JOURNAL OF BACTERIOLOGY, Aug. 2000, p. 4533–4544 Vol. 182, No. 16
0021-9193/00/$04.00+0

Role of the *Pseudomonas aeruginosa oxyR-recG* Operon in Oxidative Stress Defense and DNA Repair: OxyR-Dependent Regulation of *katB-ankB*, *ahpB*, and *ahpC-ahpF*

URS A. OCHSNER,[1]* MICHAEL L. VASIL,[1] EYAD ALSABBAGH,[2] KISLAY PARVATIYAR,[2] AND DANIEL J. HASSETT[2]

Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262,[1] and Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0524[2]

Received 11 February 2000/Accepted 19 May 2000

Pseudomonas aeruginosa **possesses an extensive armament of genes involved in oxidative stress defense, including *katB-ankB*, *ahpB*, and *ahpC-ahpF*. Transcription of these genes was regulated in response to H_2O_2, paraquat, or organic peroxides. Expression of *katB-lacZ* and the observed KatB catalase levels in *P. aeruginosa* PAO1 were induced up to 250-fold after exposure to oxidative stress-generating compounds. Also, *ahpB-lacZ* and *ahpC-lacZ* expression was 90- and 3-fold higher, respectively, upon exposure to paraquat. The dose- and time-response curves revealed that 1 μM paraquat was sufficient for half-maximal activation of each reporter fusion within 5 min of exposure. Expression of these genes was not observed in a Δ*oxyR* mutant, indicating that OxyR was essential for this response. The transcriptional start sites of *katB-ankB*, *ahpB*, and *ahpC-ahpF* were mapped, putative OxyR-binding sites were identified upstream of the −35 promoter elements, and direct binding of purified OxyR protein to these target promoters was demonstrated. The *oxyR* mutant was hypersusceptible to oxidative stress-generating agents, including H_2O_2 and paraquat, in spite of total KatA catalase activity being comparable to that of the wild type. The *oxyR* phenotype was fully complemented by a plasmid containing the *oxyR* gene, while any of the *katB*, *ahpB*, or *ahpCF* genes alone resulted in only marginal complementation. Increased *katB-lacZ* expression and higher KatB catalase levels were detected in a Δ*ahpCF* background compared to wild-type bacteria, suggesting a compensatory function for KatB in the absence of AhpCF. In *P. aeruginosa*, *oxyR* is located upstream of *recG*, encoding a putative DNA repair enzyme. *oxyR-lacZ* and *recG-lacZ* reporter activities and *oxyR-recG* mRNA analysis showed that *oxyR* and *recG* are organized in an operon and expressed constitutively with regard to oxidative stress from a single promoter upstream of *oxyR*. Mutants affected in *recG* but not *oxyR* were dramatically impaired in DNA damage repair as measured by sensitivity to UV irradiation. In conclusion, we present evidence that the *oxyR-recG* locus is essential for oxidative stress defense and for DNA repair.**

Pseudomonas aeruginosa generates metabolic energy primarily through aerobic respiration. This process, involving a four-electron reduction of molecular oxygen (O_2) to water, can be potentially dangerous to the cell. Specifically, aberrant electron flow from the electron transport chain or cellular redox enzymes to O_2 can lead to the production of reactive oxygen intermediates (ROIs). These include superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$). Furthermore, bacteria can be exposed to exogenous ROIs, especially during infection of humans, where phagocytes (e.g., neutrophils) mount a dramatic oxygen-dependent antimicrobial response (16, 38). The unchecked production or accumulation of these species can lead to cell damage, mutations, or death. The generation of $HO^\bullet$, the most destructive of the above-mentioned compounds, is in part dependent upon the presence of a transition metal, such as iron or copper, and H_2O_2. Defense against ROIs is provided by antioxidant enzymes (superoxide dismutase [SOD], catalase, and peroxidase), iron sequestration, free-radical-scavenging agents, DNA-binding proteins, and DNA repair enzymes (4, 25, 26, 32, 35, 62). *P. aeruginosa* possesses an impressive antioxidant armament for defense against ROIs, including two SODs (cofactored by either iron [Fe-SOD] or manganese [Mn-SOD] [19, 20] to disproportionate O_2^- to H_2O_2 and O_2), three catalases (KatA, KatB, and KatC) (6, 32), and four alkyl hydroperoxide reductases (AhpA, AhpB, AhpCF, and Ohr) (U. A. Ochsner, D. J. Hassett, and M. L. Vasil, unpublished data).

We have now investigated the roles of individual oxidative stress defense genes by phenotypic assessment of specific mutants and have monitored the responses of these genes to oxidative stress. It appears that redundancy of oxidative stress defense systems allows *P. aeruginosa* to optimally cope with ROIs generated by its own vigorous aerobic metabolism and to respond rapidly to exogenous ROIs. Some of the genes involved in oxidative stress defense, including *katA* (encoding the major catalase in *P. aeruginosa* [32]), and *ahpA* (encoding a ferredoxin-dependent alkyl hydroperoxide reductase [U. Ochsner and D. Hassett, unpublished data]), are expressed at high levels during aerobic growth. Their activities are maintained at such high levels that even significant oxidative stress causes only a twofold increase in expression, suggesting that high KatA and AhpA activities are critical for detoxification of ROIs produced endogenously during normal aerobic growth. On the other hand, several oxidative stress defense genes, including *katB-ankB* (24), *ahpB*, and *ahpC-ahpF*, are dramatically induced by ROI-generating agents, suggesting a specific and tightly regulated response.

* Corresponding author. Mailing address: University of Colorado Health Sci. Ctr., Department of Microbiology, B-175, 4200 E. Ninth Ave., Denver, CO 80262. Phone: (303) 315-5093. Fax: (303) 315-6785. E-mail: urs.ochsner@uchsc.edu.

Key regulators modulating the oxidative stress response in bacteria are SoxR and OxyR, both of which are activated at the posttranslational level. O_2^- activates SoxR through oxidation of its [2Fe-2S] cluster (11, 13), and oxidized SoxR induces the expression of the second transcription factor SoxS, which directly activates transcription of several genes, including *sodA* in *Escherichia coli* (27, 30, 60). H_2O_2 induces at least 30 genes in *E. coli*, and the response of a subset of these genes depends on OxyR, a 34-kDa LysR-type transcriptional activator (8, 53). *E. coli oxyR* mutants are hypersensitive to H_2O_2 and have increased rates of spontaneous mutagenesis during aerobic growth (52). OxyR-regulated genes in *E. coli* include *katG* (encoding hydroperoxidase I), *gorA* (encoding glutathione reductase), *ahpCF* (encoding alkyl hydroperoxide reductase) (52), and *fur* (for ferric uptake regulator) (66). Furthermore, *E. coli* OxyR also controls the formation of a small RNA, designated *oxyS*, that can act as a positive or negative regulator in response to oxidative stress. The abundant and relatively stable 109-nucleotide *oxyS* RNA is transcribed immediately upstream and divergently of *oxyR* in *E. coli*. Several *oxyS*-regulated genes were identified in *E. coli*, including *dps* (DNA-binding protein of stationary phase) and *rpoS* (σ^S) (1, 14). Recent biochemical studies have shed light on the molecular mechanism of OxyR activation in *E. coli*. OxyR is redox sensitive and can switch rapidly between oxidized and reduced states, but only the oxidized form of OxyR acts as a transcriptional activator (54). In the presence of H_2O_2, OxyR forms an intramolecular disulfide bond which can be deactivated by enzymatic reduction upon relief of oxidative stress (2, 65). Both the oxidized and the reduced forms of the *E. coli* OxyR protein have been shown to possess DNA binding activity (55). Oxidized OxyR recognizes a motif comprised of four ATAG elements spaced at 10-bp intervals (56).

A better understanding of the oxidative stress response in *P. aeruginosa*, a ubiquitous gram-negative opportunist, is of great industrial and clinical importance. In this work, we provide evidence for the existence of an OxyR homolog in *P. aeruginosa* and characterize three OxyR-regulated genes essential for the optimal defense against oxidative stress. We describe significant differences in the OxyR response between *P. aeruginosa* and *E. coli*, including a link to DNA repair since the *P. aeruginosa oxyR* gene is located in an operon with *recG*, and OxyR regulation of a novel type of alkyl hydroperoxide reductase (AhpB) not found in *E. coli* that is very important for resistance to H_2O_2.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and chemicals. All *P. aeruginosa* and *E. coli* strains and plasmids used in this study are listed in Table 1. Luria broth (LB) was used for strain maintenance and contained 1.5% agar (Difco) in solid media. M9 minimal medium (45) was used for cultivating *P. aeruginosa* in the presence of oxidative stress-generating agents. Liquid cultures were grown aerobically at 37°C in shake flasks or, for smaller volumes up to 2 ml, in 14-ml plastic tubes (Fisher Scientific) shaken at 250 rpm. Antibiotics were added as follows: for *E. coli*, ampicillin (Sigma Chemical Co., St. Louis, Mo.) (100 μg ml^{-1}), gentamicin (Abbott Laboratories) (15 μg ml^{-1}), kanamycin (Sigma) (100 μg ml^{-1}), and tetracycline (Sigma) (15 μg ml^{-1}); for *P. aeruginosa*, carbenicillin (Research Products International) (750 μg ml^{-1}), gentamicin (75 μg ml^{-1}), and tetracycline (150 μg ml^{-1}). Paraquat (methyl viologen), H_2O_2 (30%), cumene hydroperoxide (CHP) (80% stock solution diluted with ethanol), *t*-butyl hydroperoxide (tBHP) (70% solution), and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were from Sigma Chemical Co.). Bovine liver catalase was from Boehringer Mannheim, and concentrated protein dye (Bradford reagent) was from Bio-Rad. X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) was from Research Products International and was used at 40 μg ml^{-1}.

Oxidative stress and UV irradiation sensitivity assays. To test susceptibility of *P. aeruginosa* strains to oxidative stress agents, 100 μl of cells grown overnight in LB were inoculated into 100 ml of M9 medium, grown to mid-exponential phase (optical density at 600 nm [OD_{600} = 0.5), and split into 2-ml aliquots. Paraquat, H_2O_2, CHP, or tBHP was added at appropriate concentrations, and the subcultures were shaken aerobically. For disk inhibition assays, a culture volume representing 0.2 OD_{600} unit of cells was mixed with 3 ml of 0.7% low-melting-point M9 agarose at 37°C and poured onto M9 agar plates. Sterile filter disks containing 10 μl of either 2% H_2O_2 or 20% CHP were placed in triplicate on the top agar, the plates were incubated overnight at 37°C, and the zones of growth inhibition were recorded. For UV sensitivity assays, 100 μl of cells grown overnight in LB was diluted with 10 ml of M9 medium in an uncovered glass petri dish and shaken at 60 rpm at room temperature. Irradiation was performed with a UV lamp (Fotodyne model 3-6000) placed 5 cm above the cells. Samples were removed at 0, 10, 20, 30, 40, and 60 s of UV irradiation and serially diluted with LB in a microtiter dish. Catalase (1,300 U ml^{-1}) was added for the dilution of *oxyR* mutant strains. Appropriate dilutions (50 μl) were spotted on LB agar, and the colonies were enumerated after overnight incubation at 37°C in the dark.

General genetic procedures. PCR was performed using *Taq* polymerase and custom-made primers (Bethesda Research Laboratories, Gaithersburg, Md.) in a Perkin-Elmer Cetus thermal cycler, with 30 cycles of denaturing (1 min, 94°C), annealing (1 min, 54°C), and extending (1 min per kb of DNA, 72°C). The PCR products were purified in low-melting-point agarose gels, routinely cloned into pCRII-2.1 (Invitrogen), and sequenced with Sequenase 2.0 (United States Biochemical) and M13 primers or custom-made 18-mer oligonucleotides. Published procedures were followed for Southern blot analysis, colony hybridization, end labeling of DNA fragments, and other recombinant DNA methods (45), using DNA modifying enzymes from Bethesda Research Laboratories. Standard protocols were used for the isolation of plasmid DNA (23) and chromosomal DNA (9). Plasmids were maintained in *E. coli* DH5α-MCR (Bethesda Research Laboratories) and transformed into *P. aeruginosa* strains using the magnesium chloride method (42). RNA was isolated by the hot-phenol method and analyzed by RNase protection assays as described in detail elsewhere (3). Radiolabeled riboprobes were generated from cloned DNA fragments (Table 1), using an in vitro runoff transcription system (Promega), and excess probe was hybridized to 20 μg of total RNA.

Construction of isogenic mutant strains. Mutant strains affected in *oxyR* were constructed as follows. A 1.36-kb PCR product containing the *oxyR* region was generated with primers *oxyR*-226 (5′TGTACACCAGGTAGTCGAG) and *oxyR*-1585 (5′-GTTTCCAGGCCTACCCGAG), cloned into pCRII-2.1, sequenced, excised with *Eco*RI, and cloned into the *Eco*RI site of pUC19. A 0.62-kb *Xho*I-*Sst*II internal fragment of the *oxyR* gene was removed, and the ends were blunted with Klenow enzyme and ligated to a 1.3-kb FRT-Gm^r-FRT cassette (Gm^r) excised from pPS856 (21) with *Bam*HI and followed by end polishing. The resulting plasmid, pUCΔ*oxyR*::Gm, was digested with *Pvu*II, yielding a 2.5-kb Δ*oxyR*::Gm construct which was ligated into the *Sma*I site of the gene replacement vector pEX100T (47). *E. coli* SM10 containing pEX100T-Δ*oxyR*::Gm was used as the donor strain in a biparental mating with *P. aeruginosa* PAO1. Transconjugants were selected on brain heart infusion agar containing gentamicin (75 μg ml^{-1}) and irgasan (50 μg ml^{-1}) and subsequently plated on LB agar containing gentamicin (75 μg ml^{-1}) and 5% sucrose. Successful double-crossover events leading to the replacement of the *oxyR* gene with the Gm^r cassette in the putative Δ*oxyR*::Gm mutant strain were verified by the loss of pEX100T-encoded Cb^r and by PCR across the *oxyR* gene using the primers *oxyR*-226 and *oxyR*-1585. To obtain an unmarked Δ*oxyR* mutant strain, *E. coli* SM10 harboring pFLP2 (21) was mated into Δ*oxyR*::Gm, and Δ*oxyR*::Gm/pFLP2 was grown overnight in LB to allow excision of the Gm^r cartridge via the adjacent FRT sequences (21). Single colonies on LB-carbenicillin were then checked for the loss of Gm^r. Finally, pFLP2 was cured from Δ*oxyR* by selection for sucrose resistance, indicating the loss of the pFLP2-borne *sacB* gene, and the resulting unmarked Δ*oxyR* mutant was also checked for loss of plasmid-encoded Cb^r.

The other mutant strains used in this study were constructed by essentially the same method as described above. In brief, a 1.2-kb DNA fragment containing the *ahpB* region was PCR amplified using primers *ahpB*-1321 (5′GATGGCGCTTCAACTCGAAG) and *ahpB*-2537 (5′TGCATGCCGGTGATCAGCAG). A 0.63-kb *Hin*cII-*Sma*I fragment containing the entire *ahpB* coding sequence minus the four first codons was then replaced by a Gm^r cartridge, resulting in a Δ*ahpB*::Gm mutant. To obtain a Δ*ahpCF*::Gm mutant, a 2.1-kb region containing the *ahpC-ahpF* locus was isolated by PCR using primers *ahpC*-621 (5′GACCATCCTGGTGCTGGTC) and *ahpF*-2741 (5′TTCCAGCAGGGTCACATGG). A 1.5-kb *Hin*cII fragment containing most of the *ahpC* gene and a 5′ portion of *ahpF* was replaced by a Gm^r cartridge. A *recG*::Gm mutant was constructed by insertion of a Gm^r cartridge into the unique *Bgl*II site within the *recG* gene that had been PCR amplified with primers *recG*-1557 (5′GAGAAGCTCGCTCGGGTAG) and *recG*-3107 (5′GAAGGCTTCCATCACCACG).

Construction of *lacZ* reporter fusions. DNA fragments containing the relevant promoter regions, including the translational start sites, were PCR amplified, cloned into pCRII-2.1, sequenced, and ligated into pPZ30. To achieve an in-frame translational fusion to the promoterless *lacZ* gene, a *Pst*I site was incorporated in the primer sequence at an appropriate 3′-end position, when necessary. Specifically, plasmid pPZ-*oxyR*-126 contained a 126-bp *Eco*RI-*Pst*I fragment harboring the *oxyR* promoter region plus the first six codons of the *oxyR* gene. In pPZ-*recG*-1133 and pPZ-*recG*-360 the first 32 codons of the *recG* gene were fused to *lacZ*, and these plasmids contained increasing upstream sequence as depicted in Fig. 1. Plasmid pPZ-*katB*-480 contained the *katB* promoter plus 59 codons of the *katB* gene on a 480-bp *Eco*RI-*Pst*I fragment that had been generated by PCR with primers *katB*-38 (5′CTTGGAACTGCGCCATGCAG) and

TABLE 1. Strains and plasmids used in this study

Strains or plasmids	Genotype or characteristics[a]	Source or reference
E. coli strains		
BL21(DE3)	High-stringency T7 expression host; *hsdS* DE3	Novagen
DH5α-MCR	F$^-$ *lacZ*ΔM15 *recA1 hsdR17 supE44* Δ(*lacZYA argF*)	Bethesda Research Laboratories
SM10	Km^r; mobilizer strain	49
P. aeruginosa strains		
PAO1	Prototrophic, wound isolate	22
Δ*ahpB*::Gm	Gm^r; Δ*ahpB*::Gm mutant of PAO1	This study
Δ*ahpCF*::Gm	Gm^r; Δ*ahpCF*::Gm mutant of PAO1	This study
Δ*katA*::Gm	Gm^r; Δ*katA*::Gm mutant of PAO1	32
Δ*katB*::Gm	Gm^r; Δ*katB*::Gm mutant of PAO1	24
Δ*oxyR*::Gm	Gm^r; Δ*oxyR*::Gm mutant of PAO1	This study
Δ*oxyR*	Unmarked Δ*oxyR* mutant of PAO1	This study
Δ*recC*::Tc	Tc^r; Δ*recC*::Tc mutant of PAO1	This study
recG::Gm	Gm^r; *recG*::Gm insertion mutant of PAO1	This study
recG::Gm Δ*recC*::Tc	Gm^r Tc^r; *recG*::Gm Δ*recC*::Tc double mutant	This study
Plasmids		
pCRII-2.1	Ap^r Km^r TA cloning vector for PCR fragments	Invitrogen
pCRII-*ahpB*-606	pCRII-2.1 containing the *ahpB* promoter on a 606-bp PCR fragment generated with primers GCTTCAACTCGAAGTCCAG and GATGGGTGAACTGCGAGTC; source of T7-expressed *ahpB* riboprobe and of DNA fragment for mobility shift	This study
pCRII-*ahpC*-355	pCRII-2.1 containing the *ahpC* promoter on a 355-bp PCR fragment generated with primers GACCATCCTGGTGCTGGTC and TGCCCTTCAGGGATTCCTC; source of T7-expressed *ahpB* riboprobe and of DNA fragment for mobility shift	This study
pCRII-*omlA*-444b	pCRII-2.1 containing a 444-bp *omlA* promoter fragment; source of T7-expressed *omlA* riboprobe	39
pET-OxyR	pET14b (Novagen) carrying the *oxyR* gene on a 954-bp *Nde*I-*Bam*HI fragment for the production of His_6-tagged OxyR	This study
pEX100T	Ap^r *oriT mob sacB*	47
pFLP2	Ap^r *sacB*; broad-host-range recombination system	21
pPS856	Ap^r; source of FRT-Gm^r-FRT cassette	21
pPZ30	Ap^r; broad-host-range *lacZ* fusion vector	46
pPZ-*oxyR*-126	Ap^r; pPZ30 containing an *oxyR*::*lacZ* fusion	This study
pPZ-*recG*-1133	Ap^r; pPZ30 containing an *oxyR*::*recG*::*lacZ* fusion	This study
pPZ-*recG*-360	Ap^r; pPZ30 containing an *recG*::*lacZ* fusion	This study
pPZ-*katB*-480	Ap^r; pPZ30 containing a *katB*::*lacZ* fusion	This study
pPZ-*ahpB*-377	Ap^r; pPZ30 containing an *ahpB*::*lacZ* fusion	This study
pPZ-*ahpC*-270	Ap^r; pPZ30 containing an *ahpC*::*lacZ* fusion	This study
pPZ-*katA*	Ap^r; pPZ30 containing a *katA*::*lacZ* fusion	32
pBluescript SK+	Ap^r; *lacZ′*; cloning vector	Stratagene
pSK-*ahpC-ahpF*-484	pBluescript SK(+) containing the *ahpC-ahpF* intergenic region on a 484-bp PCR fragment generated with primers GATCAAGACCGTCGAGATC and GTCGGTCTTCAGGGTGATC; source of T7-expressed *ahpC-ahpF* riboprobe	This study
pSK-*oxyR*-384	pBluescript SK(+) containing the *oxyR* promoter on a 384-bp *Bam*HI-*Xho*I fragment; source of T7-expressed *oxyR* riboprobe and of DNA fragment for mobility shift	This study
pSK-*oxyR-recG*-365	pBluescript SK(+) containing the *oxyR-recG* overlapping region on a 365-bp PCR fragment generated with primers TGGAGTCCTCGTCGCTGGA and TGTCCTGCAGGGTTTCCAG; source of T7-expressed *oxyR-recG* riboprobe	This study
pSK-*katB*-731	pBluescript SK(+) containing the *katB* promoter on a 731-bp PCR fragment generated with primers GCTTTGAATTCACTCAGAAG and TCCTGCAGCAGCACCGAAC; source of T7-expressed *katB* riboprobe and of DNA fragment for mobility shift	This study
pUC19	Ap^r, ColE1; *E. coli* cloning vector	61
pUCP19, pUCP22	Ap^r; broad-host-range expression vectors	59
pUCP-*oxyR*	Ap^r; pUCP19 containing *oxyR* under P_{lac} control	This study
pUCP-*katB*	Ap^r; pUCP19 containing *katB* under P_{lac} control	This study
pUCP-*ahpB*	Ap^r; pUCP19 containing *ahpB* under P_{lac} control	This study
pUCP-*ahpCF*	Ap^r; pUCP19 containing *ahpCF* under P_{lac} control	This study
pUCP-*recG*	Ap^r; pUCP19 containing *recG* under P_{lac} control	This study

[a] *mob*, mobilization site; *oriT*, origin of transfer (RK2); Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; P_{lac}, *lac* promoter; FRT, Flp recombinase target.

katB-514 (5′TCCTGCAGCAGCACCGAAC [the *Pst*I site is underlined]). Construct pPZ-*ahpB*-377 harbored the first four codons of the *ahpB* gene and the *ahpB* promoter on a 377-bp *Eco*RI-*Pst*I fragment obtained with primers *ahpB*-1321 (5′GATGGCGCTTCAACTCGAAG) and *ahpB*-1697 (5′ctgCAGTACGC TCATCGCGAGG [nonmatching nucleotides in lowercase type]). A 270-bp *Eco*RI-*Pst*I fragment containing the *ahpC* promoter plus the first three codons of *ahpC* was PCR amplified with primers *ahpC*621 (5′GACCATCCTGGTGCTG GTC) and *ahpC*-890 (5′ctgCAGGGACATCAGTCGTTCCT) and cloned into

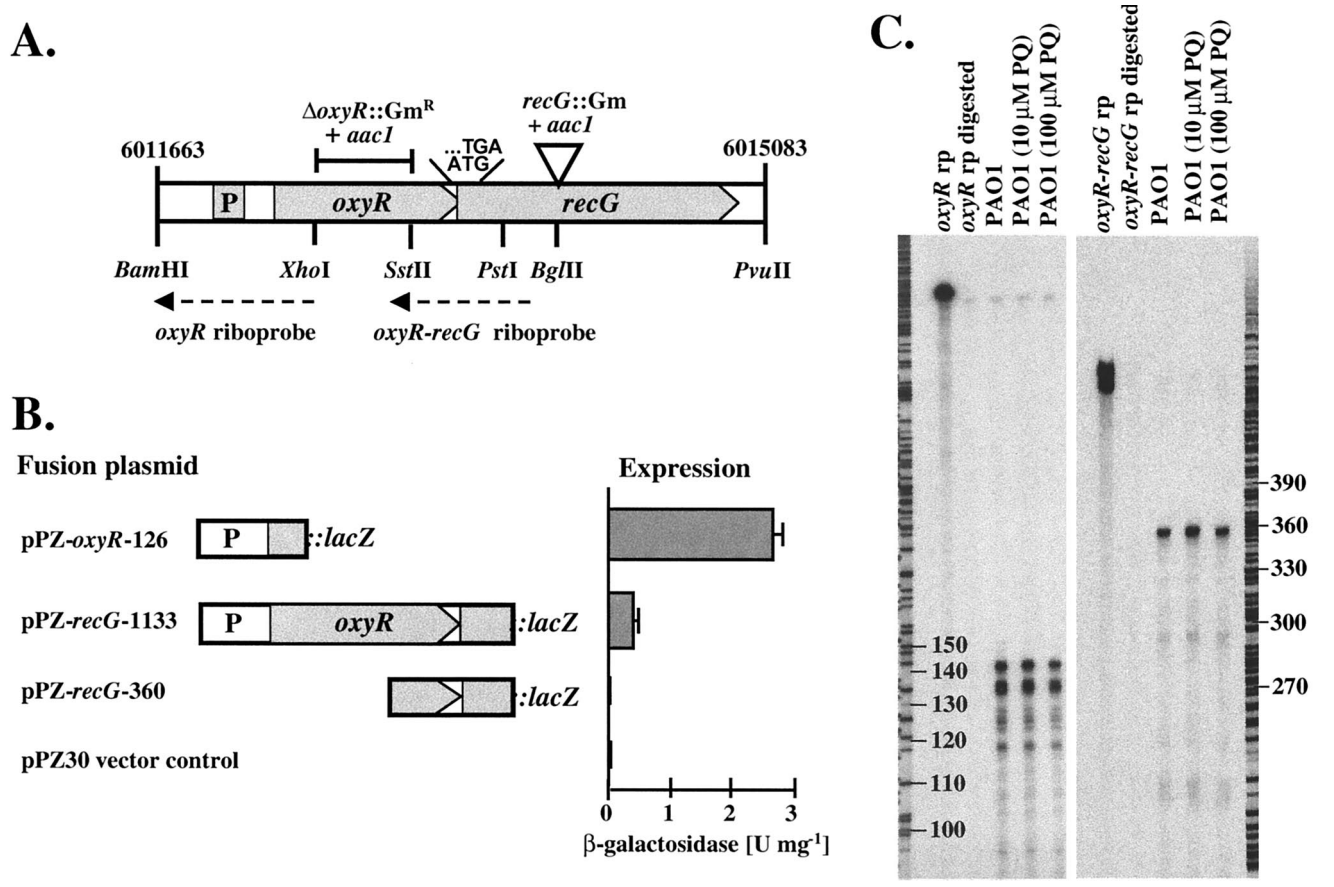

FIG. 1. Characterization of the *oxyR-recG* operon. (A) Genetic map showing the putative promoter, the overlapping TGA stop codon for *oxyR* and ATG start codon for *recG*, the locations of the riboprobes, relevant restriction sites, and the deletions and insertions made in the Δ*oxyR* and *recG* mutants. The numbers flanking the maps indicate the coordinates of these loci in the PAO1 genome (Pathogenesis Corp., 12-15-99 release). (B) Expression of *oxyR* and *recG* as fusions to *lacZ*. The portions of the *oxyR-recG* DNA sequence contained in the *lacZ* fusion plasmids are indicated, together with the corresponding β-galactosidase activities expressed from these constructs. Error bars indicate standard deviations. (C) RNase protection assays. Riboprobes specific for the *oxyR* promoter (*oxyR*-rp) and for the *oxyR-recG* overlapping region (*oxyR-recG* rp) were used to detect the corresponding transcripts in *P. aeruginosa* PAO1 total RNA isolated during the exponential growth phase in M9 medium. Paraquat (PQ) was added to final concentrations of 10 and 100 μM 1 h prior to harvest as indicated. Also shown are the digested probes in the absence of any *P. aeruginosa* RNA as a control. A DNA sequencing reaction was run in parallel and served as a size marker. Numbers indicate nucleotides.

pPZ30 linearized with *Eco*RI and *Pst*I, yielding pPZ-*ahpC*-270. Besides those mentioned above, a minilibrary of several additional genes were also tested for their dependence on OxyR, and they included *katA*, *katC*, *ahpA*, *ohr*, *fur*, *omlA*, *sodA*, *sodB*, *bfrA*, *bfrB*, *phuR*, *plcH*, *toxA*, *pvdS*, *rpoS*, *dps* and *ptxR*.

Construction of complementing plasmids. The *P. aeruginosa-E. coli* multicopy shuttle vectors pUCP19 and pUCP22 (59) were used for the construction of recombinant plasmids containing the *oxyR*, *katB*, *ahpB*, *ahpC-ahpF*, and *recG* genes under the control of the plasmid-borne *lac* promoter that drives constitutive expression in *P. aeruginosa*. The complete *oxyR* gene was PCR amplified with primers *oxyR*-460 (5′GCAGTGTAGGCGTCGAATC) and *oxyR*-1585 (5′GTTT CCAGGCCTACCCGAG), and the PCR product was cloned into pCRII-2.1 and transferred as a 1.13-kb *Eco*RI fragment into pUCP19, resulting in pUCP-*oxyR*. Plasmid pUCP-*katB* was constructed similarly using a 1.9-kb PCR product obtained with primers *katB*-38 (see above) and *katB*-1887 (5′CCAGGATTGATC GCAACCGG). The *ahpB* gene was amplified by PCR with primers *ahpB*-1321 and *ahpB*-2537 (see above) and was directionally cloned as a 1.2-kb *Hin*dIII-*Xba*I fragment from pCRII-2.1 into pUCP22, yielding pUCP-*ahpB*. The *ahpC-ahpF* region was located on a 3.5-kb *Sph*I fragment as predicted from the *P. aeruginosa* genome sequence. Accordingly, chromosomal DNA of *P. aeruginosa* PAO1 was cut with *Sph*I, and fragments of the size range of 3 to 4 kb were cloned into pUCP19. A pUCP-*ahpCF* plasmid harboring the *ahpC-ahpF* genes under *lac* promoter control was subsequently isolated by colony hybridization using a 270-bp *ahpC* promoter fragment (see above) as a radiolabeled probe. For the construction of pUCP-*recG*, it had to be considered that the native *recG* gene lacked a Shine-Dalgarno sequence due to the overlap of its ATG start codon with the *oxyR* TGA stop codon (Fig. 1). Therefore, a Shine-Dalgarno motif (underlined, see below) was incorporated 7 bp upstream of the *recG* ATG. A 2.1-kb PCR product containing *recG* was obtained with primers *recG*-1490 (5′aggagA AATAGCATGACCGAGCTGTC) and *recG*-3616 (5′GCTTCAAGACTGAGA CCTACG), cloned into pCRII-2.1, and directionally cloned as a *Hin*dIII-*Xba*I fragment into pUCP22, resulting in pUCP-*recG*.

Purification of OxyR and DNA mobility shift assays. The *oxyR* gene was PCR amplified as an *Nde*I-*Bam*HI fragment using primers (*Nde*I)-catATGACCCTC ACCGAACTGC and (*Bam*HI)-ggatCCTGGACAGCTCGGTCATG and cloned into pCRII-2.1. After verification of its sequence, the *oxyR* gene was cloned into the *Nde*I-*Bam*HI sites of pET14b (Novagen), generating an in frame fusion with the vector-encoded His tag sequence. OxyR protein with an amino-terminal His_6 tag was overexpressed from pET-OxyR in the BL21(DE3) T7 expression strain and purified through metal affinity chromatography on Ni-nitrilotriacetic acid (Qiagen). End-labeled DNA fragments (1 to 2 ng) harboring the relevant promoter sequences (Table 1) were incubated for 15 min with increasing amounts (up to 1 μM) of freshly purified His_6-OxyR protein in 20 μl of binding buffer [20 mM bis-Tris borate (pH 7.5), 40 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 100 μg of bovine serum albumin ml^{-1}, 50 μg of poly(dI-dC) ml^{-1}, 10% glycerol], and 10 μl of the mixture was loaded on a 6% polyacrylamide gel in running buffer (20 mM bis-Tris borate, pH 7.5). After electrophoresis for 3 to 4 h at 250 V, the gel was dried and autoradiographed.

Biochemical procedures. β-Galactosidase activities were determined as follows. Bacterial cell extracts from 2-ml cultures were prepared by centrifugation (10,000 × *g*, 10 min, 4°C), resuspension of the cells in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.0), and sonication for 5 s (Branson Sonifier; output level 5). The insoluble fraction was removed by centrifugation (13,000 × *g*, 10 min, 4°C), and protein concentrations were estimated by the Bradford assay using bovine serum albumin as a standard (5). β-Galactosidase assays were performed using ONPG as the substrate and expressed as international units with a millimolar extinction coefficient for ONPG of 3.1 (37). Catalase activity of normalized soluble protein samples was detected in stained 5% nondenaturing polyacrylamide gels (57).

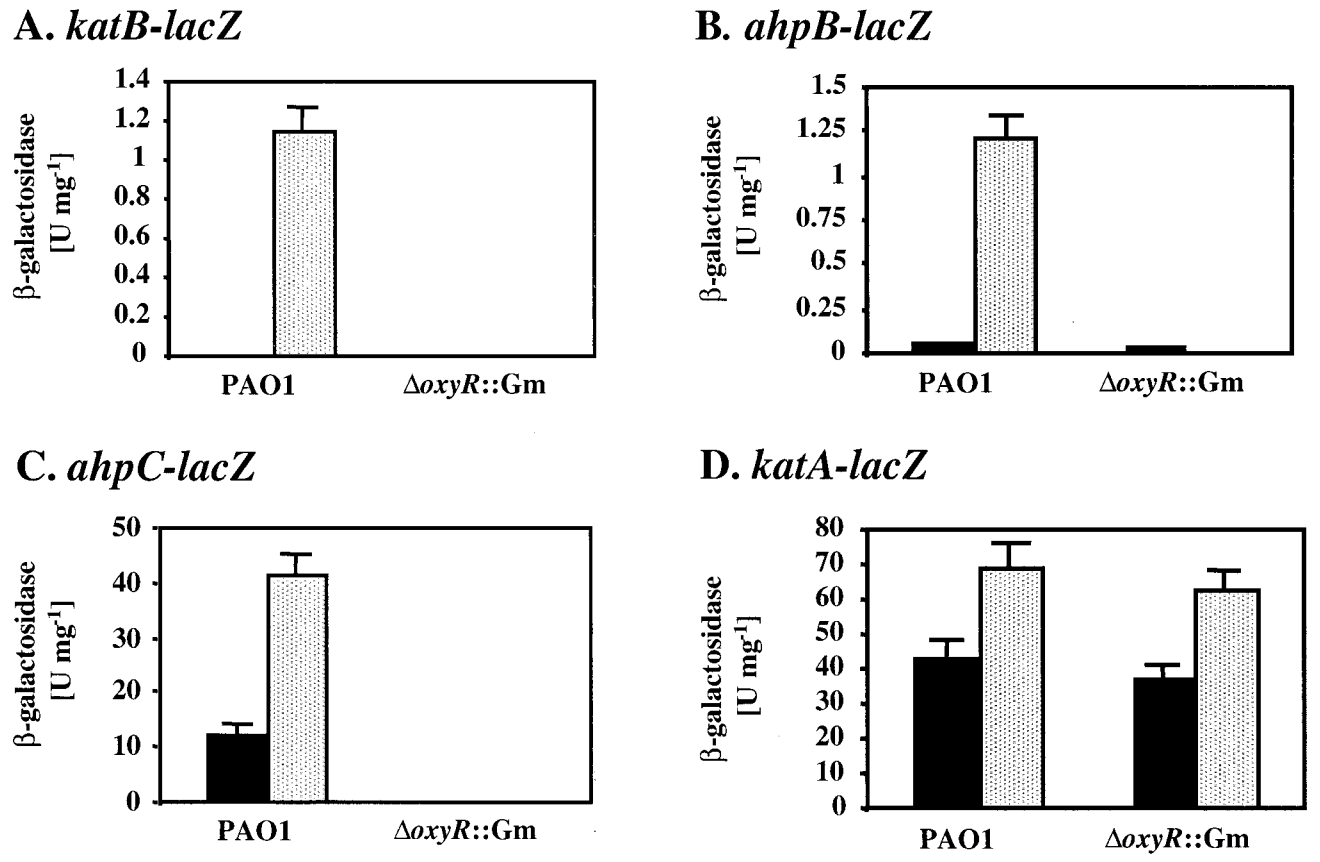

FIG. 2. OxyR-dependent gene expression in response to oxidative stress. Wild-type and Δ*oxyR* bacteria containing plasmid-borne *katB-lacZ* (A), *ahpB-lacZ* (B), *ahpC-lacZ* (C), and *katA-lacZ* (D) fusions were grown in M9 medium to mid-exponential phase. The reporter activities were measured without paraquat treatment (black bars) or after treatment with 100 μM paraquat for 1 h (stippled bars). The β-galactosidase activities are presented as international units, and the error bars represent the standard deviations from four independent experiments.

RESULTS

Characterization of the *oxyR-recG* operon in *P. aeruginosa* PAO1. A putative OxyR homolog was identified using the *E. coli* OxyR amino acid sequence to search the *P. aeruginosa* genome sequence (www.pseudomonas.com). This *P. aeruginosa* OxyR homolog is predicted to be a 34-kDa protein with 40% amino acid sequence identity to *E. coli* OxyR (8). OxyR is a positive regulator of H_2O_2-inducible genes in *E. coli* and *Salmonella enterica* serovar Typhimurium and belongs to the LysR family of bacterial regulatory proteins (8). Immediately downstream of *oxyR* was an open reading frame encoding a 76-kDa protein with 59% amino acid sequence identity to the *E. coli* RecG protein, which functions as an ATP-dependent DNA helicase involved in replication and repair of DNA (29, 31). The stop codon of *P. aeruginosa oxyR* overlapped the start codon of *recG*, suggesting that *oxyR* and *recG* are organized in an operon (Fig. 1A). Expression of *oxyR* and *recG* was monitored by translational fusions to the *lacZ* reporter gene, as depicted in Fig. 1B. *oxyR*::*lacZ* activity was detected from pPZ-*oxyR*-126 containing a promoter immediately upstream of the *oxyR* gene. *recG*::*lacZ* expression was absent in pPZ-*recG*-360, harboring roughly 300 bp of *recG* upstream sequence, but was detected in pPZ-*recG*-1133, which contained, in addition, the entire *oxyR* gene including the *oxyR* promoter region, indicating that *recG* is coexpressed from the *oxyR* promoter in an operon with *oxyR*. This finding was supported by RNase protection assays using an intergenic 365-nucleotide *oxyR-recG* riboprobe (Fig. 1A), which was entirely protected (Fig. 1C). A single transcriptional start site for the *oxyR-recG* operon was found 21 nucleotides upstream of the *oxyR* translational start, as determined by RNase protection with a 384-nucleotide probe of which 141 nucleotides were protected. The *oxyR-recG* transcription did not respond to oxidative stress (Fig. 1C) or to other stimuli, such as growth phase or iron concentration (data not shown).

OxyR-dependent activation of *katB*, *ahpB*, and *ahpC* expression. To identify OxyR-regulated genes in *P. aeruginosa*, we screened a plasmid minilibrary of *lacZ* reporter fusions of about 20 candidate genes potentially involved in oxidative stress defense, iron uptake and storage, and DNA repair, as listed in Materials and Methods. The reporter activities in the wild type and a Δ*oxyR* mutant were compared at mid-exponential growth phase in the presence and absence of 100 μM paraquat. Applying a fivefold difference in the expression levels of the candidate genes between wild-type and *oxyR* mutant cells as the cutoff, we found three fusions (pPZ-*katB*-480, pPZ-*ahpB*-377, and pPZ-*ahpC*-270) that were OxyR dependent (Fig. 2). Expression of *katB-lacZ* was not detected in unstimulated wild-type organisms and was induced 250-fold upon exposure to paraquat, while no activity was detected in the Δ*oxyR* mutant (Fig. 2A). The *ahpB-lacZ* fusion was expressed at very low levels in both wild-type and Δ*oxyR* bacteria, and a 90-fold induction by paraquat was observed in the wild type but not in the Δ*oxyR* mutant (Fig. 2B). Expression of *ahpC-lacZ* was

substantial in untreated wild-type cells and increased threefold in the presence of paraquat. In contrast, *ahpC-lacZ* was not expressed in the Δ*oxyR* mutant (Fig. 2C). All other tested fusions did not depend on OxyR; e.g., *katA-lacZ* in plasmid pPZ-*katA* was expressed at similar levels in either wild-type or Δ*oxyR* cells, although a roughly twofold response to paraquat was observed (Fig. 2D). This regulation of *katA* has been reported previously (32), and it appears that it involves a mechanism different from OxyR activation, which is, in part, controlled by quorum sensing and iron levels (18). Among other genes that were expressed independently of OxyR were *sodA* and *sodB* (encoding Fe-SOD and Mn-SOD, respectively), *dps* (encoding DNA-binding protein of stationary phase), *ahpA* (encoding alkyl hydroperoxide reductase A), *bfrA* and *bfrB* (encoding bacterioferritins A and B, respectively), *fur*, *oxyR* itself, and all additional genes of the minilibrary listed in Materials and Methods (data not shown).

Dose-response effect of OxyR-dependent expression of *katB*, *ahpB*, and *ahpC*. To obtain dose-response curves for OxyR-mediated gene activation, mid-exponential-phase cultures of wild-type bacteria containing *katB-lacZ*, *ahpB-lacZ*, and *ahpC-lacZ* were exposed for a given time to oxidative stress compounds, including paraquat, CHP, tBHP, and H_2O_2, at concentrations ranging from 0.3 μM to 1 mM (Fig. 3). The strongest induction was evoked by paraquat, which caused a half-maximal response at a concentration of 1 μM and resulted in a sustained response to concentrations of up to 300 μM, above which it became lethal. Interestingly, all three fusions were responsive to organic hydroperoxides. Approximately 100 to 300 μM CHP or tBHP was typically required to elicit a significant response, and at higher concentrations (1 mM), the cells were killed. Repeated addition of H_2O_2 caused activation of *ahpB-lacZ* and *ahpC-lacZ* at a concentration of 3 μM or higher; in contrast, at least 100 μM was required for activation of *katB-lacZ*. Generally, H_2O_2 had a less pronounced effect on OxyR-dependent gene activation than any of the other tested compounds, presumably due to rapid detoxification by endogenous catalase. Interestingly, the *ahpB-lacZ* fusion responded somewhat more strongly to lower concentrations of all of the oxidative stress compounds (e.g., 1 to 10 μM paraquat) compared to *katB-lacZ* and *ahpC-lacZ* (Fig. 3).

Time course of OxyR-mediated gene activation. The efficiency of the OxyR-mediated response was further evaluated by monitoring the timing of target gene expression. Mid-exponential-phase cultures of wild-type bacteria containing *katB-lacZ*, *ahpB-lacZ*, and *ahpC-lacZ* were treated with a fixed concentration of paraquat (100 μM) or CHP (300 μM), and the β-galactosidase reporter activities in samples taken at several time points postinduction were determined (Fig. 4). Paraquat elicited a response within 10 min of exposure, and the *katB-lacZ*, *ahpB-lacZ*, and *ahpC-lacZ* activities increased for at least 1 h. This response was expected because paraquat is not degraded and is capable of continuous redox cycling in viable aerobic bacteria. CHP caused activation within 5 min of exposure, but the response reached a plateau after 30 min and declined somewhat after that. This result was also expected, since CHP can be detoxified (e.g., by Ahp activities) and thus elicits only a transient oxidative stress response.

Genetic analysis of the OxyR-regulated genes *katB-ankB*, *ahpB*, and *ahpC-ahpF*. Genetic maps and the introduced mutations in *katB*, *ahpB*, and *ahpCF* are depicted in Fig. 5. Also shown are the results of RNase protection assays using specific riboprobes to map the individual transcriptional start sites. The *katB*, *ahpB*, and *ahpCF* transcripts were strongly induced in the presence of the oxidative stress-generating agent paraquat, and this response was dependent on OxyR. A single *katB* transcriptional start site was detected 42 nucleotides upstream of the *katB* translational initiation site. The *katB*::Gm mutation has a polar negative effect on *ankB*, which is in an operon with *katB* and encodes an ankyrin-like protein required for optimal catalase B activity (24). Transcription of *ahpB* started 60 nucleotides upstream of its start codon. The *ahpB* gene encodes a 22-kDa protein harboring a motif typical for antioxidant reductases as determined by the e-motif search (Department of Biochemistry, Stanford University) and contains a candidate membrane-spanning helix suggesting a localization in either the cytoplasmic membrane or periplasm (data not shown). The

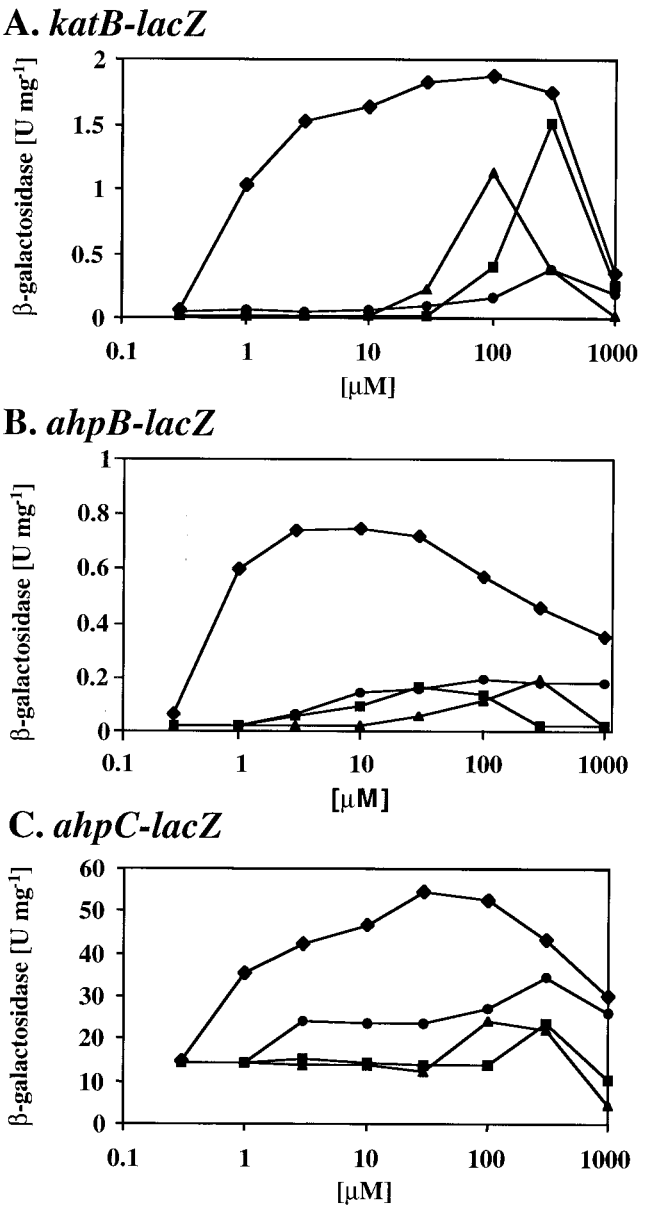

FIG. 3. Dose-response curves for OxyR-dependent gene expression. Wild-type cells containing plasmid-borne *katB-lacZ* (A), *ahpB-lacZ* (B), and *ahpC-lacZ* (C) fusions were grown in M9 medium to mid-exponential phase and treated with increasing concentrations of paraquat for 1 h (diamonds), of CHP for 30 min (squares), of tBHP for 30 min (triangles), or of H_2O_2 for 1 h (circles). The β-galactosidase activities are shown as a function of the indicated concentrations of the oxidative stress-generating compounds and are the mean values from triplicate assays.

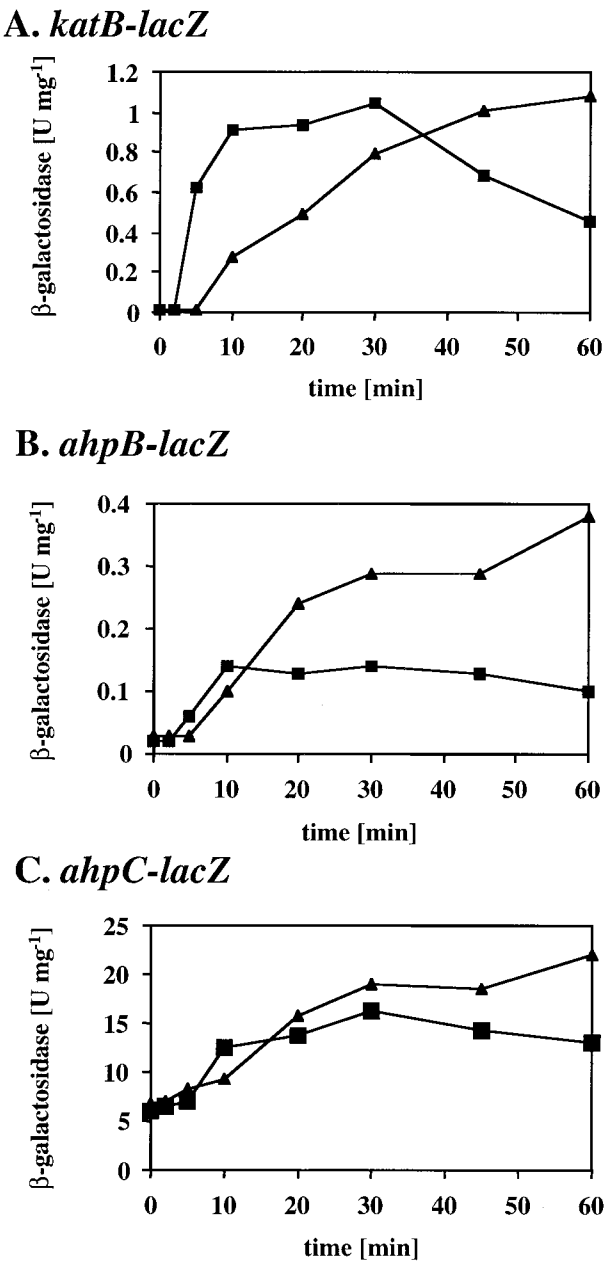

FIG. 4. Time-response curves for OxyR-dependent gene expression. Wild-type cells containing *katB-lacZ* (A), *ahpB-lacZ* (B), and *ahpC-lacZ* (C) were grown in M9 medium to mid-exponential phase and treated with 100 μM paraquat (triangles) or 300 μM CHP (squares). Samples were removed before treatment and at 2, 5, 10, 20, 30, 45, and 60 min postexposure, and their β-galactosidase activities were determined. The values are the means from triplicate experiments.

putative AhpB protein is 60% identical at the amino acid sequence level to the product of the *Legionella pneumophila* alkyl hydroperoxide reductase (*tsaA*) gene (GenBank accession number L46863) and 51% identical to a mouse thiol-specific antioxidant (GenBank accession number X82067). This type of peroxidase reduces hydroperoxides with reducing power from thioredoxin (28). Interestingly, the *ahpB* gene is located immediately upstream of an open reading frame (*tdr*) encoding a putative thioredoxin reductase (Fig. 5). The *tdr* gene, however, was not in an operon with *ahpB* but was expressed from its own promoter and independent of OxyR (data not shown). The *ahpC* mRNA start site was mapped to 43 nucleotides upstream of the *ahpC* translational start. Low levels of this transcript were also detectable in unstimulated wild-type cells and in *oxyR* mutant cells, suggesting that *ahpC* is expressed at low basal levels in an OxyR-independent way. The *ahpC* and *ahpF* coding sequences were spaced apart by a 144-bp intergenic sequence. RNase protection assays using a 484-nucleotide *ahpC-ahpF* riboprobe over this region were performed to address the question of whether *ahpC* and *ahpF* form an operon. Clearly, a fraction of the probe was protected over its entire length, suggesting an organization of *ahpC* and *ahpF* in an operon. However, additional protected RNA species of 280 and 215 nucleotides were detected, indicating that some *ahpC* transcripts may terminate within the *ahpC-ahpF* intergenic region and that *ahpF* may be transcribed from a separate promoter and independently of *ahpC*. In either case, both *ahpC* and *ahpF* transcription appeared to be OxyR responsive. The *P. aeruginosa ahpC* and *ahpF* genes encode a 21- and a 56-kDa proteins, respectively, with 59 and 66% amino acid sequence identities, respectively, to the *E. coli* AhpC and AhpF alkyl hydroperoxide reductase subunits. This type of Ahp is widely found in most bacterial species (51) and requires NADH or NADPH for activity (41).

Characterization of the *katB*, *ahpB*, and *ahpC* promoters. The mapping of the transcriptional start sites for *katB-ankB*, *ahpB*, *ahpC-ahpF*, and *oxyR* allowed the localization of the corresponding −10 and −35 elements (Fig. 6). Putative OxyR-binding sites were identified upstream of the *katB*, *ahpB*, and *ahpCF* promoters. Four ATAG elements spaced at 10-bp intervals comprise the binding sites for oxidized *E. coli* OxyR (56), and such elements were found in proper spacing and distance within the *katB*, *ahpB*, and *ahpCF* promoters. The number of bases matching the OxyR consensus binding sequence were 9 of 16 (*katB*), 13 of 16 (*ahpB*), and 12 of 16 (*ahpCF*), and in all cases, the OxyR binding motif was located exactly adjacent to the −35 promoter elements (Fig. 6). Purified His_6-tagged OxyR protein at a concentration of at least 100 μM caused a mobility shift of DNA fragments containing these target promoters, indicating direct binding of OxyR. A DNA fragment harboring the *oxyR* promoter was not shifted by OxyR (Fig. 6), which is in agreement with the finding that *oxyR* expression did not respond to oxidative stress.

Phenotypic comparison of mutants affected in *oxyR*, *katB*, *ahpB*, *ahpCF*, and *recG*. The susceptibility of a Δ*oxyR*::Gm mutant to oxidative stress compounds was compared to the phenotypes of mutants affected in single OxyR-regulated genes in order to dissect their specific roles in the oxidative stress response. Wild-type, Δ*oxyR*::Gm, *recG*::Gm, Δ*katB*::Gm, Δ*ahpB*::Gm, and Δ*ahpCF*::Gm bacteria were tested for their susceptibilities to H_2O_2 and CHP using standardized disk inhibition assays (Table 2). The Δ*oxyR*::Gm mutant containing the control plasmid pUCP19 was dramatically susceptible to both compounds. Plasmid pUCP-*oxyR* fully complemented this phenotype, while plasmid-borne copies of the *recG* gene, which is located downstream and in an operon with *oxyR* (see above), resulted in minimal complementation. These findings strongly suggest that the *oxyR* phenotype was caused by the lack of OxyR-mediated oxidative stress defense. Still, *recG* appeared to be essential for optimal resistance to H_2O_2 and CHP, and the *recG* mutant could be complemented by the *recG* gene in *trans*. Multiple copies of single OxyR-regulated genes expressed from the constitutive *lac* promoter on pUCP resulted in only marginal complementation. The *katB*::Gm mutant showed increased susceptibility to H_2O_2, and this phenotype could not be complemented by providing only *katB* in *trans*,

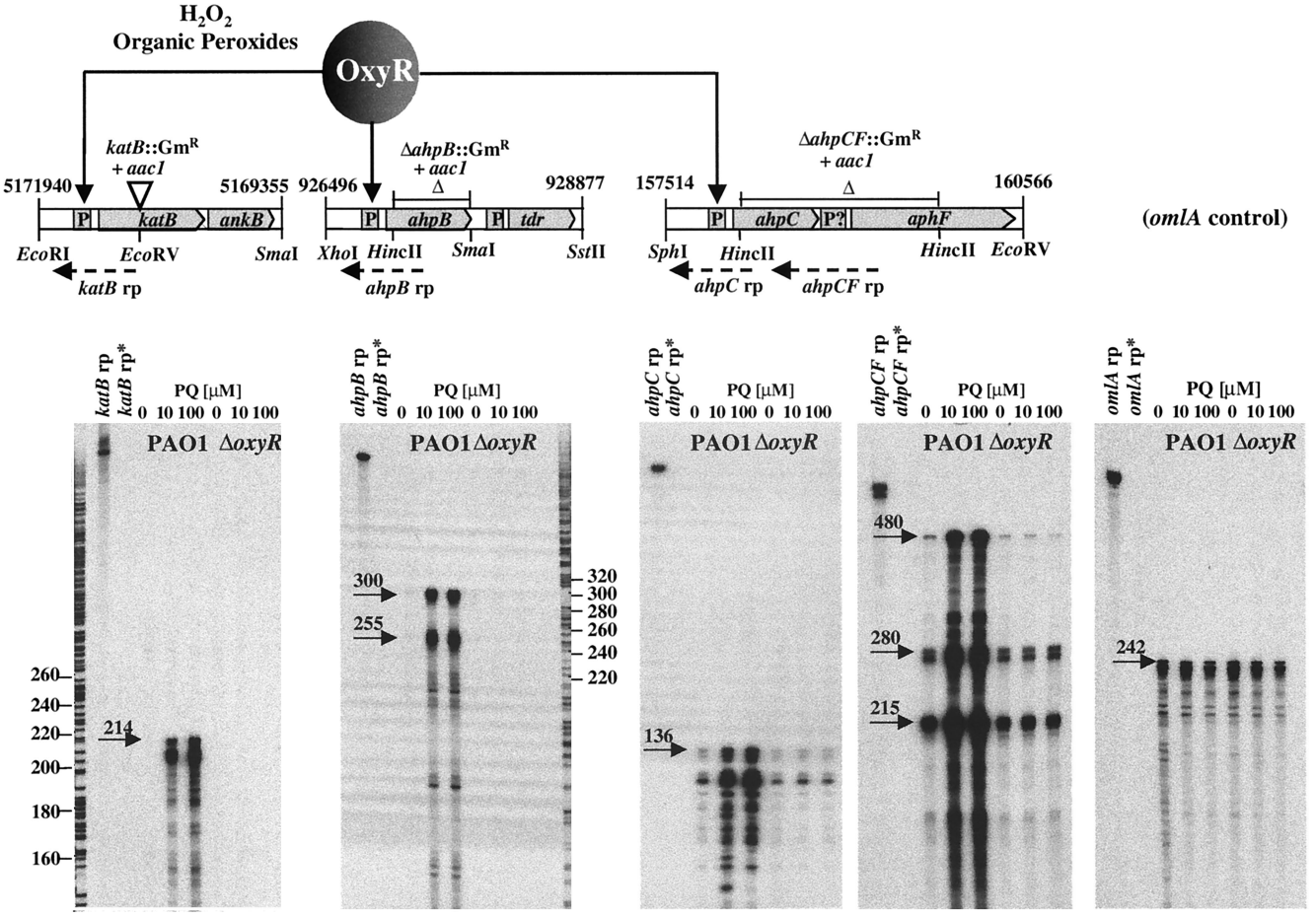

FIG. 5. Genetic maps and transcripts of OxyR-regulated genes. The *katB*, *ahpB*, and *ahpC-ahpF* loci are shown with their coordinates in the PAO1 genome (Pathogenesis Corp., 12-15-99 release), the sites of insertions or deletions in the corresponding mutants, the locations of the riboprobes (rp), and relevant restriction sites. The *katB-ankB* operon encodes a previously characterized inducible catalase (6) and an ankyrin-like factor required for optimal catalase activity (24). The *ahpB* gene encodes a thiol-specific peroxidase and is located upstream of a thioredoxin reductase (*tdr*). The *ahpC-ahpF* operon encodes the two subunits of the classic alkyl hydroperoxide reductase. The RNase protection assays were done with total RNA isolated from PAO1 wild-type or *oxyR* mutant cells in the absence or presence of paraquat (PQ) as indicated. A probe specific for the constitutively expressed *omlA* gene (39) was used as a control. Also loaded were diluted probes (rp) and the digested probes (rp*) as controls. Arrows point to the relevant protected riboprobe bands, and their approximate sizes (in nucleotides) are given.

presumably due to the polar negative effect on *ankB* (24). The Δ*ahpB*::Gm mutant was hypersusceptible to H_2O_2 but not to CHP and was successfully complemented by *ahpB* in *trans*. The Δ*ahpCF*::Gm mutant exhibited a somewhat intriguing phenotype. While Δ*ahpCF*::Gm mutant cells were hypersusceptible to CHP, they were more resistant to H_2O_2 than the wild type, and plasmid pUCP-*ahpCF* reversed that trend. Elevated KatB catalase levels were measured in the Δ*ahpCF*::Gm mutant, and the KatB catalase activity was detectable even in the absence of paraquat as an inducer (Fig. 7). In agreement with that observation was the finding that a *katB-lacZ* fusion was expressed at severalfold higher levels in a Δ*ahpCF*::Gm background than in wild-type cells (data not shown). This compensatory mechanism between *ahpCF* and *katB* expression indicates that the absence of AhpCF leads to internal oxidative stress. During experiments to measure catalase levels in various catalase-deficient and *ahp* mutant strains using activity staining, we unexpectedly observed extra bands that we suspected might reflect the ability of some alkyl hydroperoxidases to also use H_2O_2 as a substrate (Fig. 7). Wild-type organisms produced KatA and KatB activities, and two additional smaller activity bands migrated between KatA and KatB. We determined that the lower band was an electrophoretic variant of KatA, since this band was absent in a *katA* mutant. The upper band most likely represented AhpB, since it was absent in the *oxyR* and *ahpB* mutants. Both middle bands were retained in a *katB* mutant. Interestingly, the *katA* mutant possessed a catalase activity band that migrated with KatA. We determined that this band is AhpA, because it was absent in an *ahpA* mutant (data not shown). Furthermore, preliminary catalase assays indicated that both AhpB and AhpA possessed some catalase activity (data not shown), while it remained uncertain whether AhpCF had such activity.

Role of the *oxyR-recG* operon in DNA repair. Since *P. aeruginosa oxyR* was located in an operon with the *recG* gene, encoding a putative DNA helicase, a possible function of the *oxyR-recG* locus in DNA repair was investigated. Wild-type bacteria, an unmarked Δ*oxyR* mutant harboring an in-frame deletion of *oxyR* with an unlikely polar effect on *recG*, a *recG*::Gm mutant, and a Δ*recC*::Tc mutant were compared for their sensitivity to UV irradiation-induced DNA damage (Fig. 8). Wild-type cells and the Δ*oxyR* mutant showed similar killing patterns, characterized by roughly 3 to 4 log units of killing over 60 s of UV irradiation. The *recG*::Gm mutant was hyper-

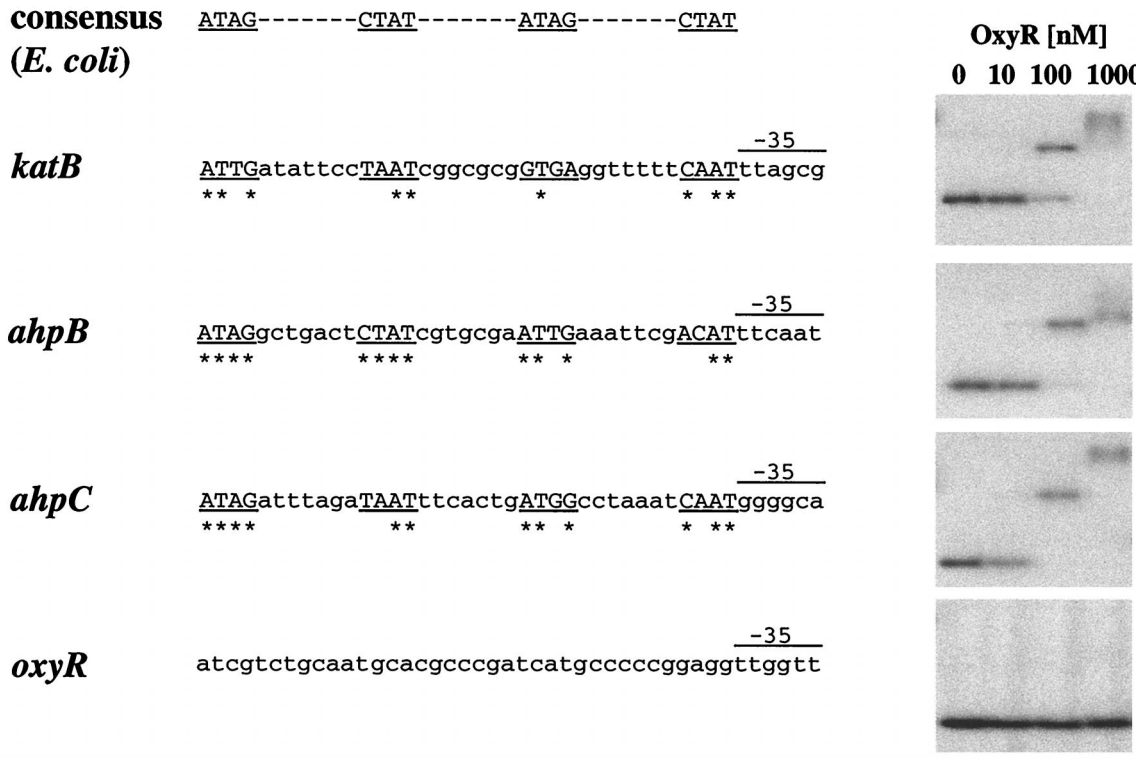

FIG. 6. Binding of OxyR to the *katB*, *ahpB*, and *ahpC* promoters. The alignment of the OxyR-regulated promoters indicates four putative OxyR-binding tetranucleotide sequences (underlined), the residues matching the consensus sequence derived from *E. coli* OxyR-regulated promoters (asterisks), and the −35 promoter elements in proper distance of the mapped transcriptional start sites. The gel mobility shift assays of radiolabeled DNA fragments containing the corresponding promoter regions were performed with purified His_6-tagged OxyR protein at the given concentrations.

sensitive to UV and was killed by more than 5 log units within 20 s of irradiation. The UV sensitivity of *recG*::Gm was more dramatic than that of the Δ*recC*::Tc mutant. Expression of the *recG* gene in *trans* partially restored UV tolerance in the *recG*::Gm mutant. A *recG*::Gm Δ*recC*::Tc double mutant was slightly more sensitive than the *recG*::Gm single mutant, suggesting an additive effect. Clearly, our data show that *recG* plays an important role in DNA damage repair.

DISCUSSION

H_2O_2 is a powerful antimicrobial agent commonly used in health care as a topical anti-infective, as well as in industry for the treatment of problematic bacterial biofilms. Also, human phagocytes produce H_2O_2 as a natural weapon during the respiratory burst to combat microbial infections (38). Thus, it is not surprising that microbes have evolved several strategies to cope with oxidative stress. In *P. aeruginosa*, the primary defense against H_2O_2 involves a constitutively expressed catalase, KatA (12, 17, 32), but little is known about a specific response of *P. aeruginosa* to oxidative stress agents. While the existence of a second, H_2O_2- or paraquat-inducible, catalase (KatB) has been described (6), the regulatory mechanism governing this response remained unknown. In this report, we characterize a regulatory gene, *oxyR*, and present evidence that the OxyR protein is involved in transcriptional activation of at least three genes encoding antioxidants. The OxyR-mediated stress response has been well studied in *E. coli* by both genetic and biochemical means (52–56). Also, a possible role of OxyR to combat host defense systems has been investigated in numerous pathogenic bacteria, including *Enterococcus faecalis* (44), *Haemophilus influenzae* (33), and *Mycobacterium tuberculosis* (10, 50). In *M. tuberculosis*, the OxyR-regulated genes *katG* and *ahpC* play crucial roles in isoniazid resistance, since isoniazid requires activation by KatG to exert lethal effects, while AhpC could play a detoxifying role (63, 64). Interest-

TABLE 2. Oxidative stress susceptibility of mutant strains[a]

Strain/plasmid[b]	Zone of growth inhibition (mm)[c] with:	
	2% H_2O_2	20% CHP
PAO1/pUCP19	20 ± 1	19 ± 1
Δ*oxyR*::Gm/pUCP19	46 ± 4	31 ± 3
Δ*oxyR*::Gm/pUCP-*oxyR*	19 ± 1	19 ± 1
Δ*oxyR*::Gm/pUCP-*recG*	29 ± 3	25 ± 2
Δ*oxyR*::Gm/pUCP-*katB*	38 ± 3	26 ± 3
Δ*oxyR*::Gm/pUCP-*ahpB*	33 ± 2	25 ± 2
Δ*oxyR*::Gm/pUCP-*ahpCF*	37 ± 1	25 ± 1
Δ*recG*::Gm/pUCP19	25 ± 1	27 ± 2
Δ*recG*::Gm/pUCP-*recG*	21 ± 1	22 ± 1
Δ*katB*::Gm/pUCP19	24 ± 1	18 ± 1
Δ*katB*::Gm/pUCP-*katB*	24 ± 1	19 ± 1
Δ*ahpB*::Gm/pUCP19	28 ± 3	19 ± 2
Δ*ahpB*::Gm/pUCP-*ahpB*	21 ± 1	19 ± 1
Δ*ahpCF*::Gm/pUCP19	18 ± 1	24 ± 2
Δ*ahpCF*::Gm/pUCP-*ahpCF*	24 ± 3	20 ± 1

[a] The strains were grown overnight in M9 medium, and 0.2 OD_{600} unit of culture was mixed with 3 ml of 0.8% low-melting-point agarose in M9 medium containing carbenicillin and poured onto M9 agar. Triplicate filter disks containing 10 μl of 2% H_2O_2 or 20% CHP were immediately placed on the plates, and zones of growth inhibitions were measured after overnight incubation at 37°C.

[b] Genes were cloned into pUCP19 in the orientation of the plasmid P_{lac} promoter, which is constitutively expressed in *P. aeruginosa*.

[c] Results are means and standard deviations.

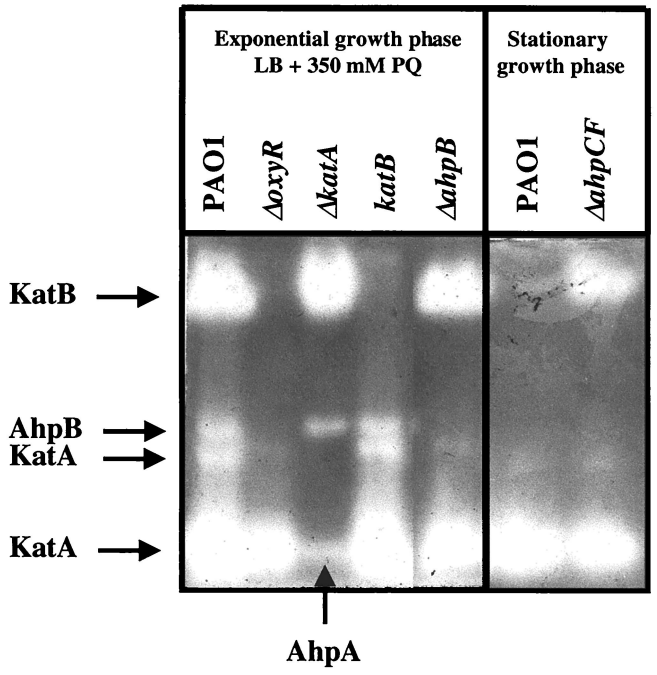

FIG. 7. Catalase activity gel of soluble cell extracts. Bacterial cultures of PAO1 wild-type and *oxyR*, *katA*, *katB*, and *ahpB* mutant cells were grown under aerobic conditions in LB to mid-exponential phase and then exposed to 350 μM paraquat (PQ) for 1 h. PAO1 and the *ahpCF* mutant were grown to stationary phase (16 h) as indicated. Normalized amounts (15 μg) of the soluble protein fractions were separated on a nondenaturing gel and stained for catalase activity. The arrows indicate the positions of KatA, KatB, AhpA, and AhpB activities.

ingly, the *oxyR* gene in members of the *M. tuberculosis* complex is located next to *ahpC*; however, *oxyR* is nonfunctional, due to numerous deletions and point mutations (10). *M. tuberculosis katG* mutant strains were found to have acquired a compensatory mutation resulting in an upregulation of AhpC, and it has been shown that this protein confers resistance to isoniazid and protection against H_2O_2, even in the absence of adequate catalase and peroxidase activities (48). We found a compensatory cross-regulation of OxyR-dependent *katB-ankB* and *ahpC-ahpF* expression in *P. aeruginosa*. A Δ*ahpCF*::Gm mutant strain was more resistant to H_2O_2, and this phenotype correlated with higher levels of KatB observed in a catalase activity gel. Such an increased resistance due to elevated expression from all peroxide regulon promoters has been reported for a *Bacillus subtilis ahpC* mutant (7). Similarly, the lack of AhpC-AhpF peroxidase expression in *E. coli* has been shown to lead to constitutive OxyR activation due to the accumulation of endogenous oxidants (43). Somewhat surprising was the hypersusceptibility to H_2O_2 of strains harboring the *ahpCF* genes on a multicopy plasmid. However, a similar phenomenon has been observed upon overexpression of *ahpCF* in *S. enterica* serovar Typhimurium (51). The reasons for this are unclear, but possible explanations are that multiple copies of the *ahpCF* promoter titrate out OxyR or that increased AhpC-AhpF hydroperoxide reductase activity could somehow interfere with the proper sensing of oxidative stress, e.g., by maintaining the oxidized state of the OxyR protein.

In the course of this study, we learned that *P. aeruginosa* possesses multiple lines of OxyR-dependent, inducible oxidative stress defense systems with potentially overlapping functions. The expression of the three identified OxyR targets, *katB-ankB*, *ahpB*, and *ahpC-ahpF*, responded to any of the exogenously added oxidative stress compounds, including H_2O_2, paraquat, and organic hydroperoxides, suggesting that all of these agents or products derived from their action cause oxidation of the OxyR protein, which then indiscriminately activates the target promoters. However, one of the OxyR target genes, *ahpB*, responded to significantly lower concentrations of oxidative stress agents than the other targets. The reason for this dose-response shift is unknown, but interestingly, the putative OxyR-binding site in the *ahpB* promoter had a higher identity to the consensus "OxyR box" than the OxyR boxes in the *katB* and *ahpCF* promoters. The strength of an OxyR-binding site could possibly determine the affinity of OxyR to a target promoter and could allow the sequential activation of antioxidant genes with regard to the extent of oxidative stress encountered. Furthermore, we found that not only the KatB catalase, but also AhpB, which belongs to the alkyl hydroperoxide reductase family, possesses catalase activity. In fact, a Δ*ahpB*::Gm mutant strain exhibited a more pronounced hypersusceptibility to H_2O_2 than to organic hydroperoxides. These findings suggest overlapping functions of KatB, AhpB, and AhpC-AhpF in the detoxification processes. Clearly, detailed biochemical studies on purified KatB, AhpB, and AhpC-AhpF are needed to investigate their potential broad substrate specificities. Multiple enzymatic activities have been demonstrated for mycobacterial KatG, which can act both as a catalase-peroxidase (34) and as a peroxynitritase (58). The observed redundancy of overlapping oxidative stress defense systems in *P. aeruginosa* may also be explained, in part, by the localization of the antioxidant enzymes in different cellular compartments. While KatA is found in the cytoplasm and in the extracellular milieu (see the accompanying paper by Hassett et al. [15]), KatB is found in the cytoplasm, cytoplasmic membrane, and periplasm (24). AhpB possesses a single cytoplasmic membrane-spanning domain, suggesting a function in the protection of membrane-bound respiratory chain components from H_2O_2.

We present evidence that expression of *katB-ankB*, *ahpB*, and *ahpC-ahpF* depends on OxyR. Putative OxyR boxes were

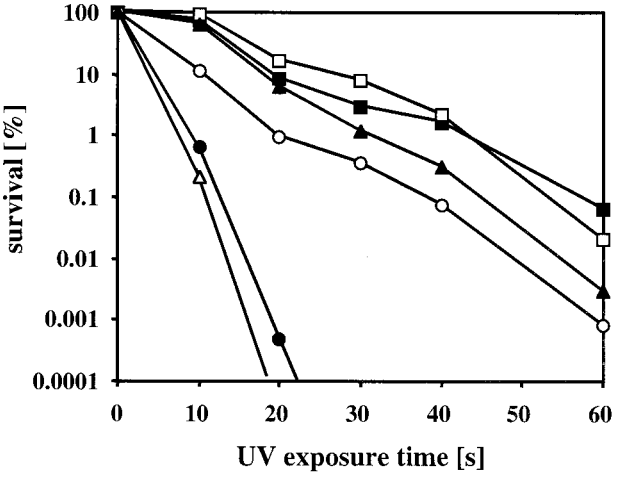

FIG. 8. Kill curves upon exposure to UV irradiation. Wild-type PAO1 (■), an unmarked nonpolar Δ*oxyR* mutant (□), a *recG*::Gm mutant (●), a *recG*::Gm mutant complemented with pUCP-*recG* (○), a Δ*recC*::Tc mutant (▲), and a *recG*::Gm Δ*recC*::Tc double mutant (△) were grown overnight in LB. The cells were UV irradiated while shaking, and aliquots were removed at 10-s intervals. Serial dilutions were plated on L agar to determine the viable cell counts. The UV killing assays were performed five times with independent cultures, and the outcome of one representative experiment is shown.

found at the proper location within the *ahpB* and *ahpC* promoters, and binding of OxyR to these target promoters was demonstrated, indicating a direct activation of these genes by OxyR.

The phenotypes of a Δ*oxyR* mutant included a dramatic susceptibility to oxidative stress agents and a low plating efficiency (see also the accompanying paper by Hassett et al. [15]). The oxidative stress susceptibility of *P. aeruginosa* was significantly increased in a low-iron environment compared to iron-rich conditions (data not shown). Although the presence of iron is known to trigger the formation of $HO^{\bullet}$, which has deleterious effects on the cells, iron is required for the function of the heme-containing antioxidant enzymes. The hypersusceptibility to oxidative stress in low-iron media was even more drastic in a Δ*oxyR* mutant, suggesting a potential role of OxyR in iron metabolism. Moreover, none of the OxyR-regulated factors characterized in this study was capable of fully complementing the Δ*oxyR* mutant phenotype, suggesting the existence of additional members of the OxyR regulon. Some genes, including *fur* and *dps* of *E. coli*, are expressed in both OxyR-dependent and OxyR-independent ways, and the situation in *P. aeruginosa* may be similar. In our screening of a minilibrary of translational fusions to the *lacZ* gene, we pulled out those genes that were expressed at at least a fivefold higher level in wild-type compared to *oxyR* mutant cells under oxidative stress conditions. As a consequence, we did not pick up those genes that are expressed OxyR independently but can be further upregulated by OxyR. Also, the possibility of indirect OxyR regulation exists and could involve the small RNA *oxyS*, which has been shown to regulate several genes in *E. coli* (1). However, we have not found an *oxyS*-like gene in a search of the *P. aeruginosa* genome (www.pseudomonas.com). A future goal is the isolation of other OxyR-regulated genes, through an in vitro cycle selection procedure that has been successful in the past to identify Fur-regulated genes of *P. aeruginosa* (40).

Antioxidant enzymes represent the first line of defense in the battle against oxidative stress. A second strategy to survive these harsh conditions is to maintain an efficient DNA repair system. Interestingly, the *P. aeruginosa oxyR* gene was found in an operon with *recG*, encoding a homolog of the *E. coli* RecG DNA helicase, which is an ATP-dependent DNA recombinase implicated in DNA replication, recombination, and repair (29, 31). To our knowledge, *P. aeruginosa* is the first microorganism for which such a genetic link between an oxidative stress gene and a DNA repair gene has been identified. In some other organisms, including mycobacteria, the *oxyR* gene is located in a cluster with genes encoding antioxidant enzymes, but we did not find any alkyl hydroperoxide reductases or catalases encoded near *P. aeruginosa oxyR* (data not shown). The precise role of RecG is somewhat elusive, but it has been postulated that the DNA binding and unwinding activities of RecG are involved in promoting branch migration by catalyzing the formation of four-strand Holliday junctions from three-strand junctions (36). Clearly, we demonstrated that a *recG*::Gm mutant of *P. aeruginosa* was hypersensitive to UV irradiation-induced DNA damage, indicating that *recG* is essential for optimal DNA repair. Also, the *P. aeruginosa recG*::Gm mutant was hypersusceptible to oxidative stress agents, thus directly demonstrating the DNA-damaging effects of ROIs. Taken together, the two coordinately expressed factors encoded by the *oxyR-recG* operon play a crucial role the survival in response to environmental challenges.

ACKNOWLEDGMENTS

This work was supported by grant AI-15490 (to M.L.V.) from the National Institutes of Health and by Public Health Service grant AI-40541 (to D.J.H.) and a Pilot Grant from the Cystic Fibrosis Foundation (to D.J.H.).

REFERENCES

1. **Altuvia, S., D. Weinstein-Fischer, A. Zhang, L. Postow, and G. Storz.** 1997. A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. Cell **90:**43–53.
2. **Aslund, F., M. Zheng, J. Beckwith, and G. Storz.** 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. Proc. Natl. Acad. Sci. USA **96:**6161–6165.
3. **Barton, H. A., Z. Johnson, C. D. Cox, A. I. Vasil, and M. L. Vasil.** 1996. Ferric uptake regulator mutants of *Pseudomonas aeruginosa* with distinct alterations in the iron-dependent repression of exotoxin A and siderophores in aerobic and microaerobic environments. Mol. Microbiol. **21:**1001–1017.
4. **Beyer, W., J. Imlay, and I. Fridovich.** 1991. Superoxide dismutases. Prog. Nucleic Acid Res. Mol. Biol. **40:**221–253.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brown, S. M., M. L. Howell, M. L. Vasil, A. J. Anderson, and D. J. Hassett.** 1995. Cloning and characterization of the *katB* gene of *Pseudomonas aeruginosa* encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. J. Bacteriol. **177:**6536–6544.
7. **Bsat, N., L. Chen, and J. D. Helmann.** 1996. Mutation of the *Bacillus subtilis* alkyl hydroperoxide reductase (*ahpCF*) operon reveals compensatory interactions among hydrogen peroxide stress genes. J. Bacteriol. **178:**6579–6586.
8. **Christman, M. F., G. Storz, and B. N. Ames.** 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA **86:**3484–3488.
9. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Deretic, V., W. Philipp, S. Dhandayuthapani, M. H. Mudd, R. Curcic, T. Garbe, B. Heym, L. E. Via, and S. T. Cole.** 1995. *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid. Mol. Microbiol. **17:**889–900.
11. **Ding, H., and B. Demple.** 1998. Thiol-mediated disassembly and reassembly of [2Fe-2S] clusters in the redox-regulated transcription factor SoxR. Biochemistry **37:**17280–17286.
12. **Elkins, J. G., D. J. Hassett, P. S. Stewart, H. P. Schweizer, and T. R. McDermott.** 1999. Protective role of catalase in *Pseudomonas aeruginosa* biofilm resistance to hydrogen peroxide. Appl. Environ. Microbiol. **65:**4594–4600.
13. **Gaudu, P., and B. Weiss.** 1996. SoxR, a [2Fe-2S] transcription factor, is active only in its oxidized form. Proc. Natl. Acad. Sci. USA **93:**10094–10098.
14. **Gonzalez-Flecha, B., and B. Demple.** 1999. Role for the *oxyS* gene in regulation of intracellular hydrogen peroxide in *Escherichia coli*. J. Bacteriol. **181:**3833–3836.
15. **Hassett, D. J., E. Alsabbagh, K. Parvatiyar, M. L. Howell, R. W. Wilmott, and U. A. Ochsner.** 2000. A protease-resistant catalase, KatA, released upon cell lysis during stationary phase is essential for aerobic survival of a *Pseudomonas aeruginosa oxyR* mutant at low cell densities. J. Bacteriol. **182:**4557–4563.
16. **Hassett, D. J., and M. S. Cohen.** 1989. Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. FASEB J. **3:**2574–2582.
17. **Hassett, D. J., J. G. Elkins, J. F. Ma, and T. R. McDermott.** 1999. *Pseudomonas aeruginosa* biofilm sensitivity to biocides: use of hydrogen peroxide as model antimicrobial agent for examining resistance mechanisms. Methods Enzymol. **310:**599–608.
18. **Hassett, D. J., J.-F. Ma, J. G. Elkins, T. R. McDermott, U. A. Ochsner, S. E. H. West, C.-T. Huang, J. Fredericks, S. Burnett, P. S. Stewart, G. McPheters, L. Passador, and B. H. Iglewski.** 1999. Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Mol. Microbiol. **34:**1082–1093.
19. **Hassett, D. J., H. P. Schweizer, and D. E. Ohman.** 1995. *Pseudomonas aeruginosa sodA* and *sodB* mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. J. Bacteriol. **177:**6330–6337.
20. **Hassett, D. J., W. A. Woodruff, D. J. Wozniak, M. L. Vasil, M. S. Cohen, and D. E. Ohman.** 1993. Cloning and characterization of the *Pseudomonas aeruginosa sodA* and *sodB* genes encoding manganese- and iron-cofactored superoxide dismutase: demonstration of increased manganese superoxide dismutase activity in alginate-producing bacteria. J. Bacteriol. **175:**7658–7665.
21. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.

22. **Holloway, B. W.** 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. **33:**419–443.
23. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114:**193–197.
24. **Howell, M. L., E. Alsabbagh, J.-F. Ma, U. A. Ochsner, M. G. Klotz, T. J. Beveridge, K. M. Blumenthal, E. C. Niederhoffer, R. E. Morris, D. Needham, G. E. Dean, M. A. Wani, and D. J. Hassett.** 2000. AnkB, a periplasmic ankyrin-like protein in *Pseudomonas aeruginosa*, is required for optimal catalase B (KatB) activity and resistance to hydrogen peroxide. J. Bacteriol. **182:**4545–4556.
25. **Imlay, J. A., and S. Linn.** 1988. DNA damage and oxygen radical toxicity. Science **240:**1302–1309.
26. **Imlay, J. A., and S. Linn.** 1987. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. J. Bacteriol. **169:**2967–2976.
27. **Jair, K. W., W. P. Fawcett, N. Fujita, A. Ishihama, and R. E. Wolf, Jr.** 1996. Ambidextrous transcriptional activation by SoxS: requirement for the C-terminal domain of the RNA polymerase alpha subunit in a subset of *Escherichia coli* superoxide-inducible genes. Mol. Microbiol. **19:**307–317.
28. **Jeong, W., M. K. Cha, and I. H. Kim.** 2000. Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family. J. Biol. Chem. **275:**2924–2930.
29. **Kalman, M., H. Murphy, and M. Cashel.** 1992. The nucleotide sequence of *recG*, the distal *spo* operon gene in *Escherichia coli* K-12. Gene **110:**95–99.
30. **Li, Z., and B. Demple.** 1994. SoxS, an activator of superoxide stress genes in *Escherichia coli*. Purification and interaction with DNA. J. Biol. Chem. **269:** 18371–18377.
31. **Lloyd, R. G., and C. Buckman.** 1991. Genetic analysis of the *recG* locus of *Escherichia coli* K-12 and of its role in recombination and DNA repair. J. Bacteriol. **173:**1004–1011.
32. **Ma, J.-F., U. A. Ochsner, M. G. Klotz, V. K. Nanayakkara, M. L. Howell, Z. Johnson, J. Posey, M. L. Vasil, J. J. Monaco, and D. J. Hassett.** 1999. Bacterioferritin A modulates catalase A (KatA) activity and resistance to hydrogen peroxide in *Pseudomonas aeruginosa*. J. Bacteriol. **181:**3730–3742.
33. **Maciver, I., and E. J. Hansen.** 1996. Lack of expression of the global regulator OxyR in *Haemophilus influenzae* has a profound effect on growth phenotype. Infect. Immun. **64:**4618–4629.
34. **Marcinkeviciene, J. A., R. S. Magliozzo, and J. S. Blanchard.** 1995. Purification and characterization of the *Mycobacterium smegmatis* catalase-peroxidase involved in isoniazid activation. J. Biol. Chem. **270:**22290–22295.
35. **Martinez, A., and R. Kolter.** 1997. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J. Bacteriol. **179:**5188–5194.
36. **McGlynn, P., and R. G. Lloyd.** 1999. RecG helicase activity at three- and four-strand DNA structures. Nucleic Acids Res. **27:**3049–3056.
37. **Miller, J. H.** 1992. A short course in bacterial genetics, p. 72–74. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
38. **Miller, R. A., and B. E. Britigan.** 1997. Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. **10:**1–18.
39. **Ochsner, U. A., A. I. Vasil, Z. Johnson, and M. L. Vasil.** 1999. *Pseudomonas aeruginosa fur* overlaps with a gene encoding a novel outer membrane lipoprotein, OmlA. J. Bacteriol. **181:**1099–1109.
40. **Ochsner, U. A., and M. L. Vasil.** 1996. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc. Natl. Acad. Sci. USA **93:**4409–4414.
41. **Poole, L. B., and H. R. Ellis.** 1996. Flavin-dependent alkyl hydroperoxide reductase from *Salmonella typhimurium*. 1. Purification and enzymatic activities of overexpressed AhpF and AhpC proteins. Biochemistry **35:**56–64.
42. **Potter, A. A.** 1985. Gene cloning in *Pseudomonas aeruginosa*, p. 147–156. *In* J. A. Dillon, A. Nasim, and E. R. Nestman (ed.), Recombinant DNA methodology. John Wiley and Sons, New York, N.Y.
43. **Rosner, J. L., and G. Storz.** 1994. Effects of peroxides on susceptibilities of *Escherichia coli* and *Mycobacterium smegmatis* to isoniazid. Antimicrob. Agents Chemother. **38:**1829–1833.
44. **Ross, R. P., and A. Claiborne.** 1997. Evidence for regulation of the NADH peroxidase gene (npr) from *Enterococcus faecalis* by OxyR. FEMS Microbiol. Lett. **151:**177–183.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Schweizer, H. P.** 1991. Improved broad-host-range lac-based plasmid vectors for the isolation and characterization of protein fusions in *Pseudomonas aeruginosa*. Gene **103:**87–92.
47. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:** 15–22.
48. **Sherman, D. R., K. Mdluli, M. J. Hickey, T. M. Arain, S. L. Morris, C. E. Barry, 3rd, and C. K. Stover.** 1996. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. Science **272:**1641–1643.
49. **Simon, R., U. Priefer, and A. Puehler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
50. **Sreevatsan, S., X. Pan, Y. Zhang, V. Deretic, and J. M. Musser.** 1997. Analysis of the *oxyR-ahpC* region in isoniazid-resistant and -susceptible *Mycobacterium tuberculosis* complex organisms recovered from diseased humans and animals in diverse localities. Antimicrob. Agents Chemother. **41:** 600–606.
51. **Storz, G., F. S. Jacobson, L. A. Tartaglia, R. W. Morgan, L. A. Silveira, and B. N. Ames.** 1989. An alkyl hydroperoxide reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: genetic characterization and cloning of *ahp*. J. Bacteriol. **171:**2049–2055.
52. **Storz, G., and L. A. Tartaglia.** 1992. OxyR: a regulator of antioxidant genes. J. Nutr. **122:**627–630.
53. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. The OxyR regulon. Antonie Leeuwenhoek **58:**157–161.
54. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. Science **248:** 189–194.
55. **Tartaglia, L. A., C. J. Gimeno, G. Storz, and B. N. Ames.** 1992. Multidegenerate DNA recognition by the OxyR transcriptional regulator. J. Biol. Chem. **267:**2038–2045.
56. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell **78:**897–909.
57. **Wayne, L. G., and G. A. Diaz.** 1986. A double staining method for differentiating between two classes of mycobacterial catalase in polyacrylamide gels. Anal. Biochem. **157:**89–92.
58. **Wengenack, N. L., M. P. Jensen, F. Rusnak, and M. K. Stern.** 1999. *Mycobacterium tuberculosis* KatG is a peroxynitritase. Biochem. Biophys. Res. Commun. **256:**485–487.
59. **West, S. E., H. P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **148:**81–86.
60. **Wu, J., and B. Weiss.** 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. J. Bacteriol. **173:**2864–2871.
61. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
62. **Zhang, Q. M., T. Takemoto, S. Mito, and S. Yonei.** 1996. Induction of repair capacity for oxidatively damaged DNA as a component of peroxide stress response in *Escherichia coli*. J. Radiat. Res. (Tokyo) **37:**171–176.
63. **Zhang, Y., S. Dhandayuthapani, and V. Deretic.** 1996. Molecular basis for the exquisite sensitivity of *Mycobacterium tuberculosis* to isoniazid. Proc. Natl. Acad. Sci. USA **93:**13212–13216.
64. **Zhang, Y., B. Heym, B. Allen, D. Young, and S. Cole.** 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature **358:**591–593.
65. **Zheng, M., F. Åslund, and G. Storz.** 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science **279:**1718–1721.
66. **Zheng, M., B. Doan, T. D. Schneider, and G. Storz.** 1999. OxyR and SoxRS regulation of *fur*. J. Bacteriol. **181:**4639–4643.