

# Genetic and Physiological Characterization of *ohr*, Encoding a Protein Involved in Organic Hydroperoxide Resistance in *Pseudomonas aeruginosa*

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The *ohr* (organic hydroperoxide resistance) gene product of *Pseudomonas aeruginosa* was essential for optimal resistance to organic hydroperoxides (OHPs) but not to hydrogen peroxide or paraquat. A  $\Delta ohr$  mutant was hypersusceptible to OHPs in disk inhibition assays and showed enhanced killing by OHPs in liquid culture. The *ohr* gene product was demonstrated to contribute to the decomposition of OHPs. Transcription of *ohr* was induced up to 15-fold upon exposure to OHPs, and this induction was independent of OxyR. Somewhat enhanced *ohr-lacZ* activity was detected in mutant strains affected in *ohr*, *ahpC*, and *oxyR*, and this phenotype correlated with hypersusceptibility to OHPs, suggesting overlapping or compensatory functions of the *ohr* and *ahpC* gene products. A single transcriptional start site for *ohr* was determined, and *ohr* transcripts were abundant in cells treated with a sublethal dose of OHPs but not in cells treated with paraquat. An 84-bp portion upstream of the *ohr* mRNA start site was sufficient for *ohr* induction by OHPs. Thus, the *ohr* gene appears to encode an antioxidant enzyme that is not part of the OxyR regulon yet is specifically induced by OHPs.

Aerobic respiration in *Pseudomonas aeruginosa* leads to the production of toxic metabolic byproducts, including the superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ), the hydroxyl radical ( $HO^{\cdot}$ ), and organic hydroperoxides (OHPs). Sublethal

levels of  $O_2^{\cdot-}$  and  $H_2O_2$  are detoxified by two superoxide dismutases (Fe-SOD and Mn-SOD) and two catalases (KatA and KatB), respectively (1, 3–7, 10). Although much is known of systems that combat  $O_2^{\cdot-}$  and  $H_2O_2$  stress in *P. aeruginosa*

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics <sup>a</sup>	Source or reference
<i>E. coli</i>		
DH5 $\alpha$ -MCR	F <sup>-</sup> <i>lacZ</i> $\Delta$ M15 <i>recA1 hsdR17 supE44</i> $\Delta$ ( <i>lacZYA argF</i> )	Bethesda Research Laboratories
SM10	Km <sup>r</sup> , mobilizer strain	19
<i>P. aeruginosa</i>		
PAO1	Prototrophic, wound isolate	8
PAO1 $\Delta ohr::Tc$	Tc <sup>r</sup> , PAO1 harboring a 516-bp deletion of the <i>ohr</i> locus	This study
PAO1 $\Delta ahpCF::Gm$	Gm <sup>r</sup> , $\Delta ahpCF::Gm$ mutant of PAO1	13
PAO1 $\Delta oxyR::Gm$	Gm <sup>r</sup> , $\Delta oxyR::Gm$ mutant of PAO1	This study
PAO1 $\Delta ohr \Delta ahpCF$	Gm <sup>r</sup> Tc <sup>r</sup> , $\Delta ohr \Delta ahpCF$ double mutant of PAO1	This study
PAO1 $\Delta ahpA \Delta ahpB$	Gm <sup>r</sup> , Tc <sup>r</sup> , $\Delta ahpA \Delta ahpB$ double mutant of PAO1	This study
Plasmids		
pCRII-2.1	Ap <sup>r</sup> Km <sup>r</sup> TA cloning vector for PCR fragments	Invitrogen
pCRII- <i>ohr</i> -220	pCRII-2.1 containing a 220-bp 5' <i>ohr</i> portion generated with primers CCAACGATTTGTCTTGCGCAC and ACCTGTCTGATTTGTACGTTAAG; source of T7-expressed <i>ohr</i> riboprobe	This study
pCRII- <i>omlA</i> -444b	pCRII-2.1 containing a 444-bp <i>omlA</i> promoter fragment; source of T7-expressed <i>omlA</i> riboprobe	12
pEX100T	Ap <sup>r</sup> <i>oriT mob sacB</i>	18
pEX100T- $\Delta ohr::Tc$	pEX100T containing a $\Delta ohr::Tc$ cassette; used for deleting <i>ohr</i> from bases 203999 to 204505	This study
pPZTC	Ap <sup>r</sup> , pPZ30-based broad-host-range vector used for transcriptional <i>lacZ</i> fusions	17 and this study
pOHR593	pUCP22 containing the <i>ohr</i> gene and <i>ohr</i> promoter, from bases 203847 to 204439	This study
pPZ-P <sub><i>ohr</i></sub>	pPZTC containing portions of the <i>ohr</i> promoter as indicated in Fig. 5; series of <i>ohr-lacZ</i> fusions	This study
pUCP22	Ap <sup>r</sup> , multicopy broad-host-range expression vector	23

<sup>a</sup> *mob*, mobilization site; *oriT*, origin of transfer (RK2); *sacB*, encodes a levansucrase; Ap<sup>r</sup>, ampicillin resistance; Km<sup>r</sup>, kanamycin resistance; Gm<sup>r</sup>, gentamicin resistance; Tc<sup>r</sup>, tetracycline resistance.

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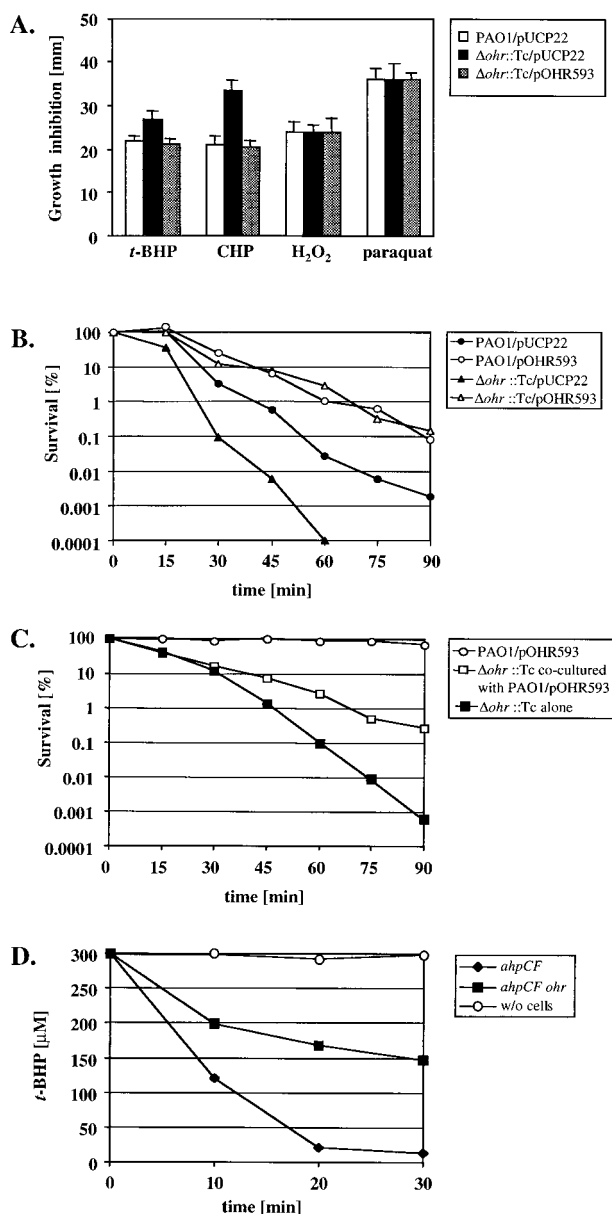


FIG. 1. Susceptibility of a *P. aeruginosa ohr* mutant to oxidative stress agents. (A) Inhibition assay of PAO1/pUCP22, PAO1  $\Delta ohr::Tc/pUCP22$ , and PAO1  $\Delta ohr::Tc/pOHR593$  growth around disks containing 10  $\mu$ l of 1% (114 mM) *t*-BHP, 20% (1.25 M) CHP, 0.5% (145 mM) H<sub>2</sub>O<sub>2</sub>, or 0.5% (20 mM) paraquat. Normalized amounts of cells (0.2  $A_{600}$  unit, roughly  $6 \times 10^8$  cells) grown in M9-0.4% glucose medium were mixed with 3 ml of M9 top agarose and poured onto M9 agar. The zones of growth inhibition were measured after incubation for 16 h at 37°C. The error bars represent the standard deviations of quadruplicate assays. (B) Killing of *P. aeruginosa* by CHP. PAO1/pUCP22, PAO1/pOHR593, PAO1  $\Delta ohr::Tc/pUCP22$ , and PAO1  $\Delta ohr::Tc/pOHR593$  were grown at 37°C to mid-exponential phase ( $A_{600} = 0.5$ ) and then exposed to a sublethal concentration (300  $\mu$ M) of CHP for 15 min, after which CHP was added to a final concentration of 3 mM. The number of viable cells was determined in serially diluted samples taken every 15 min postexposure. The experiment was performed separately four times, and one representative set of data is shown. (C) Killing of the *ohr* mutant in coculture with an *ohr* overproducer strain. Mid-log-phase PAO1/pOHR593 and  $\Delta ohr::Tc$  cells were washed, and 0.2  $A_{600}$  unit of each  $ml^{-1}$  (roughly  $6 \times 10^8$  cells  $ml^{-1}$ ) were cocultured in fresh antibiotic-free M9 medium containing 1 mM CHP. Viable cells of the two strains in the mixed culture were

and of regulators that facilitate such control (7, 10), little is known of those involved in resistance to OHPs. Bacterial OHP resistance is mediated by alkyl hydroperoxide reductases (Ahrs) that detoxify the peroxide by reducing it to an alcohol (9, 21). In *Salmonella enterica* serovar Typhimurium, activity is mediated by a dimer composed of AhrC and AhrF subunits (14). Recently, a novel gene from *Xanthomonas campestris* restored wild-type resistance to *t*-butyl hydroperoxide (*t*-BHP) in an *ahpCF* mutant of *Escherichia coli* (11). This gene was designated *ohr* for OHP resistance and, strikingly, showed no significant homology to any known bacterial genes. In this study, we describe the *ohr* locus in *P. aeruginosa*, specifically focusing on its genetic regulation and role in OHP resistance.

**Identification and cloning of a *P. aeruginosa ohr* homolog.** The amino acid sequence of the *X. campestris* Ohr protein was used to search the complete *P. aeruginosa* genome (<http://www.pseudomonas.com>). An *ohr*-like gene (Pa-*ohr*), composed of 426 bp encoding a predicted 14.5-kDa hydrophilic protein, was identified at bases 3203997 to 3204425. Pa-Ohr was 68% identical to the Ohr protein of *X. campestris* (GenBank accession no. AF036166) and 62% identical to an unknown protein from *Acinetobacter calcoaceticus* (GenBank accession no. Y09102) (data not shown). Using the available sequence information of the unfinished microbial genome projects, additional putative homologs of Ohr with at least 50% identity to Pa-Ohr were detected in several eubacteria, including *Pseudomonas putida*, *Legionella pneumophila*, *Bacillus subtilis*, *Deinococcus radiodurans*, *Caulobacter crescentus*, *Shewanella putrefaciens*, *Vibrio cholerae*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Each of these proteins possessed two conserved cysteine residues that are postulated to participate in the proper function of Ohr (11).

**Enhanced sensitivity of an *ohr* mutant to OHPs.** To determine the physiological role of Ohr in *P. aeruginosa*, a  $\Delta ohr::Tc$  mutant was constructed from strain PAO1. Replacement of the *ohr* gene by a tetracycline resistance cassette was achieved through a biparental mating using *E. coli* SM10 harboring pEX100T- $\Delta ohr::Tc$  (Table 1), which allowed sucrose counterselection (16, 18). The deletion of the *ohr* locus ranged from 7 bp upstream of the *ohr* start codon to 80 bp downstream of the stop codon and was verified by Southern blotting (data not shown). Wild-type bacteria containing the control plasmid pUCP22 (23) and the  $\Delta ohr::Tc$  mutant harboring either pUCP22 or the complementing plasmid pOHR593 (Table 1) were analyzed for their susceptibilities to oxidative stress-generating agents in disk inhibition assays as described before (13). The  $\Delta ohr::Tc$  mutant was hypersusceptible to the organic

determined by plating on agar containing carbenicillin (PAO1/pOHR593) or tetracycline (PAO1  $\Delta ohr::Tc$ ). Also shown is the kill curve for  $\Delta ohr::Tc$  cells in a monoculture at a comparable cell density ( $A_{600} = 0.4$ ). (D) Decomposition of *t*-BHP. An *ohr* mutant in an *ahpCF* background and its parental strain were grown to stationary phase in M9 medium and then exposed to 300  $\mu$ M *t*-BHP. Hydroperoxide concentrations were determined at 10-min intervals by mixing 100  $\mu$ l of supernatant with 900  $\mu$ l of a freshly prepared solution of 25 mM sulfuric acid, 100  $\mu$ M xylenol orange, and 100  $\mu$ M ferrous sulfate in methanol-water (9:1), allowing the color to develop for 15 min, and measuring the  $A_{560}$ . A standard curve was previously established using known concentrations of *t*-BHP (data not shown).

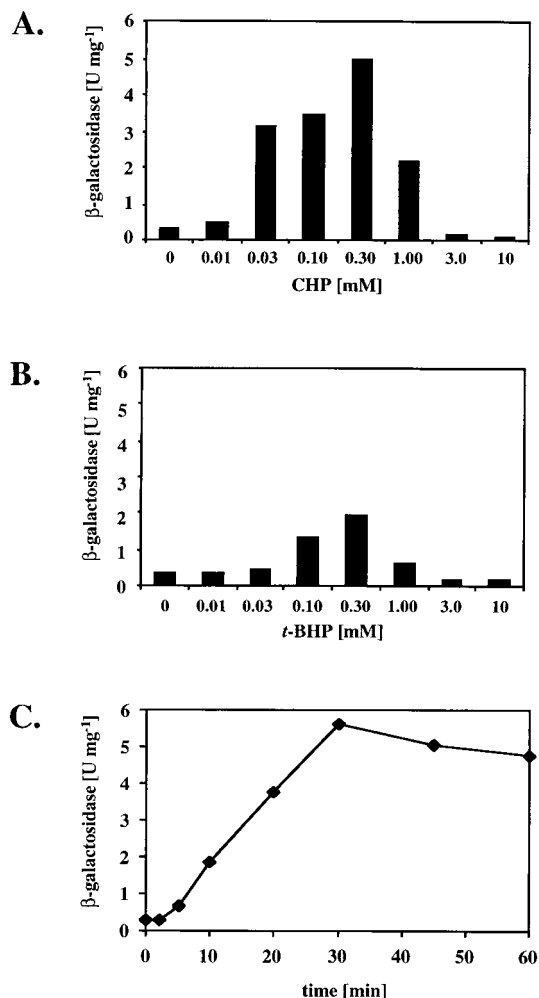


FIG. 2. Response of *ohr-lacZ* expression to OHPs. (A) Induction by CHP. *P. aeruginosa* PAO1 harboring pPZ-*P<sub>ohr</sub>*-99 was grown aerobically in 50 ml of M9-0.4% glucose medium at 37°C and split into 2.5-ml subcultures at mid-exponential phase ( $A_{600} = 0.5$ ), to which increasing concentrations of CHP ranging from 0 to 10 mM were added. The cells were shaken for 1 h, and the  $\beta$ -galactosidase reporter activities were determined as previously described (13). (B) Induction by *t*-BHP. Cells were grown as for panel A and exposed to increasing concentrations of *t*-BHP prior to analysis of  $\beta$ -galactosidase reporter activity. (C) Time response curve of *ohr-lacZ* expression. Mid-exponential-phase cultures ( $A_{600} = 0.5$ ) of PAO1/pPZ-*P<sub>ohr</sub>*-99 in M9 medium at 37°C were exposed to 100  $\mu$ M CHP, and  $\beta$ -galactosidase activities were determined in samples taken at the indicated time points. The values are the means of triplicate experiments.

peroxides *t*-BHP and cumene hydroperoxide (CHP) but not to H<sub>2</sub>O<sub>2</sub> or paraquat, and this phenotype was fully complemented by plasmid pOHR593 containing the entire *ohr* gene (Fig. 1A). The susceptibilities to CHP of the  $\Delta$ *ohr*::Tc mutant and wild-type bacteria were also compared in liquid medium containing 3 mM CHP. The corresponding kill curves indicated that  $\Delta$ *ohr*::Tc mutant cells were hypersusceptible to CHP (Fig. 1B). Furthermore, wild-type or  $\Delta$ *ohr*::Tc mutant cells containing plasmid pOHR593 were more resistant to killing by CHP than cells containing the pUCP22 vector control (Fig. 1B). The calculated rates during the exponential killing by CHP, expressed in logarithmic units per hour, were 4.8 (PAO1/

pUCP22), 7.4 (PAO1  $\Delta$ *ohr*::Tc/pUCP22), and 2.5 (PAO1/pOHR593 and PAO1  $\Delta$ *ohr*::Tc/pOHR593). These data strongly suggest a protective role for the *ohr* gene product in the defense against oxidative stress specifically imposed by OHPs. This hypothesis is supported by the finding that an *ohr* over-producer strain (PAO1/pOHR593) partially protected a co-cultured  $\Delta$ *ohr* mutant from killing by 1 mM CHP (Fig. 1C). Furthermore, the contribution of the *ohr* gene product to the overall decomposition of OHP was assessed in  $\Delta$ *ahpCF* mutant background. The concentration of *t*-BHP diminished by >95% within 30 min when added to a culture of a  $\Delta$ *ahpCF* mutant but decreased only by 50% in a culture of  $\Delta$ *ohr*  $\Delta$ *ahpCF* double mutant, indicating that the *ohr* gene product is involved in detoxifying OHPs (Fig. 1D). The  $\Delta$ *ohr* mutation did not affect the susceptibility to H<sub>2</sub>O<sub>2</sub> or paraquat. In fact, the total enzymatic activities of catalase and of SOD in  $\Delta$ *ohr*::Tc mutant cells were identical to the levels measured in wild-type cells. Total catalase activities were determined as described elsewhere (3) and were  $71 \pm 12$  U mg<sup>-1</sup> (PAO1) and  $72 \pm 9$  U mg<sup>-1</sup> ( $\Delta$ *ohr*::Tc). Total SOD activities were measured as previously described (6) and were  $98 \pm 3.5$  U mg<sup>-1</sup> (PAO1) and  $95 \pm 2.4$  U mg<sup>-1</sup> ( $\Delta$ *ohr*::Tc).

**Inducible expression of *ohr*.** Expression of *ohr* was studied by using plasmid pPZ-*P<sub>ohr</sub>*-99 containing a transcriptional fusion of the *ohr* promoter to the promoterless *lacZ* report-

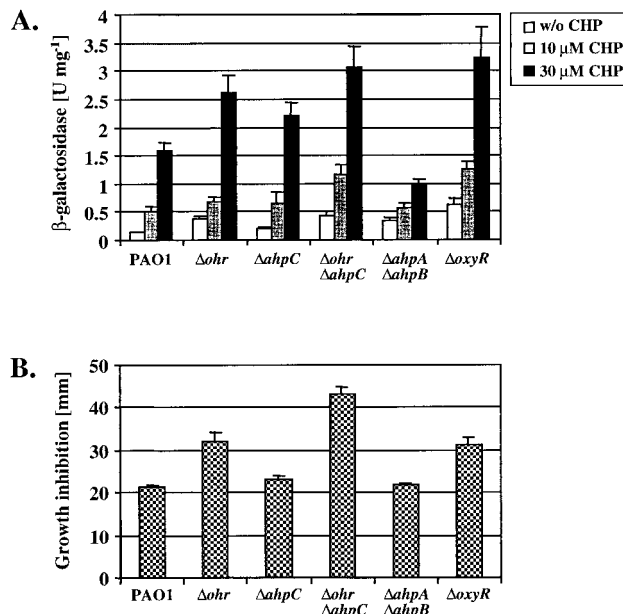


FIG. 3. Role of *ohr* in mutant strains affected in Ahp reductase genes. (A) Expression of *ohr-lacZ* in various mutant strains. Cultures of PAO1, single mutants ( $\Delta$ *ohr*::Tc,  $\Delta$ *ahpCF*::Gm, and  $\Delta$ *oxyR*::Gm), and double mutants ( $\Delta$ *ohr*::Tc  $\Delta$ *ahpCF*::Gm and  $\Delta$ *ahpA*::Tc  $\Delta$ *ahpB*::Gm) harboring pPZ-*P<sub>ohr</sub>*-99 were grown in 15 ml of M9 medium at 37°C and split into 2.5-ml subcultures at mid-exponential phase ( $A_{600} = 0.5$ ), to which various concentrations (0, 10, and 30  $\mu$ M) of CHP were added. The cells were harvested 30 min postexposure and  $\beta$ -galactosidase activities were determined. The error bars show the standard deviations of triplicate experiments. (B) Growth inhibition of mutant strains by CHP. The PAO1 wild type and the indicated single and double mutants were assayed for their susceptibilities to 20% (1.25 M) CHP in a disk inhibition assay as described for Fig. 1.

er gene.  $\beta$ -Galactosidase activity was detectable at low levels ( $<0.5$  U  $\text{mg}^{-1}$ ) in cell extracts from untreated PAO1/pPZ- $P_{ohr}$ -99 cultures. This basal *ohr-lacZ* expression was only marginally affected by the carbon source added to M9-based medium. The presence of 1% citrate resulted in a twofold increase, while 1% corn oil resulted in a twofold decrease compared to reporter activity measured in M9-1% glucose cultures (data not shown). Reporter activity increased up to 15-fold upon treatment of bacteria with 0.03 to 0.30 mM CHP (Fig. 2A). Up to fivefold greater  $\beta$ -galactosidase activity was detected in cells exposed to 0.10 or 0.30 mM *t*-BHP (Fig. 2B). Due to cell death, CHP or *t*-BHP concentrations equal to or higher than 1 mM did not result in such a response (data not shown). In contrast, the addition of  $\text{H}_2\text{O}_2$  (1 mM) and the  $\text{O}_2^{\cdot-}$  generating compound paraquat (0.35 mM), both of which lead to induction of other antioxidant genes in *P. aeruginosa*, such as *katB*, *ahpB*, and *ahpCF*, at these sublethal concentrations (13), or the presence of a high iron concentration [100  $\mu\text{M}$  Fe(III)], did not result in higher *ohr-lacZ* expression (data not shown). Thus, *ohr* expression appeared to respond specifically to OHPs. The reason for the observed difference in maximal *ohr* induction upon exposure to either CHP or *t*-BHP is unclear. It may be a consequence of different uptake efficiencies or reactivities of these two compounds. In fact, CHP is poorly water soluble and therefore may readily bind to the cell surface, while the more water-soluble *t*-BHP remains dissolved in the medium. The expression of *ohr* in response to OHP exposure was further studied by analyzing extracts of PAO1/pPZ- $P_{ohr}$ -99 at various time points after the addition of 100  $\mu\text{M}$  CHP to mid-exponential-phase cultures ( $A_{600} = 0.5$ ). A roughly twofold increase in  $\beta$ -galactosidase activity was observed after 5 min. Then the response was linear until 30 min postexposure (Fig. 2C), with a calculated  $\beta$ -galactosidase production rate of 0.20 U  $\text{mg}^{-1}$   $\text{min}^{-1}$ . After 30 min,  $\beta$ -galactosidase activity reached a plateau, presumably due to a new steady-state level of *ohr* expression or due to complete detoxification of CHP, as shown above.

**Role of Ohr in compensating for an absence of Ahps.** Since *ohr* expression was inducible by OHPs, Ohr may function similarly to Ahps. Therefore, expression of *ohr* was monitored using pPZ- $P_{ohr}$ -99 in various mutant strains affected in single or multiple *ahp* genes that were described earlier (13). Basal expression of *ohr* was threefold higher in the  $\Delta\text{ohr}::\text{Tc}$  mutant than in wild-type cells, and induction by low concentrations of CHP (10 and 30  $\mu\text{M}$ ) also resulted in higher activities (Fig. 3A). In the  $\Delta\text{ahpCF}::\text{Gm}$  mutant, *ohr* expression was somewhat higher than in wild-type cells, and in the  $\Delta\text{ohr}::\text{Tc}$   $\Delta\text{ahpCF}::\text{Gm}$  double mutant, *ohr* was expressed at higher levels than in the single mutants, suggesting an additive effect (Fig. 3A). Similarly,  $\Delta\text{oxyR}::\text{Gm}$  mutant cells, which are affected in the production of at least AhpB, AhpC-AhpF, and KatB-AnkB (13), expressed *ohr* at higher levels than wild-type cells. In contrast, a  $\Delta\text{ahpA}::\text{Tc}$   $\Delta\text{ahpB}::\text{Gm}$  double mutant had a somewhat higher basal expression of *ohr* but did not respond to CHP to the extent observed in the other mutants. The susceptibilities of these mutant strains to CHP were determined using a disk inhibition assay as described above (Fig. 3B). Strikingly, the susceptibilities correlated well with the observed *ohr-lacZ* activities, with the  $\Delta\text{ohr}::\text{Tc}$   $\Delta\text{ahpCF}::\text{Gm}$  double mutant being the most susceptible. Taken together, the data suggest over-



FIG. 4. RNase protection assay of *ohr* mRNA. A riboprobe specific for the 5' portion of *ohr* (*ohr* rp) was generated by runoff in vitro transcription from the T7 promoter on pCRII-*ohr*-220 and used to detect *ohr* transcripts. RNA was isolated from wild-type cells and from  $\Delta\text{oxyR}::\text{Gm}$  mutant cells grown aerobically to mid-exponential phase in M9 medium at 37°C. Parallel cultures were treated with 100  $\mu\text{M}$  CHP or 100  $\mu\text{M}$  paraquat (PQ) as indicated. Normalized samples (20  $\mu\text{g}$ ) of total RNA were used. Aliquots of the same RNA batches were subjected to control hybridizations to a riboprobe specific for the constitutively expressed *omlA* gene (12), as shown in the boxed part at the bottom.

lapping functions of Ohr and AhpC-AhpF. However, the AhpA, AhpB, AhpC-AhpF, and Ohr proteins of *P. aeruginosa* have not yet been characterized biochemically, especially regarding their potential for use of multiple substrates. Some of the observed *ohr* expression levels and the CHP susceptibilities of the mutant strains may be caused by compensatory induction of alternate antioxidant genes, as has been described for other bacteria (2, 15) and for *P. aeruginosa* (13). Specifically, mutants affected in either *ohr*, *ahpC*, or both exhibited higher *ohr* expression and induction levels, presumably due to higher oxidative stress in these backgrounds, while the *ahpA* *ahpB* double mutant showed lower *ohr* induction, since it may respond to this defect with a compensatory upregulation of *ahpC-ahpF*.

**A genetic analysis of the *ohr* promoter.** The transcriptional start site of *ohr* was mapped to a single T at bp  $-56$  relative to the ATG translational start codon. Specific *ohr* transcripts were detected using an *ohr* riboprobe covering the 5' end of the *ohr* transcript in RNase protection assays as described elsewhere (13). Total RNA of untreated wild-type cells contained barely detectable levels of *ohr* mRNA, while a dramatic

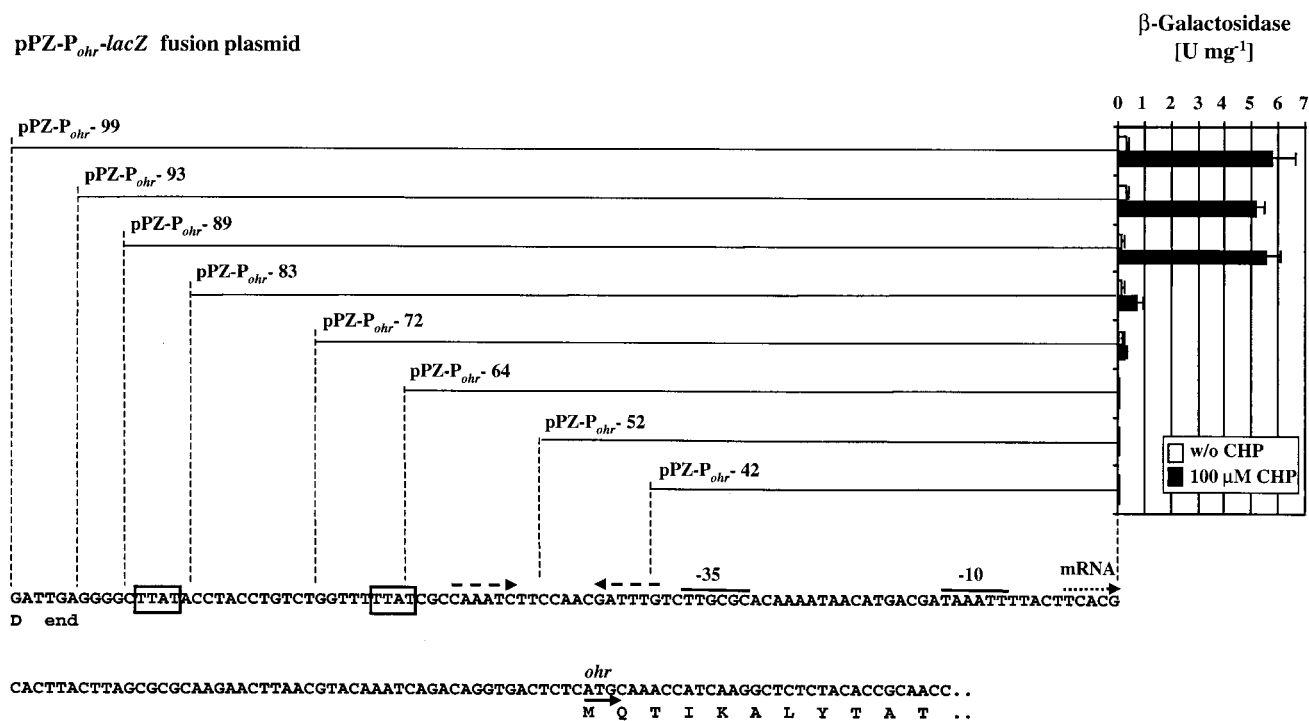


FIG. 5. Analysis of the *ohr* promoter. A series of pPZ-P<sub>ohr</sub>-lacZ fusions containing the indicated portions of the *ohr* promoter and upstream sequence was constructed. Their reporter activities were measured in triplicate cultures of wild-type cells in the absence or presence of 100 μM CHP. Beside the indicated -35 and -10 promoter elements and the mapped mRNA start site, elements potentially involved in *ohr* regulation are shown, including two TTAT motifs (boxed) and a stem-loop structure (dashed arrows). Also shown are the translational start of *ohr* and the end of the coding sequence of the unrelated upstream gene.

increase in *ohr* mRNA was detected in cells that had been exposed to 100 μM CHP for 30 min (Fig. 4). The presence of 100 μM paraquat did not affect the level of *ohr* mRNA. An identical pattern of *ohr* expression was observed in ΔoxyR mutant cells. These findings indicate that *ohr* induction occurred at the transcriptional level and was independent of OxyR, which is in agreement with the results obtained using *ohr-lacZ* reporter fusions. To determine whether other known regulators of the *P. aeruginosa* oxidative stress machinery were involved in *ohr* regulation, *ohr-lacZ* expression was also measured in mutant strains affected in *soxR*, *rpoS*, and *rhlR lasR*. In these cases, induction by 100 or 300 μM CHP was not affected (data not shown). Therefore, *ohr* gene expression appeared to be upregulated by a novel mechanism involving a yet-to-be-identified positive or negative factor responding specifically to OHPs. As a first step toward the identification of such a regulatory factor, the *ohr* promoter was analyzed using a series of pPZ-P<sub>ohr</sub>-lacZ fusions containing systematically shorter upstream sequences (Fig. 5). A sequence of at least 84 bp upstream of the mapped transcriptional start site was required for full induction of the *ohr* promoter (pPZ-P<sub>ohr</sub>-89). A 6-bp-shorter promoter fragment (pPZ-P<sub>ohr</sub>-83) resulted in normal basal expression but responded only marginally to the presence of CHP. Constructs containing only 59 bp or less of upstream sequence did not result in any detectable *ohr-lacZ* expression, although they contained the -35 and -10 promoter elements (Fig. 5). These results demonstrate that the 84-bp sequence located upstream of the mRNA start site was essential for the stress response. This sequence contained inverted repeats (CA

AATC-N<sub>7</sub>-GATTTG) with the potential to form a stem-loop structure ( $\Delta G_0 = -3.9 \text{ kcal mol}^{-1}$ ) and contained two short direct repeats (TTAT) spaced 21 bp apart. Deletion of the upstream TTAT element resulted in the loss of inducibility (Fig. 5). The spacing of the two TTAT motifs suggested a location on the same face of the DNA, spaced two turns apart, and may represent the target site of a positive activator of *ohr*. Similar *cis*-acting regulatory elements have been demonstrated to play a crucial role in antioxidant gene activation by the well-characterized OxyR protein (13, 20, 22). We therefore postulate the existence of a *trans*-acting regulatory protein involved in *ohr* regulation, and future approaches to identify such a regulator could include the screening of a mutant library for the lack of *ohr-lacZ* induction or affinity purification of a putative regulator on its immobilized target sequence. Such studies are currently under way.

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