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 (H2O2) 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 H2O2 was abrogated in the P. aeruginosa PA14 oxyR null mutant. Promoter deletion analyses revealed that H2O2-mediated induction was dependent on a region of DNA −76 to −36 upstream of the H2O2-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 H2O2-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 H2O2, as assessed by free thiol trapping using 4-acetamido-4-maleimidodistyrlbene-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 H2O2 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 H2O2 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 (O2) 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 O2, which can lead to the successive production of reactive oxygen species (ROS) (17), such as superoxide radical (O2−), hydrogen peroxide (H2O2), and hydroxyl radical (HO·), 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 H2O2 in E. coli and Salmonella enterica serovar Typhimurium (1, 13). OxyR senses H2O2 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 H2O2, 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-
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 H2O2-sensing transcriptional regulator (43). It is also known that typical antioxidant enzymes for the defense against H2O2 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 H2O2 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 H2O2 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 H2O2 resistance and osmoprotection (36). KatA is critical for the adaptive response to H2O2 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 H2O2-scavenging enzyme in H2O2 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 H2O2 stress in P. aeruginosa strain PA14. We identified the katA promoter, whose transcription was induced by H2O2 exposure. Transcriptional activation was found to require the H2O2-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 H2O2 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 × 10^6 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; carbenicillin, 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 H2O2. The H2O2-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 RNasey 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-32P]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 [γ-32P]ATP 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 −56), or katA-N22 (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 Ncol 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 antiseraum 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 Na2HPO4, and 2 mM KH2PO4, pH 7.0) was injected into five mice (ICR females) at intervals of 2 days.
weeks. Antiseras 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 proteins were tagged with a FLAG epitope as described previously (37), using PCR primers oxyR-N1 and oxyR-FLAG-C0. The DNA fragments were end labeled with [γ-32P]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 MgCl2, 10 ng/ml bovine serum albumin [BSA], and 10% 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-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.
The DNA-protein mixture was treated with DNase I as described elsewhere (52). Briefly, 50 μl of a CaCl₂-MgCl₂ solution (50 mM CaCl₂ and 10 mM MgCl₂) 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 digestion reaction was stopped with 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml tRNA). 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 cells were sedimented by centrifugation at 8,000 rpm for 5 min at 4°C, 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 1 M 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 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 incubated for 2 h at 4°C to reduce the nonspecific associations. 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 (50 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 washing buffer consisting of 50 μM 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 NaHCO₃, 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 Sall 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 288th cysteine residues to serine by a TGC-to-TCC mutation) (C25S, C199S, and C288S) 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 pUC18-T-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 of the atTn7 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 H₂O₂-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 H₂O₂ 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. The 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$_7$- and O$_{20}$-truncated ORE (N22) or missing the entire ORE (N23). Cells were treated with (+) or without (−) 1 mM H$_2$O$_2$ 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.

spectrum, 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$_2$O$_2$, 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$_2$) 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$_2$O$_2$ 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$_2$O$_2$-induced LacZ activity from the lacZ fusions was relatively modest, we found that the deletion of the IR (N21) did not alter H$_2$O$_2$-induced promoter activity, whereas the deletion of the first two operator sites (N22) of the ORE partially abolished H$_2$O$_2$-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$_2$O$_2$ treatment. These results lead us to hypothesize that the ORE is involved in the H$_2$O$_2$-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 katA in response to H$_2$O$_2$. We introduced the katAp-lacZ fusion (pQF-N10) in the oxyR null mutant and other mutants lacking quorum-sensing circuitry (lasR rhlR and myR), stationary-phase adaptation sigma factor (pooS), 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$_2$O$_2$-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 katA. The introduction of a mini-Tn7-based chromosome copy of the wild-type OxyR (mTn7-oxyR) into the oxyR null mutant containing the katAp-lacZ fusion fully restored H$_2$O$_2$-mediated katA activity, while the previously elevated basal level decreased back to wild-type levels (Fig. 3A).

We next verified the ablation of the H$_2$O$_2$-mediated induction of katA 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$_2$O$_2$ 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$_2$O$_2$.

OxyR-dependent regulation of katAp transcription is direct, requiring DNA-binding activity to the ORE. To test the hypothesis that the regulation of katA is mediated by direct
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. 3. H$_2$O$_2$-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, mvrF, and oxyR mutants complemented with mTn7-oxyR) treated with (+) or without (−) 1 mM H$_2$O$_2$ 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$_2$O$_2$. Total protein (50 μg) was prepared from the cells harvested before treatment and at 10, 20, and 30 min after the H$_2$O$_2$ treatment, followed by Western blotting with anti-KatA and anti-RpoA antisera.

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 S' 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$_5$) 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$_2$O$_2$ 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.
Involvement of OxyR cysteine residues in H$_2$O$_2$-mediated oxidation of OxyR. To investigate the potential involvement of the cysteine (Cys) residues in H$_2$O$_2$-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 bacterial 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$, 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 katA transcription. We have assessed how the conserved cysteine (Cys) residues of OxyR affect katA transcription, given the results outlined above as well as the knowledge that the thiol residues of E. coli OxyR play pivotal roles in H$_2$O$_2$ 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 Tn7 integration site (attTn7) 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$_2$O$_2$ 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$_2$O$_2$ 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, C208S, and C199A C208S mutants, even without the H$_2$O$_2$ treatment, whereas those in the C208S mutant were not significantly increased in response to H$_2$O$_2$ (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
was elevated in response to H$_2$O$_2$ stress but rapidly decreased, C199S C208S mutants) that had been grown to the mid-logarithmic growth phase (OD$_{600}$ = 0.3) with (+) or without (−) 1 mM H$_2$O$_2$ treatment for 20 min were analyzed by Western blotting. Fifty micrograms of total RNA was used for S1 nuclease mapping analyses. They reported that the $katA$ transcription in response to H$_2$O$_2$ 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 (PO)-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$_2$O$_2$ 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$_2$O$_2$, the promoter and the cis-acting elements under H$_2$O$_2$ 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$_2$O$_2$ 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 differential involvement of OxyR in the H$_2$O$_2$-induced regulation of both catalase genes (61).

Then, we investigated the time course of $katA$ transcription upon H$_2$O$_2$ 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 $katA$. The $katA$ transcription in the C208S mutant was elevated in response to H$_2$O$_2$ 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$_2$O$_2$ 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.
KatA protein as the primary H$_2$O$_2$-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$_2$O$_2$ 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 sulfonic (−SO$_3$H) or sulfonic (−SO$_2$OH) 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 mutants 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$_2$O$_2$ oxidation, composed of the derepression by Cys 199 oxidation and the activation by further modification, which could accompany structural changes to activate the cystic 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$_2$O$_2$-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 and the subsequent alteration by rapidly and transiently responding to H$_2$O$_2$ 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 cystic 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 explained by the slower-migrating species in the C199S mutein at 10 min after H$_2$O$_2$ 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 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.

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