-

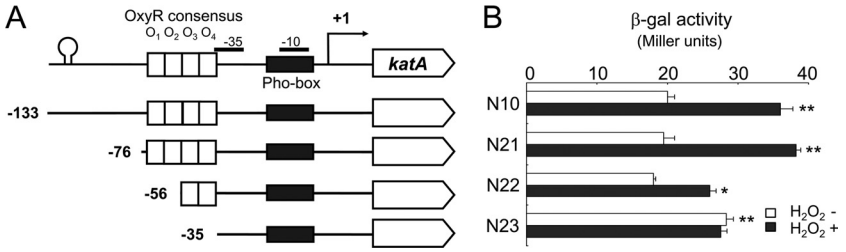

FIG. 2. Effects of deletions on the *katA* promoter activity. (A) Schematic representations of promoter deletions, with the OxyR consensus (ORE) and PhoB box (Pho-box) shown. (B) β-Galactosidase (β-gal) assay. The β-galactosidase activities were determined for the wild-type cells harboring one of the pQF50-derived *lacZ* fusions with the full-length promoter construct (N10) containing a potential inverted repeat (Ω) and for deletion mutants encompassing the intact ORE (N21) or the O_1- and O_2-truncated ORE (N22) or missing the entire ORE (N23). Cells were treated with (+) or without (−) 1 mM H_2O_2 for 20 min at the mid-logarithmic growth phase. β-Galactosidase activities from the mutant promoters are expressed as Miller units, with standard deviations from the three independent experiments. The statistical significance based on Student's *t* test is indicated as follows: *, $P < 0.005$; **, $P < 0.001$.

script, most likely due to the lack of maximal phosphate limitation under our experimental conditions (data not shown).

Based on the transcription start site at the T-155 position, the transcription of which was highly elevated in response to H_2O_2 stress, the core promoter and the potential *cis*-acting regulatory elements could be identified from a careful inspection of the upstream region. We identified the potential promoter elements (two hexamers separated by 18 nt) and similar elements of the *E. coli* OxyR tetramer binding sites (O_1 to O_4) that overlap with the proposed −35 box, as for *E. coli* OxyR-dependent promoters (Fig. 1B). Although the O_3 site of the *katA* gene displays weak similarity to the ATAG consensus as well as to the proposed operator sequences from the *P. aeruginosa* OxyR-dependent promoters (*katB*, *ahpB*, and *ahpC*), the overall context based on the sequence similarity and the arrangement of the operators suggests that this region could be involved in the peroxide-induced transcription of the *katA* promoter (*katAp*), which may recruit OxyR or other related transcriptional regulators.

Differential involvement of the *katA cis*-acting element (ORE) in the transcription at *katAp*. In addition to the potential OxyR-binding sites (herein termed ORE, for OxyR-responsive element) and the previously identified PhoB box (60), we found a potential inverted repeat (IR) located about 100 bp upstream of the *katAp* transcription start site, which might act as the intrinsic transcriptional termination signal for the *rplQ* gene (39). We created a series of promoter deletion constructs, depicted in Fig. 1B and 2A. Transcription from these promoters was measured using plasmids harboring *katAp* transcriptional *lacZ* fusions. As shown in Fig. 2, the N10 fusion showed only about a 2-fold increase upon H_2O_2 exposure. One possible explanation for this modest effect compared to the change in transcript level shown in Fig. 1A is that pQF50 is a medium-copy-number plasmid with a pRO1600 replicon in *P. aeruginosa*. Although the H_2O_2-induced LacZ activity from the *lacZ* fusions was relatively modest, we found that the deletion of the IR (N21) did not alter H_2O_2-induced promoter activity, whereas the deletion of the first two operator sites (N22) of the ORE partially abolished H_2O_2-mediated induction (to about 60% of the level for the wild type) (Fig. 2B). Interestingly, the uninduced *katAp* activity was elevated about 35% in bacteria harboring the entire ORE deletion (N23) and was not inducible by H_2O_2 treatment. These results lead us to hypothesize that the ORE is involved in the H_2O_2-mediated transcription initiation at *katAp* in a negative as well as a positive fashion.

OxyR is required for the peroxide-mediated activation of *katAp* transcription. Since the ORE is required for the transcriptional regulation of the *katA* promoter, we were prompted to examine whether OxyR is required for the transcriptional regulation of *katAp* in response to H_2O_2. We introduced the *katAp-lacZ* fusion (pQF-N10) in the *oxyR* null mutant and other mutants lacking quorum-sensing circuitry (*lasR rhlR* and *mvfR*), stationary-phase adaptation sigma factor (*rpoS*), or phosphate limitation response (*phoB*), each of which have been shown to be or potentially are involved in the regulation of the *katA* gene (23, 56, 60). H_2O_2-mediated transcriptional activation of *katA* was completely impaired in the *oxyR* mutant, in contrast to each of the other mutants tested (Fig. 3A). Basal *katA* transcription was elevated about 30% in the *oxyR* mutant, which is most likely due to the absence of the repressor-like activity of OxyR on *katAp*. The introduction of a mini-Tn*7*-based chromosome copy of the wild-type OxyR (mTn7-oxyR) into the *oxyR* null mutant containing the *katAp-lacZ* fusion fully restored H_2O_2-mediated *katAp* activity, while the previously elevated basal level decreased back to wild-type levels (Fig. 3A).

We next verified the ablation of the H_2O_2-mediated induction of *katAp* in the *oxyR* null mutant by Western blotting using anti-KatA antiserum (51). As shown in Fig. 3B, KatA protein expression was highly elevated at 10 min and gradually increased up to 30 min, whereas no change was observed for the *oxyR* mutant. Furthermore, the KatA expression level in the *oxyR* mutant in the absence of H_2O_2 treatment was higher than that in wild-type organisms (Fig. 3B, lanes 1 and 5). Although we did not quantitatively assess the amount of the KatA protein, the difference between the uninduced levels of the *oxyR* mutant versus the wild-type bacteria was even greater on a protein level (Western blotting) than by measuring *lacZ* reporter fusion activity (Fig. 3), which is most likely attributed to the unusual metastability of the KatA protein (51). The aforementioned results confirm the involvement of OxyR in both the positive and the negative regulation of the *katA* gene in response to H_2O_2.

OxyR-dependent regulation of *katAp* transcription is direct, requiring DNA-binding activity to the ORE. To test the hypothesis that the regulation of *katAp* is mediated by direct

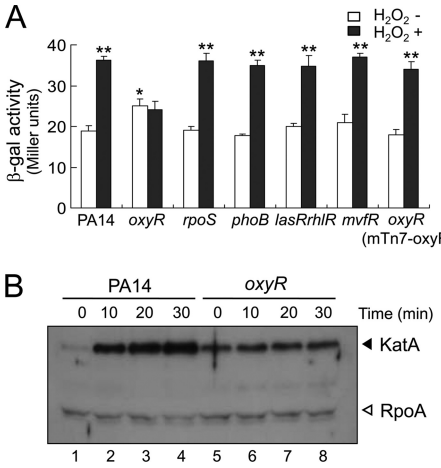

FIG. 3. H_2O_2-induced transcription of *katAp* requires OxyR. (A) β-Galactosidase (β-gal) activities driven from the *katA* N10 promoter (Fig. 2) were determined for PA14 (WT) and various mutants (*oxyR*, *rpoS*, *phoB*, *lasR rhlR*, *mvfR*, and *oxyR* mutants complemented with mTn7-oxyR) treated with (+) or without (−) 1 mM H_2O_2 for 20 min. The cells were grown as described in the legend for Fig. 2. The β-galactosidase activities are represented in Miller units, with standard deviations from the three independent experiments. The statistical significance based on Student's *t* test is indicated as follows: ∗, $P < 0.005$; ∗∗, $P < 0.001$. (B) The KatA protein level in the *oxyR* mutant was determined by Western blot analysis. Both PA14 (WT) and *oxyR* mutant cells were grown to the mid-logarithmic growth phase ($OD_{600} = 0.3$) and treated with 1 mM H_2O_2. Total protein (50 μg) was prepared from the cells harvested before treatment and at 10, 20, and 30 min after the H_2O_2 treatment, followed by Western blotting with anti-KatA and anti-RpoA antisera.

binding of OxyR, we performed an electrophoretic mobility shift assay using purified His-tagged OxyR (His-OxyR). Purified OxyR proteins exhibited increased mobility compared to that expected under nonreducing conditions, suggesting that an intramolecular disulfide bond(s) was formed by autooxidation. To prevent autooxidation of OxyR during the gel shift assay, we used 1 mM DTT (43) and detected a specific gel mobility shift by OxyR for both the *ahpC* and the *katA* promoter fragment (Fig. 4A; also, data not shown). The unbound DNA was not detected by adding 3 pmol of OxyR, which corresponded to a final concentration of 150 nM. The binding complex was not observed for the *katA* promoter fragment lacking the entire ORE (N23) (Fig. 4B), and the OxyR protein could bind to the 50-bp ORE fragment, although the binding affinity appeared to differ from that of the full-length fragment (N21) (Fig. 4A and C). To further substantiate the interaction between OxyR and the ORE, we analyzed OxyR binding through DNase I footprinting under the same binding condition used for the gel mobility shift assay. Increasing amounts of OxyR were incubated with the *katA* DNA fragment (339 bp, from −225 to +114) labeled at the bottom strand. Figure 4D shows that OxyR protected a relatively long region spanning about a 60-bp (−79 to −22) region at the bottom strand, which contains the ORE in the middle and overlaps with the −35 box. Therefore, this result as well as the electrophoretic mobility shift assay results demonstrates that OxyR may directly regulate the *katA* gene by binding to the ORE.

Furthermore, we examined whether OxyR could bind to the *katA* promoter region *in vivo*, as assessed by chromatin immu-

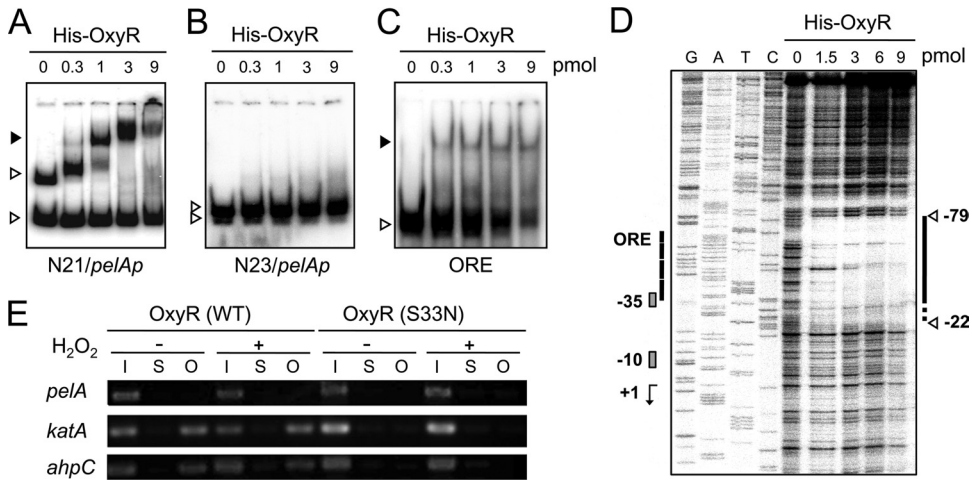

FIG. 4. OxyR binds the *katA* promoter region *in vitro* and *in vivo*. (A, B, and C) The indicated amounts of the purified OxyR proteins were treated with 1 mM dithiothreitol and then incubated with 10 fmol of the radiolabeled *katA* promoter fragments N21 (340 bp) and N23 (199 bp) and the synthetic ORE consensus oligonucleotide (50 bp). The radiolabeled *pelA* promoter fragment (164 bp) was included as the negative control in the binding reaction. The open arrowheads indicate the free probes, and the filled arrowheads indicate the OxyR-bound complexes. The numbers indicate the amounts of OxyR (pmol) in 20 μl of binding buffer, with 9 pmol corresponding to 450 nM. (D) DNase I footprinting analysis of OxyR, tested under the same conditions described above. The DNA probes (339 bp) radiolabeled at the 5′ end of the bottom strand were incubated with increasing amounts of OxyR as indicated, followed by DNase I treatment. The samples were run on a 6% polyacrylamide sequencing gel with the corresponding sequencing ladder. The region protected by oxidized OxyR is indicated by a thick solid line, with the slightly protected region overlapping with the −35 box indicated by a dashed line. The ORE (O_1 to O_4) and the promoter elements (−35 and −10 boxes and +1 site) are designated. (E) The wild-type and the *oxyR* DNA-binding domain (S33N) mutant bacteria were grown to the late logarithmic growth phase ($OD_{600} = 0.7$), treated with (+) or without (−) 10 mM H_2O_2 for 1 min, and then subjected to the chromatin immunoprecipitation assay as described in Materials and Methods. The samples were precipitated with either anti-OxyR antiserum (O) or preimmune serum (S) (as the negative template control). Then, the nonprecipitated, input samples (I) and the precipitated samples were analyzed by PCR targeting the *katA*, *ahpC* (positive control), and *pelA* (negative control) promoter regions.

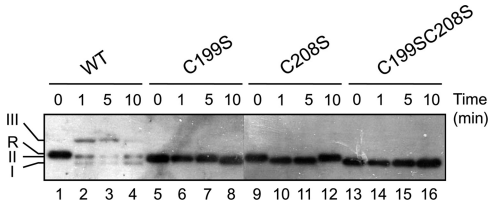

FIG. 5. H_2O_2-induced modification of OxyR *in vivo*. Total protein (50 μg) from the *oxyR* null mutants containing either the FLAG-tagged OxyR protein (WT) or one of the FLAG-tagged OxyR muteins with mutations in the conserved Cys residues (C199S, C208S, and C199S C208S) that were harvested before (0 min) or after (1, 5, and 10 min) treatment with 1 mM H_2O_2 and alkylated using 10 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) was used to monitor the OxyR status, based on Western blotting using the anti-FLAG antiserum. The single band for the WT protein in the uninduced condition (0 min) (~36 kDa, species R), the two faster-migrating bands (species I and II), and the slower-migrating band (~50 kDa, species III) under the H_2O_2-induced conditions are designated.

noprecipitation (ChIP) using an anti-OxyR antibody (Fig. 4E). For the ChIP experiment, we used the *oxyR* deletion mutant that had been complemented with a mini-Tn*7*-based chromosome copy of an *oxyR* allele with a point mutation (S33N) at the potential DNA binding domain, analogous to *E. coli* OxyR (30). The binding was observed only from the cells with DNA binding-competent OxyR. Taken together, these results suggest that *P. aeruginosa* OxyR can bind to the *katA* promoter region under both noninducing and H_2O_2-inducing conditions *in vivo*.

Involvement of OxyR cysteine residues in H_2O_2-mediated oxidation of OxyR. To investigate the potential involvement of the cysteine (Cys) residues in H_2O_2-mediated oxidation of OxyR *in vivo*, we monitored the mobility shift of the wild-type OxyR as well as the OxyR mutant proteins (muteins). The multiple alignment of the OxyR homologs from 20 proteobacterial species enabled us to determine that Cys 25 (C25) is highly conserved (19 out of 20), whereas Cys 199 (C199) and Cys 208 (C208) are perfectly conserved, among the 20 bacteria (data not shown). C199 and C208 are well known Cys residues which form a disulfide bond under oxidizing conditions, stabilizing the conformational state of OxyR in its transcriptionally activating form (31). We tagged the wild-type OxyR and the OxyR muteins with mutations in the two conserved cysteine (Cys) residues (C199S, C208S, and C199S C208S) with a FLAG epitope at their C termini. The FLAG-tagged variants were introduced into the *oxyR* deletion mutant by use of a multicopy plasmid (pUCP18) and verified as functionally equivalent to the corresponding single-copy, native OxyR proteins in terms of oxidant sensitivity and target gene regulation (data not shown). Cells were harvested after H_2O_2 treatment using 10% trichloroacetic acid (TCA) to prevent the free thiols from being oxidized; the free thiols were alkylated with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS), and the mobility shift was assessed by Western blotting using an anti-FLAG antibody.

As shown in Fig. 5, three distinct bands were observed upon H_2O_2 exposure for up to 10 min, with mobilities different from that determined under the uninduced condition (species R): two faster-migrating bands (species I and II) and a slower-migrating band (~50 kDa, species III) were evident (in contrast to the oxidized *E. coli* OxyR, showing only one faster-migrating band by formation of an intramolecular disulfide bond) (1, 35). Based on the mobility changes, species I is supposed to contain a disulfide bond between C199 and C208, as for *E. coli* OxyR, since it was not observed with the C199S, C208S, and C199S C208S mutants. Species II may contain a single Cys oxidation, with three AMS moieties added. The single Cys oxidation can occur preponderantly at C199, since the appearance of such a species was delayed with the C199S mutant (Fig. 5, lane 8) but not with the C208S mutant. Species III, with apparent molecular mass of ~50 kDa, could be a dimer or a complex with another protein(s) whose formation involves both C199 and C208, since it was not observed with the Cys mutants. All of these results suggest that *P. aeruginosa* OxyR undergoes more than one oxidation state or forms relatively stable intermediates in response to H_2O_2, involving a single Cys residue (C199) as the more sensitive peroxidatic Cys and double Cys residues (C199 and C208) to form a disulfide bond or undergo other modifications.

Involvement of OxyR cysteine residues in *katAp* transcription. We have assessed how the conserved cysteine (Cys) residues of OxyR affect *katAp* transcription, given the results outlined above as well as the knowledge that the thiol residues of *E. coli* OxyR play pivotal roles in H_2O_2 sensing (31). Furthermore, we wished to elucidate potential differences between *P. aeruginosa* OxyR and *E. coli* OxyR, since *P. aeruginosa* OxyR may act as both a positive and a negative regulator on the same target, having multiple oxidation states observed *in vivo* (Fig. 5). To this end, we created five Cys-to-serine and Cys-to-alanine point mutants (C25S, C199S, C199A, C208S, and C208A mutants), which were introduced into the *oxyR* deletion mutant at the Tn*7* integration site (*att*Tn*7*) of the chromosome of the *oxyR* null mutant. All of the point mutants could complement the aerobic serial dilution defect, as described initially by Hassett et al. (20) (data not shown). We excluded the C25S mutant from further experimentation, because the C25S mutant did not differ from the wild type in any aspect of the known *oxyR* mutant defective phenotypes, such as virulence attenuation in acute infections (33) and target gene regulation (data not shown) (31, 44). The C199S, C199A, C208S, and C208A mutants displayed similar amounts of OxyR protein, whereas the amount of OxyR protein in the C25S mutant was slightly smaller than that in the wild type (Fig. 6A; also, data not shown). Furthermore, H_2O_2 treatment did not affect the amount of OxyR protein in any of the point mutants described above (Fig. 6A), indicating the absence of autoregulation by OxyR in *P. aeruginosa* in response to H_2O_2 and/or Cys modifications, which differs from the result for OxyR in *E. coli* (14, 61). We created two C199 and C208 double mutants (C199S C208S and C199A C208S mutants) as well.

Then, we examined whether the expression of KatA was affected in the Cys mutants. The protein level as well as the activity of KatA was highly elevated in the C199S, C199A, C199S C208S, and C199A C208S mutants, even without the H_2O_2 treatment, whereas those in the C208S mutant were not significantly increased in response to H_2O_2 (Fig. 6A). Induction of KatB expression, which is also controlled by OxyR (20, 43), was evident only in the wild-type bacteria, as verified by S1 nuclease protection (Fig. 6B). These results substantiate the

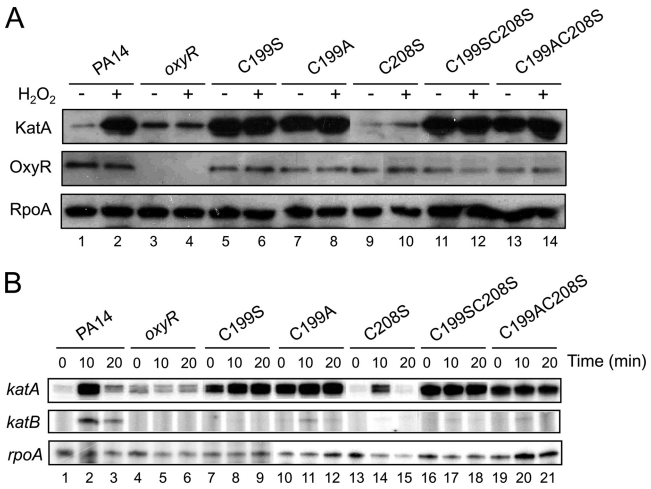

FIG. 6. *katA* transcription in *oxyR* cysteine mutants. (A) Total proteins from the wild type (PA14) and the *oxyR* mutants (*oxyR* null, C199A, C199S, C208S, C199A C208S, and C199S C208S mutants) grown to the mid-logarithmic growth phase (OD_{600} = 0.3) with (+) or without (−)1 mM H_2O_2 treatment for 20 min were analyzed by Western blotting. Fifty micrograms of total proteins was used for Western blot analysis using anti-KatA, anti-RpoA, or anti-OxyR antiserum. (B) Fifty micrograms of total RNA from the wild type (PA14) and the *oxyR* mutants (*oxyR* null, C199A, C199S, C208S, C199A C208S, and C199S C208S mutants) that had been grown to the mid-logarithmic growth phase (OD_{600} = 0.3) with 1 mM H_2O_2 and harvested before treatment and at 10 and 20 min after H_2O_2 treatment was used for the *katA*, *katB*, and *rpoA* transcript analysis by S1 nuclease protection. The probes were prepared as described in Materials and Methods.

differential involvement of OxyR in the H_2O_2-induced regulation of both catalase genes (61).

Then, we investigated the time course of *katAp* transcription upon H_2O_2 treatment in the Cys mutants. As shown in Fig. 6B, the basal transcription in the C199S mutant was elevated. These data suggest that the free thiol of C199 is required for the negative regulation of *katAp*. The *katAp* transcription in the C208S mutant was elevated in response to H_2O_2 stress but rapidly decreased, resulting in an induced level at 10 min lower than that of wild-type bacteria (Fig. 6B). This apparent smaller increase at 10 min after H_2O_2 exposure and the rapid decrease might be due to the Cys 199 oxidation upon H_2O_2 treatment and its rereduction into the active repressor form, respectively, concomitant with the alteration of the C208S mutein mobility (Fig. 5).

In contrast to the delayed H_2O_2 induction of *katAp* transcription in the C199S mutant, the C199A, C199S C208S, and C199A C208S mutants exhibited a high basal transcription of *katAp*, which was apparently uninduced upon H_2O_2 exposure (Fig. 6B). This result suggests that the slow induction upon H_2O_2 in the C199S mutant compared to that for the C199A mutant and the double mutants requires the functional C208, since no change in OxyR mobility was observed upon H_2O_2 exposure in either the C199A or the C199S C208S mutant (Fig. 5; also, data not shown).

DISCUSSION

In this study, we present two features in regard to the response of *P. aeruginosa* toward H_2O_2 stress. OxyR, the primary H_2O_2-sensing transcriptional regulator in this bacterium, was already known to activate three predominant genes encoding the back-up catalase KatB, the periplasmic alkylhydroperoxide reductase AhpB, and the cytoplasmic alkylhydroperoxide reductase AhpC. One unique feature of this study is that the H_2O_2-mediated induction of the *katA* gene, encoding the major "housekeeping" catalase, is regulated by the primary H_2O_2-responsive global transactivator OxyR in *P. aeruginosa* as well. The other feature is that *P. aeruginosa* OxyR displays interesting aspects due to its potential for both positive and negative regulatory modes on a target gene, as recently exemplified by *Neisseria meningitidis* OxyR (26), which involves the conserved Cys residues in a manner different from that known for *E. coli* OxyR, with only one or two oxidation states (29, 61).

KatA is a highly stable enzyme that is released upon cell lysis into the extracellular milieu (20). Its inherent stability may be critical for its putative role in biofilm growth and presumably for chronic infections caused by *P. aeruginosa* (15, 51). This unusual metastability of KatA is one of the contributors responsible for the high-level activity under normal aerobic and anaerobic growth conditions, and thus KatA has been regarded as a constitutively expressed catalase in *P. aeruginosa* (21). Although KatA protein activity is relatively high and not dramatically changed under exposure to H_2O_2, several lines of evidence support that the transcription of the *katA* gene is induced upon H_2O_2 treatment (45). Nevertheless, little is known about the specific regulatory mechanism that underlies *katA* transcription in response to H_2O_2 challenge. Previous work by Ochsner et al. (43) describing the role of OxyR in the oxidative stress response in *P. aeruginosa* strain PAO1 revealed that inactivation of the *oxyR* gene impaired the paraquat (PQ)-induced transcription of several genes, including *katB*, *ahpB*, and *ahpC*, as assessed by *lacZ* reporter fusion analysis and S1 nuclease mapping analyses. They reported that the *katA-lacZ* translational fusion was still induced by PQ in the *oxyR* mutant. However, we have shown that the translational fusion may not be entirely appropriate to determine whether the *katA* transcription is dependent on OxyR. The apparent discrepancy between the present and the previous studies might be due to the different experimental conditions, which may include the difference in the strains (PA14 versus PAO1), the *oxyR* mutations (in-frame deletion versus marked replacement), the inducing conditions (H_2O_2 for less than 30 min versus PQ for 1 h), the methods for the promoter assay (S1 mapping and transcriptional fusion versus translational fusion), or others. We could exclude that the strain difference was an issue, since we obtained the same results using the PAO1 *oxyR* in-frame deletion mutant (data not shown). From our comprehensive transcription study with the determination of the +1 site in response to H_2O_2, the promoter and the *cis*-acting elements under H_2O_2 stress, and the fact that all of the currently known inducible catalase genes in bacteria are under the control of primary peroxide-sensing transcription factors, such as OxyR and PerR, we were able to determine that the *P. aeruginosa* *katA* gene is also regulated by OxyR in response to H_2O_2 stress. However, much remains to be elucidated concerning the potential for multiple regulatory mechanisms that could be exerted sophisticatedly not only at the level of transcription but also at the posttranscriptional and posttranslational levels, considering the unusual metastability and versatile roles of the

KatA protein as the primary H_2O_2-detoxifying enzyme in *P. aeruginosa*.

We suggest here that the oxidation cycle of *P. aeruginosa* OxyR *in vivo* could be a little more complicated than that of *E. coli* OxyR. It is generally accepted that a disulfide bond forms between two cysteines, with the peroxidatic thiol (e.g., Cys 199 in OxyR and Cys 30 in DsbA) firstly oxidized by oxidants. The sulfenic acid (−SOH) generated by thiol oxidation at the peroxidatic thiol was susceptible to nucleophilic attack by the resolving thiol (e.g., Cys 208 in OxyR and Cys 33 in DsbA) (19, 61). In *P. aeruginosa*, however, based on the multiple oxidized species of OxyR upon H_2O_2 exposure, the sulfenic acid of C199, which could be formed transiently, is prone to be either resolved by C208 or modified in other ways, such as further oxidation to sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid. Thus, the modification of C199 under oxidizing conditions, which includes C199-C208 disulfide formation, plays a key role in activation of *P. aeruginosa* OxyR. This hypothesis was in part supported by the AMS modification experiments using OxyR muteins *in vivo* but needs to be verified further by detailed characterization of the OxyR redox cycle *in vitro* as well as *in vivo*, which may involve both C199 and C208 residues.

One of the most strongly supported conclusions from this study may be that *P. aeruginosa* OxyR could act as both the activator and the repressor at the *katA* promoter. We suggest that *P. aeruginosa* OxyR might undergo more than two-step activation by H_2O_2 oxidation, composed of the derepression by Cys 199 oxidation and the activation by further modification, which could accompany structural changes to activate the cryptic activating region for *katA* that resides in the OxyR protein, since the *oxyR* null mutant displayed only a slight elevation of basal *katAp* transcription compared to the level of H_2O_2-induced transcription observed for the wild-type cells. There is still controversy regarding the activation mechanism of *E. coli* OxyR, i.e., whether the OxyR activation involves the two-step disulfide bond formation or only the first step of oxidation at Cys 199 (29, 35, 61). According to our proposed model for *P. aeruginosa* OxyR activation, C199 oxidation to sulfenic acid or the subsequent alteration by rapidly and transiently responding to H_2O_2 exposure is potentially involved in the derepression, and further modification of C199 sulfenic acid to a disulfide bond species or others is involved in further activation by derepressing the cryptic activating region. It can be hypothesized that the C199A mutant, as well as the double mutants with mutations in C199 and C208, mimics fully activated OxyR, whereas the C199S mutant mimics partially activated OxyR. The lower induced expression in the C208S mutant can be attributed to the rapid reduction of the fully activated OxyR into less active states. Furthermore, the slow induction of the *katAp* transcription in the C199S mutant may indicate the slow/inefficient activation that leads to the structural change that mimics fully activated OxyR, which could involve the alternative mechanisms to sense oxidizing conditions, as observed for the slower-migrating species in the C199S mutein at 10 min after H_2O_2 exposure. These hypotheses need to be addressed in future studies to elucidate the relationship between the multiple oxidation states and the multiple activation states. A deeper understanding of the novel properties that *P. aeruginosa* OxyR possesses and its ever-broadening physiological roles in the oxidative stress responses may not only lead us to new insights into the molecular mechanisms of sensing and regulation based on thiol oxidation in a variety of bacterial species of diverse ecological niches but also provide a new therapeutic target to control the virulence of this opportunistic pathogen under both acute- and chronic-infection conditions, which entails proper redox regulation.

ACKNOWLEDGMENTS

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