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ohrR and *ohr* Are the Primary Sensor/Regulator and Protective Genes against Organic Hydroperoxide Stress in *Agrobacterium tumefaciens*

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The genes involved in organic hydroperoxide protection in *Agrobacterium tumefaciens* were functionally evaluated. Gene inactivation studies and functional analyses have identified *ohr*, encoding a thiol peroxidase, as the gene primarily responsible for organic hydroperoxide protection in *A. tumefaciens*. An *ohr* mutant was sensitive to organic hydroperoxide killing and had a reduced capacity to metabolize organic hydroperoxides. *ohr* is located next to, and is divergently transcribed from, *ohrR*, encoding a sensor and transcription regulator of organic hydroperoxide stress. Transcription of both *ohr* and *ohrR* was induced by exposure to organic hydroperoxides but not by exposure to other oxidants. This induction required functional *ohrR*. The results of gel mobility shift and DNase I footprinting assays with purified OhrR, combined with in vivo promoter deletion analyses, confirmed that OhrR regulated both *ohrR* and *ohr* by binding to a single OhrR binding box that overlapped the *ohrR* and *ohr* promoters. *ohrR* and *ohr* are both required for the establishment of a novel cumene hydroperoxide-induced adaptive response. Inactivation or overexpression of other Prx family genes (*prx1*, *prx2*, *prx3*, *bcp1*, and *bcp2*) did not affect either the resistance to, or the ability to degrade, organic hydroperoxide. Taken together, the results of biochemical, gene regulation and physiological studies support the role of *ohrR* and *ohr* as the primary system in sensing and protecting *A. tumefaciens* from organic hydroperoxide stress.

Agrobacterium tumefaciens is a soil bacterium that causes crown gall disease in a wide range of plants by transferring some of its DNA (T-DNA) into the plant host. The T-DNA is then stably integrated into the plant genome, where its expression leads to the synthesis of plant hormones that stimulate tumor growth (5). In general, soil bacteria are exposed to hydroperoxides from various sources, such as their own aerobic metabolism and exposure to other soil bacteria and fungi that produce hydroperoxides. In addition, during plant microbe interactions, bacterial phytopathogens are exposed to reactive oxygen species (ROS), including H_2O_2, superoxide anions, and lipid hydroperoxides, that are generated as part of active plant defense responses. Although the levels of plant lipoxygenases that catalyze the formation of fatty acid hydroperoxides from fatty acid precursors have been shown to increase in response to microbial invasion (12), the role of ROS during *Agrobacterium*-plant interactions is not clear.

In order to grow and proliferate, bacterial phytopathogens and soil bacteria must overcome these ROS. In regard to the protection against organic hydroperoxide toxicity, there are two major families of enzymes, peroxiredoxins (Prx) and Ohr, that have been shown to be important in many bacteria (3, 16, 26). AhpC (alkyl hydroperoxide reductase), an enzyme of the Prx family that catalyzes the reduction of organic hydroperoxides to their corresponding alcohols, has been well characterized biochemically and genetically (26). The enzyme not only detoxifies organic hydroperoxides but is also involved in the degradation of low concentrations of intracellular H_2O_2 (29). The physiological functions and biochemical properties of other members of the Prx family, such as Tpx (thiol peroxidase), bactoferritin comigratory protein (BCP), 1-Cys Prx, and 2-Cys Prx, are less clear partly due to their limited distribution in only a few bacterial species (4, 13, 26, 34). Nonetheless, they have been shown to be capable of metabolizing organic hydroperoxide. Ohr (organic hydroperoxide resistance protein), a thiol peroxidase, was initially discovered in *Xanthomonas campestris* due to its ability to complement organic hydroperoxide-sensitive phenotypes in an *Escherichia coli ahpC* mutant (21). Ohr is uniquely regulated, and its expression is highly induced only by organic hydroperoxides. Purified Ohr has hydroperoxide peroxidase activity and catalyzes the reduction of organic hydroperoxides to their corresponding alcohols (6, 16). Both *ahpC* and *ohr* are found in diverse species of bacteria (3, 10, 16, 21, 23, 30). They have similar biochemical properties but differ in both their physiological function and pattern of gene expression in response to stresses. In many bacteria, the expression of *ahpC* is regulated by OxyR, a peroxide sensor and transcription regulator (17, 31); however, in a number of bacteria *ahpC* is regulated by the peroxide sensing repressor, PerR (19). *ohr* is controlled by OhrR, an organic hydroperoxide-inducible transcription repressor (3, 19, 20, 32).

The aim of the present study was to functionally evaluate the roles of genes predicted, based on sequence homology, to be involved in organic hydroperoxide resistance. The analysis of the biochemical properties of *ohrR* and *ohr* mutants and the

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expression patterns of *ohrR* and *ohr* indicate that this system plays a primary role in sensing and protecting *A. tumefaciens* from organic hydroperoxides.

MATERIALS AND METHODS

Bacterial growth conditions. *A. tumefaciens* NTL4 (18) and mutant strains were grown aerobically in Luria-Bertani (LB) medium at 30°C with continuous shaking at 150 rpm. To ensure synchronous growth, overnight cultures were inoculated into fresh LB medium to give an optical density at 600 nm (OD_{600}) of ca. 0.1. Exponential-phase (OD_{600} of ~0.6, after 4 h of growth) and stationary-phase (OD_{600} of ~5.0, after 30 h of growth) cells were used in all experiments. The peroxide induction experiments were executed with exponential treated with various concentrations of peroxides for 15 and 30 min for Northern analysis and enzymatic assays, respectively. The organic hydroperoxides, *tert*-butyl hydroperoxide and cumene hydroperoxide were obtained from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany), respectively. Linoleic acid hydroperoxide was prepared from linoleic acid (Sigma, St. Louis, MO) as described by Evans et al. (9).

Molecular biology techniques. General molecular genetics techniques, including genomic DNA preparation, plasmid preparation, restriction endonuclease digestions, ligation, transformation in *E. coli*, agarose gel electrophoresis, and Southern and Northern blot analyses were performed according to standard protocols (28). Plasmid purification for DNA sequencing was performed by using the QIAGEN Miniprep kit. DNA was sequenced by using a BigDye terminator cycle sequencing kit (PE Biosystems) and run on an ABI 310 automated DNA sequencer. Routinely, *A. tumefaciens* was transformed by electroporation as previously described (18).

Purification of OhrR. A 472-bp PCR fragment containing *ohrR*, in which an NcoI site overlapping the start codon had been introduced, was generated by using pOhrR as a template and the specific oligonucleotide primers BT992 and BT486. The NcoI-digested fragment was cloned into NcoI-HincII-digested pETBlue-2 (Novagen), yielding pETohrR.

E. coli harboring pETohrR was grown to mid-log phase before 1 mM IPTG was added, followed by incubation for 3 h. The cultures were harvested by centrifugation, and cell pellets were resuspended in 50 mM PB, sonicated, and then spun at 10,000 × *g* for 15 min. The cleared lysate was then loaded onto an Affi-Gel heparin column (Bio-Rad), followed by extensive washing with column buffer (25 mM Tris-HCl [pH 8], 25 mM NaCl, 2 mM EDTA). The protein was eluted by the addition of elution buffer (25 mM Tris-HCl [pH 8], 500 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol). The eluted fraction was dialyzed against 25 mM Tris-HCl [pH 8]–100 mM NaCl–2 mM EDTA–1 mM dithiothreitol. The purity of the protein was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Gel mobility shift and DNase I protection assays. ^{32}P-labeled DNA fragments were prepared by PCR with the oligonucleotide primers BT536 and BT537 (see Table 1) and *A. tumefaciens* NTL4 genomic DNA as the template to generate a 363-bp fragment spanning the *ohr* and *ohrR* promoter region. Gel mobility shift assays were performed as previously described (20). Gel mobility shift reactions contained 3 fmol of labeled probe in 25 μl of reaction buffer (20 mM Tris [pH 7.0], 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μg of bovine serum albumin ml^{-1}, 5 μg of calf thymus DNA ml^{-1}, 0.5 mg of poly(dI-dC) ml^{-1}, 400 ng of purified OhrR). DNase I footprinting assays with the 336-bp PCR-generated DNA fragment spanning the *ohr-ohrR* intergenic region and purified OhrR were performed as described previously (20).

Construction of *A. tumefaciens bcp*, *prx*, *ohr*, and *ohrR* mutants. The specific primers used for PCR amplification of gene internal fragments of *A. tumefaciens bcp1*, *bcp2*, *prx1*, *prx2*, *prx3*, *ohr*, and *ohrR* were designed based on the nucleotide sequences corresponding to putative open reading frames (ORFs) Atu1830 (BT907 and BT908), Atu3655 (BT911 and BT912), Atu1480 (BT532 and BT533), Atu0779 (BT1173 and BT1174)), Atu2399 (BT1319 and BT1320), Atu0847 (BT538 and BT539), and Atu0846 (BT546 and BT547), respectively, in the *A. tumefaciens* genome sequence (35) (Table 1). The PCR products were ligated into pDrive prior to the subcloning of the EcoRI fragments into pKNOCK-Gm or pKNOCK-Km and insertion mutants were constructed by using a protocol previously described (1). Mutants were confirmed by PCR with two primers flanking the insertion site and by Southern blot analysis.

Construction of pBcp1, pBcp2, pPrx1, pPrx2, pPrx3, pOhr, and pOhrR. The full-length genes were PCR amplified from *A. tumefaciens* genomic DNA by using specific pairs of primers (BT909 and BT910 for *bcp1*; BT913 and BT914 for *bcp2*; BT574 and BT575 for *prx1*;BT1046 and BT1047 for *prx2*; BT1317 and BT1318 for *prx3*; BT487 and BT488 for *ohr* and BT485 and BT486 for *ohrR*) (Table 1) and *Pfu* polymerase. The PCR products were cloned into pCR-Blunt (Invitrogen), sequenced, and subcloned into the broad-host-range plasmid pBBR1MSC-4 (15) to generate the high-expression plasmids pBcp1, pBcp2, pPrx1, pPrx2, pPrx3, pOhr, and pOhrR.

Organic hydroperoxide degradation assay. The degradation of organic hydroperoxides was measured as previously described (23, 30) with some modifications. Overnight cultures of various *A. tumefaciens* strains were inoculated into 20 ml of LB medium at a final OD_{600} of 0.1. Exponential-phase cultures (after 4 h of growth) were adjusted to an OD_{600} of 0.5 with fresh medium prior to addition of *tert*-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CuOOH), or linoleic acid hydroperoxide (LOOH) at a concentration of 200 μM. Residual organic hydroperoxide concentrations were determined at 10-min intervals using a xylenol orange-iron reaction. At various time intervals, 1 ml of the culture was removed, and the cells were pelleted. A total of 100 μl of the cleared supernatant was then added to 400 μl of 25 mM sulfuric acid in a 1-ml cuvette. A total of 500 μl of freshly prepared reaction buffer (200 μM ammonium ferrous sulfate, 200 μM xylenol orange, and 25 mM sulfuric acid) was then added to the mixture. After 10 min of incubation at room temperature, the absorbance at 540 nm was determined. The concentration of residual organic hydroperoxide in the culture was calculated from a standard curve generated by using LB medium containing known organic hydroperoxide concentrations.

Determination of oxidant resistance by inhibition zone and plate sensitivity assays. The resistance levels of *A. tumefaciens* strains to oxidants were determined by using either growth inhibition zone (21) or a plate sensitivity assay as previously described (27). Briefly, 1 ml of exponential-phase cells were mixed with 10 ml of molten top agar (LB containing 0.7% agar) prewarmed at 50°C and overlaid onto LB plates (14-cm-diameter petri dishes containing 40 ml of LB agar). The plates were left at room temperature for 15 min to let the top agar solidify. Sterile 6-mm-diameter disks (prepared from Whatman filter paper no. 3) soaked with either 5 μl of 1.0 M H_2O_2, 1.0 M tBOOH, or 0.5 M CuOOH were placed on the cell lawn, and zones of growth inhibition were measured after 24 h of incubation at 30°C. For plate sensitivity assay, serial dilutions of exponential phase cells were made in LB medium and 10 μl of each dilution was spotted onto a LB agar plate containing either 200 μM CuOOH or 800 μM tBOOH. The plates were incubated at 30°C for 24 h before bacterial colonies were scored.

Determination of adaptive protection to CuOOH. Induced adaptive resistance to CuOOH killing was measured by adding 50 μM CuOOH to exponential-phase cultures of *A. tumefaciens* strains prior to treatment with lethal concentrations of CuOOH (1, 5, and 10 mM) for 30 min. After treatment, the cells were washed with fresh LB medium, and the number of viable cells was determined as described previously (33). The surviving fraction was defined as the number of CFU recovered after treatment divided by the CFU prior to treatment. Three independent experiments were performed in each case.

β-Galactosidase assay. Crude bacterial lysates were prepared, and protein assays were performed as previously described (21). In brief, 20 ml of exponential-phase cultures were harvested and washed once with 50 mM sodium phosphate buffer (pH 7.0; PB). Bacterial suspensions in 0.5 ml of PB containing 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor, were lysed by intermittent sonication, followed by centrifugation at 10,000 × *g* for 20 min. The total protein concentration was determined for each of the cleared lysates prior to their use in enzyme assays. β-Galactosidase was assayed as described earlier (25).

RESULTS AND DISCUSSION

Physiological analysis of *ohr* and *ohrR* mutants. As a first step in investigating the role of the *ohrR-ohr* system in oxidative stress defense, *Agrobacterium* NTL4 strains carrying insertions in either *ohr* or *ohrR* were constructed by using the pKNOCK system (1), and their ability to resist exposure to oxidants was evaluated. Inactivation of either *ohrR* or *ohr* had no effect on aerobic growth rate or colony formation on a complex medium and an *ohr* mutant was less resistant, as shown by zones of growth inhibition of 26.5 ± 1.0 mm and 25.5 ± 1.2 mm to the organic hydroperoxides, CuOOH and tBOOH, respectively, compared to the wild-type strain NTL4 (20.0 ± 0.7 mm and 21.5 ± 1.0 mm). However, the sensitivity of the *ohr* mutant to CuOOH was more pronounced, suggesting that the *A. tumefaciens ohrR-ohr* system has evolved to be more efficient at sensing and protecting the bacteria from mod-

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain plasmid, or primer	Description or sequence (5′ to 3′)	Source or reference
A. tumefaciens strains		
NTL4	pTiC58-cured derivative of strain C58 Δtet_{C58}	18
bcp1	*bcp1*::pKNOCK-Gm	This study
bcp2	*bcp2*::pKNOCK-Gm	This study
prx1	*prx1*::pKNOCK-Gm	This study
prx2	*prx2*::pKNOCK-Gm	This study
prx3	*prx3*::pKNOCK-Gm	This study
ohr	*ohr*::pKNOCK-Km	This study
ohrR	*ohrR*::pKNOCK-Gm	This study
ohr bcp1	*ohr*::pKNOCK-Km, *bcp1*::pKNOCK-Gm	This study
ohr prx1	*ohr*::pKNOCK-Km, *prx1*::pKNOCK-Gm	This study
Plasmids		
pKNOCK	Broad-host-range suicide vector; RP4 *oriT*, R6K γ-*ori*	1
pBBR1MCS-4	Broad-host-range cloning vector; *rep, mob, lacZ*α, Ap^R	15
pBcp1	pBBR1MCS-4 containing *A. tumefaciens bcp1*	This study
pBcp2	pBBR1MCS-4 containing *A. tumefaciens bcp2*	This study
pPrx1	pBBR1MCS-4 containing *A. tumefaciens prx1*	This study
pPrx2	pBBR1MCS-4 containing *A. tumefaciens prx2*	This study
pPrx3	pBBR1MCS-4 containing *A. tumefaciens prx3*	This study
pOhr	pBBR1MCS-4 containing *A. tumefaciens ohr*	This study
pOhrR	pBBR1MCS-4 containing *A. tumefaciens ohrR*	This study
pP_{ohr}	pUFR027 containing *ohr*::*lacZ*	This study
pP_{ohrR}	pUFR027 containing *ohrR*::*lacZ*	This study
Primers		
BT485	TGGAGCAGGAAAATGGAC	
BT486	GCCTATGGCCGGGAGAGA	
BT487	GAAGGAGTAAATGCCATG	
BT488	TAAGCCCGCTTTATCAGG	
BT532	ATCGGGTAAGTGAGGACC	
BT533	ATTCGCACCGCGACCGGC	
BT536	CTGCGCGTAAAGGGCAA	
BT537	GAGCGTGACGTCGAGAAC	
BT538	CGGAACAGCTTTTCGCGG	
BT539	TTCTTCGGCTTTCTCACG	
BT546	TAGGCGTCAGCGCCACCA	
BT547	TCGACCCGATCGGGGCTCA	
BT574	GGAGAAAGCACACTATGA	
BT575	TTCGTTAGCAGCTTAGCC	
BT907	ATCGACTTCAGCGCGCTC	
BT908	TTCGGCGATCTTGCCATC	
BT909	GCGAAAGAAAGGTAGAAT	
BT910	GGCTGGATGGCCCCTCAG	
BT911	CACCTCAATCTTGCGCTT	
BT912	CACCCGCTTCAAACATTG	
BT913	TGCCAGGGATGGAAATGC	
BT914	ATTGGCCAAGACACTCAT	
BT1046	TAAGAATGGGCTTGTTAAA	
BT1047	GTCTCCTCGTGCCTGTACT	
BT1173	CGCTTAGAGCGCACACCAA	
BT1174	TCTCGAAAATCGCGACGCC	
BT1236	TAATTGTACGCTATAAGG	
BT1317	AGAACAGGAGACAAGACATC	
BT1318	AACCGAGATCAGGCCGCAGC	
BT1319	CTATTGCCTTTCGGTCAACG	
BT1320	ACTGCTCCACCAGACCGTCA	

erately complex organic hydroperoxides such as CuOOH than to the simple organic hydroperoxide, tBOOH, or to linoleic acid hydroperoxide (LOOH), a more complex organic hydroperoxide. The increased sensitivity to CuOOH, in the *ohr* mutant, could be complemented by the introduction of plasmid-borne *ohr*. The *ohr* mutant harboring pOhr gave 18.5 ± 0.5 mm zone of inhibition with 0.5 M CuOOH compared to 26.5 ± 1.0 mm for the mutant and 20.0 ± 0.7 mm for wild-type NTL4.

In a few bacteria, *ohr* has been implicated in H_2O_2 protection and metabolism (6, 16). In *A. tumefaciens*, it is unlikely that *ohr* plays any protective role against H_2O_2 since *ohr* mutant or high-level expression of *ohr* on an expression vector in NTL4 had no effect on resistance to either H_2O_2 or the super-

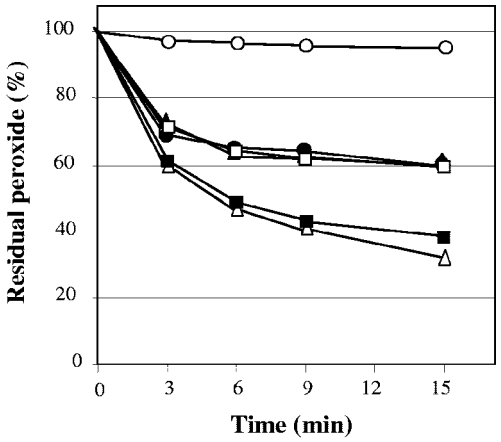

FIG. 1. Degradation of CuOOH by various *A. tumefaciens* strains. The rates of CuOOH degradation in culture medium containing 200 μM CuOOH by *A. tumefaciens* parental NTL4 (■), *ohr* mutant (□), *ohr prx1* mutant (▲), *ohr bcp1* mutant (●), and *ohr* mutant harboring pOhr (△) are indicated. The levels of CuOOH remaining in the culture medium at the various time points are reported, along with those of a medium control without bacteria (○).

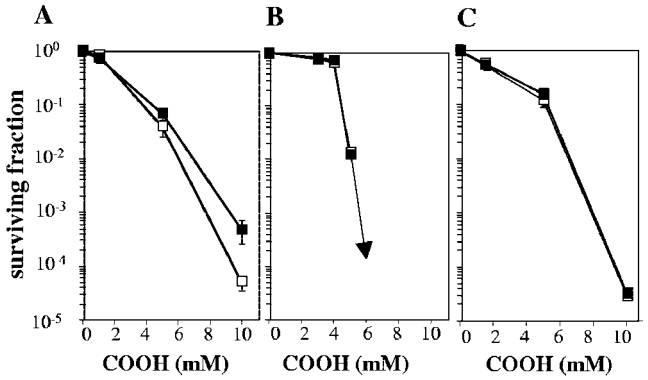

FIG. 2. Induced adaptive protection to CuOOH in *A. tumefaciens* requires functional *ohr* and *ohrR*. CuOOH-induced adaptive response experiments were performed by incubating exponential-phase cultures of *A. tumefaciens* (A), *ohr* mutant (B), and *ohrR* mutant (C) in 50 μM CuOOH for 30 min before treatment with the indicated concentrations of CuOOH for 30 min. Cells that survived various treatments were scored after 48 h of incubation. The CuOOH survival curves against CuOOH concentration are plotted. Symbols: ■, CuOOH-induced; □, uninduced cultures. Values presented are the means and the standard deviations (SD) of four replicate experiments.

oxide generator, menadione (data not shown). Inactivation of *ohrR* led to a small increase in the resistance level to CuOOH as shown by zone of growth inhibition of 18.5 ± 0.8 mm for the mutant compared to 20.0 ± 0.7 mm in NTL4. This was probably due to increased expression of *ohr*. Furthermore, no changes in the resistance levels to inorganic oxidants were detected in an *ohrR* mutant (data not shown).

The *ohr* insertion mutant was further evaluated for its ability to degrade CuOOH in the culture media. The *ohr* mutant, along with the wild-type strain NTL4 and the *ohr* mutant strain carrying the Ohr expression plasmid, pOhr, were incubated with CuOOH and the rate of hydroperoxide degradation was determined. The results, shown in Fig. 1, indicate that the wild-type strain NTL4 rapidly metabolized CuOOH, whereas only the *ohr* mutant showed a significant reduction in the ability to metabolize CuOOH. After 15 min of incubation in medium containing CuOOH, the amount of CuOOH remaining was 60% for the *ohr* mutant and 40% for wild-type strain NTL4 (Fig. 1). The reduced capacity to metabolize CuOOH in the *ohr* mutant could be complemented by the introduction of plasmid-borne *ohr* in pOhr, resulting in a rate of CuOOH degradation that was similar to that in NTL4 (Fig. 1). These observations indicate that *ohr* is the major detoxification enzyme involved in organic hydroperoxide degradation in *A. tumefaciens*.

***ohrR*- and *ohr*-mediated adaptive response.** The ability to adapt to stress is crucial for bacterial survival under stressful conditions. It has often been observed that low-level exposure to a particular stress can elicit an adaptive response that results in an increased resistance to a subsequent high-level exposure to the same stress. An adaptive response to H_2O_2 exposure has been observed in many bacteria, including *A. tumefaciens* (33). Although adaptive responses to organic hydroperoxide are rare, an adaptive response to lipid hydroperoxide involving the *ohrR*/*ohr* system has been reported in *X. campestris* pv. phaseoli (14). We previously reported the lack of an adaptive response in *A. tumefaciens* to the organic hydroperoxide, tBOOH (33). In light of the physiological data concerning the role played by the *ohrR*/*ohr* system in organic hydroperoxide defense, the adaptive response of *A. tumefaciens* to CuOOH was investigated. The results indicate that preexposure to a low concentration (50 μM) of CuOOH conferred a 10-fold increase in resistance to subsequent exposure to killing concentrations (1, 5, and 10 mM) of CuOOH relative to uninduced cells (Fig. 2). Moreover, inactivation of either *ohr* or *ohrR* resulted in a complete loss of the CuOOH adaptive response (Fig. 2), indicating that the establishment of an adaptive response to CuOOH in *A. tumefaciens* requires the *ohr*/*ohrR* system.

Regulation of *ohrR* and *ohr* expression in response to stresses. Given the primary role played by Ohr in organic oxidant defense, studies were conducted to investigate the regulatory mechanism of the *ohrR*/*ohr* system in *Agrobacterium*. The expression patterns of genes involved in stress protection should correlate with their physiological roles. Thus, regulators of these genes must have mechanisms to sense and respond to changes in the levels of the appropriate stresses. In general, genes involved in stress protection are tightly regulated, and their expression is highly induced by stresses. In order to determine the regulatory pattern of *ohr* transcription, the levels of *ohr* mRNA were determined under uninduced and oxidant-induced growth conditions by Northern analysis. Compared to uninduced cultures, the levels of *ohr* mRNA in strain NTL4 markedly increased during growth in the presence of 200 μM tBOOH, 50 μM CuOOH, or 50 μM LOOH by 20-, 30-, or 15-fold, respectively, as determined by densitometry (Fig. 3A). The observed pattern of oxidant-induced *ohr* expression was similar to the pattern observed in several other microorganisms, where *ohr* expression was highly induced only by organic hydroperoxides (21–23) and is consistent with *ohr*'s proposed physiological role as the major protective system against organic hydroperoxide toxicity.

An increase in *ohr* expression upon exposure to an organic

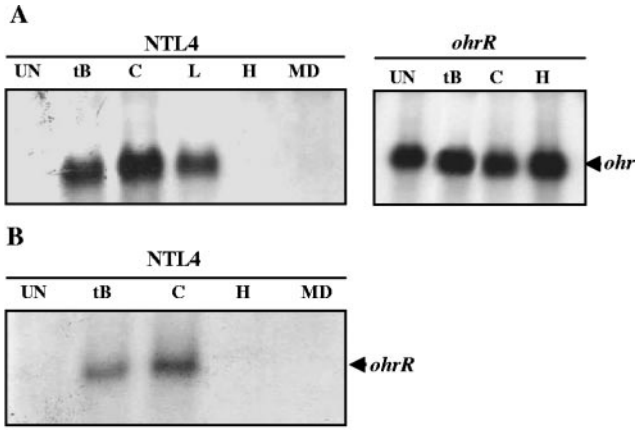

FIG. 3. Organic hydroperoxide-induced gene expression of *ohr* and *ohrR*. Northern blots of total RNA extracted from exponential-phase cultures of *A. tumefaciens* parental strain NTL4 and an *ohrR* mutant under uninduced conditions (UN) and after exposure to 200 μM tBOOH (tB), 50 μM CuOOH (C), 50 μM LOOH (L), 200 μM H_2O_2 (H), and 200 μM menadione (M) and then hybridized with a radioactively labeled *ohr* (A)- and *ohrR* (B)-specific probe.

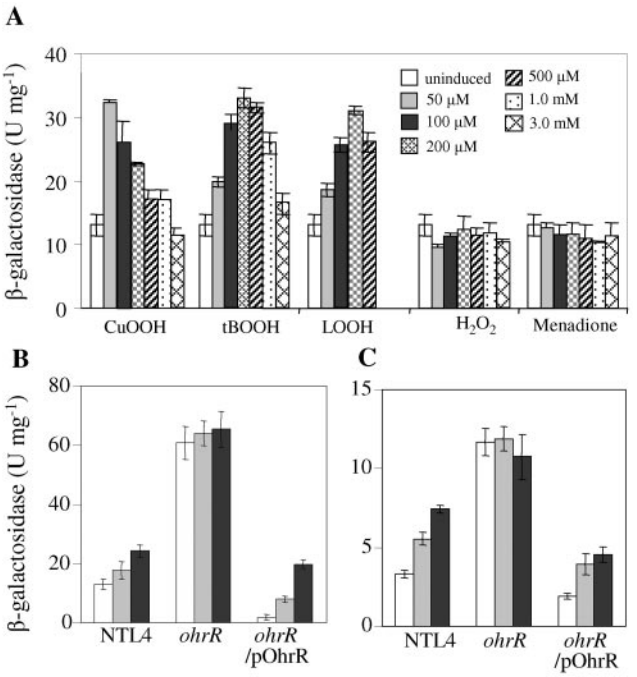

FIG. 4. In vivo characterization of the *ohr* and *ohrR* promoters. The β-galactosidase activity of exponential-phase cultures of *A. tumefaciens* strains, containing either an *ohr* or an *ohrR* promoter-*lacZ* transcriptional fusion plasmid, exposed to CuOOH, tBOOH, LOOH, H_2O_2, or menadione at various concentrations was determined. (A) *A. tumefaciens* harboring pP_{ohr}; (B) *A. tumefaciens* (NTL4), *A. tumefaciens ohr* mutant (*ohr*), and *A. tumefaciens ohr* mutant containing pOhrR (*ohr*/pOhrR) harboring pP_{ohr} exposed to tBOOH (▨), CuOOH (■), or unexposed (□). (C) Experiments were performed as described in panel B but with *A. tumefaciens* strains containing the *ohrR* promoter-*lacZ* fusion plasmid, pP_{ohrR}. Values are the means and the SD of four replicate experiments.

hydroperoxide (an Ohr substrate) would certainly contribute to bacterial survival under this stress condition. It should be noted that treatment of *A. tumefaciens* cultures with inorganic oxidants, including a superoxide generator (200 μM menadione), and 200 μM H_2O_2 failed to induce *ohr* expression (Fig. 3A). The lack of H_2O_2-induced expression of *ohr* is at odds with results in *Pseudomonas* and other bacteria showing that treatment with high concentrations of H_2O_2 resulted in low-level induction of *ohr* expression (22, 24), leading to the suggestion that *ohr* may also play some role in H_2O_2 protection (16). In these cases it is unclear whether the inducer is H_2O_2 or some by-product of H_2O_2 treatment such as organic hydroperoxides that are produced during exposure to high concentrations of H_2O_2. If *ohr* induction is due to the accumulation of by-products resulting from H_2O_2 exposure, then lack of induction in *A. tumefaciens* could be a reflection of the organism's ability to rapidly detoxify H_2O_2.

In other organisms, such as *Bacillus subtilis*, OhrR has been shown to be an organic hydroperoxide responsive repressor of *ohr* and *ohrR* transcription (10). Under reducing conditions the repressor is active and binds to the *ohr* and *ohrR* promoters. Exposure to organic oxidants renders the repressor incapable of DNA binding through the reversible oxidation of conserved cysteine residues (11). In order to assess the role of the peroxide-sensing repressor *ohrR* in regulating *ohr* expression, an *ohrR* insertion mutant was constructed and the mutation's effects on *ohr* transcription during uninduced and oxidant induced conditions were investigated. The results in Fig. 3A clearly demonstrate that *A. tumefaciens ohrR* is a repressor of *ohr* expression since its inactivation resulted in constitutively high expression of *ohr* that was unaffected by oxidant exposure. The expression analysis was extended to determine the pattern of oxidative stress induced expression of *ohrR*. *ohrR* expression was highly induced (10- to 15-fold) by treatments with the organic hydroperoxides, tBOOH and CuOOH, but not the superoxide generator menadione or H_2O_2 (Fig. 3B). Thus, *ohrR* shares a similar organic hydroperoxide inducible expression profile with *ohr*.

Further analysis of *ohr* regulation was done by using strains carrying *ohr* promoter-*lacZ* fusion constructs. A 363-bp fragment (PCR with BT536 and BT537 primers) containing the *ohr* promoter was transcriptionally fused to a promoterless *lacZ* in the promoter probe vector pUFR027*lacZ*, a derivative of pUFR027 (7) to yield pP_{ohr}. pP_{ohr} was then used to monitor *ohr* promoter activity in response to inducing concentrations of hydroperoxides and the superoxide generator, menadione in wild-type strain NTL4 and an *ohrR* mutant. The results shown in Fig. 4A mirror those of the Northern analyses and indicate that the organic hydroperoxides CuOOH, tBOOH, and LOOH were potent inducers of *ohr* promoter activity, with magnitudes of induction ranging from 2.5- to 3-fold. The increases in promoter activity appeared to be dose dependent in the low-dosage range (i.e., 200 μM and below) for tBOOH and LOOH. However, as the inducing concentrations of the various organic hydroperoxides increased, significant reductions in *ohr* promoter activity were observed (Fig. 4A). This was most likely due to organic hydroperoxide toxicity resulting in growth arrest and cell death.

It has recently been reported that expression of the *X. campestris ohrR*/*ohr* system is more responsive toward low con-

centrations of the complex organic hydroperoxide, LOOH, compared to the simple organic hydroperoxide, tBOOH (14). In *A. tumefaciens*, the *ohr* promoter was more responsive to the moderately complex hydroperoxide, CuOOH, than to either LOOH or tBOOH, suggesting that the relative sensitivity of regulatory system to respond to different organic hydroperoxides is organism specific. The differences in the sensitivity to the various organic hydroperoxide inducers between the two bacteria are probably due to differences in the structure of the OhrRs. *A. tumefaciens* and *X. campestris* pv. phaseoli OhrRs each contain a cysteine (Cys) residue at position 21 that is absolutely conserved among all OhrRs and has been shown to be required for sensing organic hydroperoxide (11, 25). *X. campestris ohrR* also contains additional Cys residues at positions 127 and 131, and there is evidence that Cys-127 interacts with Cys-21 during peroxide sensing (25a). *A. tumefaciens* OhrR lacks Cys-127, suggesting that the hydroperoxide sensing mechanisms of the *A. tumefaciens* and *X. campestris* proteins may be different. Minor differences in the efficiency of different types of organic hydroperoxides in inducing *ohr* could be advantageous to *A. tumefaciens* when it encounters mixtures of organic hydroperoxides of various toxicity. In any case, organic hydroperoxides were still much more efficient inducers of the system than either the inorganic oxidant H_2O_2 or the superoxide generator, menadione, regardless of concentration (Fig. 4A).

As expected, inactivation of *ohrR* resulted in *ohr* promoter activity that was constitutively high and unaffected by organic hydroperoxide treatments (Fig. 4B). Furthermore, *ohr* promoter activity in the *ohrR* mutant was twofold higher than the fully induced level observed in the wild-type strain (Fig. 4B), suggesting that, even under fully induced conditions, some OhrR probably still bound to the *ohr* promoter. This could provide additional fine-tuning of the expression of OhrR regulated genes. Finally, high-level expression of *ohrR* from an expression vector led to the repression of *ohr* promoter activity, and this effect could be negated by CuOOH treatment (Fig. 4B). This observation is consistent with the idea that OhrR acts as the transcription repressor of the *ohr* promoter.

The in vivo promoter analyses were extended to the *ohrR* promoter. The *ohrR* promoter activity was induced by organic hydroperoxide treatments, but was unaffected by either H_2O_2 or menadione treatment (data not shown). The pattern of sensitivity of the *ohrR* promoter to induction by organic hydroperoxides was similar to the pattern for the *ohr* promoter. CuOOH was the most potent inducer, followed by LOOH and tBOOH. The organic hydroperoxide inducibility of the *ohrR* promoter was lost in an *ohrR* mutant background with absolute levels of *ohrR* promoter activity that were higher than those in wild-type strain NTL4 (Fig. 4C). Moreover, complementation with plasmid-borne *ohrR* in pOhrR restored the normal pattern of hydroperoxide inducibility (Fig. 4C). These observations indicate that OhrR negatively autoregulated its own expression. Consistent with the results of the Northern blotting experiments (Fig. 3B), comparative analyses of induced *ohr* and *ohrR* promoter activities showed that the *ohr* promoter was the stronger of the two, with up to ninefold higher promoter activity under a given condition.

Mapping of regulatory elements within the *ohr* and *ohrR* promoters. As a first step in the characterization of both *ohrR* and *ohr* promoters, primer extension experiments were performed to determine the transcription start sites of both genes. The results in Fig. 5A show that *ohr* transcription initiates at a C residue, 21 bases upstream from the translation initiation codon. Immediately upstream of the *ohr* transcription start site were found *E. coli* RNA polymerase δ^{70}-like −10 (TATAAG) and −35 (TTGCGT) sequence elements that were separated by 17 bases (Fig. 5A). The transcriptional start site of *ohrR* was mapped to a G residue 81 bases upstream of the ATG codon. Examination of the region upstream of the transcription start also revealed the presence of *E. coli* RNA polymerase δ^{70}-like −10 and −35 sequence motifs TTGAAT and GATAAT, respectively, separated by 17 bases (Fig. 5A). Quantitative analysis of *ohr* and *ohrR* primer extension products indicated that transcription initiation from these promoters was highly induced by CuOOH (Fig. 5A). Thus, the increase in *ohr* and *ohrR* transcripts in response to CuOOH treatment detected in Northern experiments was due to increases in transcription initiation.

Genetic evidence indicates that *ohrR* regulates its own expression in addition to that of *ohr*. This fact, combined with the

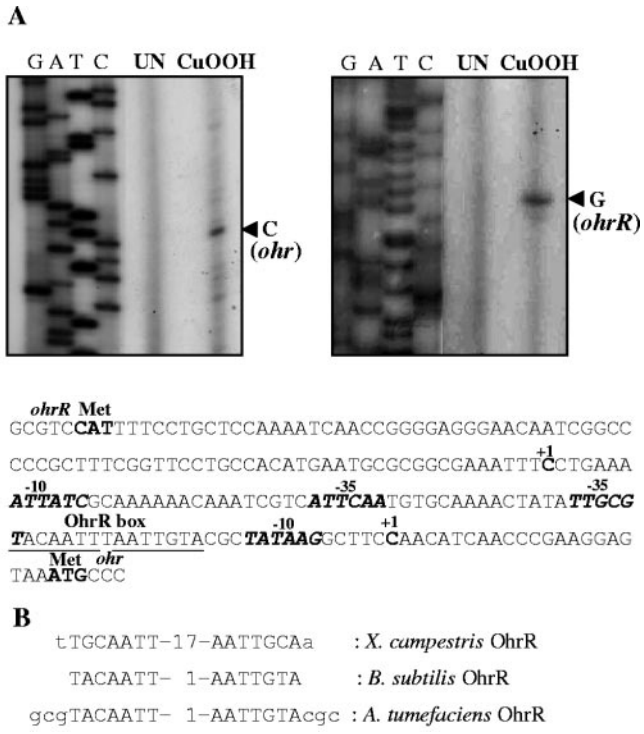

FIG. 5. Localization of *ohr* and *ohrR* promoters and alignment of OhrR binding box. (A) Primer extension of RNA extracted from uninduced (UN) and CuOOH-induced cultures. The experiment was performed with a ^{32}P-labeled oligonucleotide primer as described in Materials and Methods. The C, T, A, and G lanes of a dideoxy sequencing ladder using the same primer as that used for the primer extension are shown. The *ohr* and *ohrR* transcription start sites are marked by arrowheads in the primer extension autoradiographs and as "+1" in the accompanying sequence. Putative −35 and −10 regions are shown in boldface italics. The translation initiation codons (ATG) are in boldface. The putative OhrR box is underlined. (B) Alignment of putative OhrR binding sites from *X. campestris* (32), *B. subtilis* (10), and *A. tumefaciens*. The numbers indicate the number of intervening nucleotides.

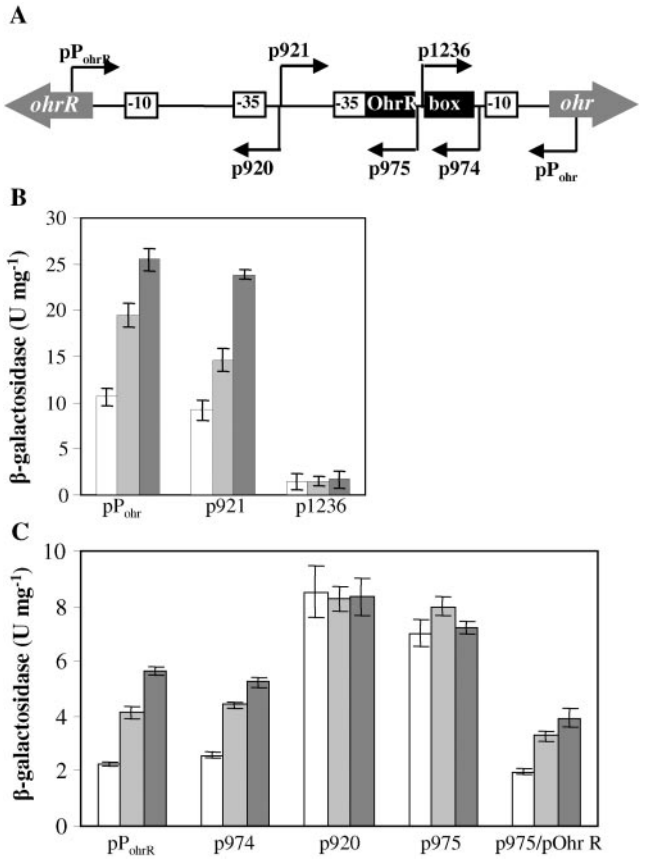

FIG. 6. *ohr* and *ohrR* promoter deletion analyses. (A) Map of the *ohr-ohrR* intergenic region showing the upstream end points of promoter fragments used to construct the various promoter-*lacZ* fusion plasmids; (B) β-galactosidase activity of *A. tumefaciens* harboring the *ohr* promoter-*lacZ* fusion pP_{ohr} or its deletions p921 and p1236; (C) β-galactosidase activity of *A. tumefaciens* harboring pP_{ohrR} or its deletions. p975/pOhrR represents *A. tumefaciens* containing p975 and carrying pOhrR for the expression of *ohrR*. Cells were cultured to exponential phase before induction with tBOOH (▨), CuOOH (■), or uninduced (□). Values are the means and SD from four replicate experiments.

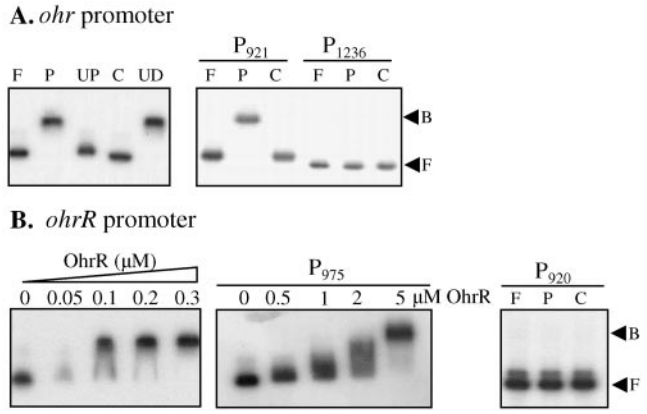

FIG. 7. OhrR binds the *ohr* and *ohrR* promoters. The results of DNA mobility shift assays with ^{32}P-labeled *ohr* (A) and *ohrR* (B) promoter fragments and purified OhrR. F, free probe; P, a reaction containing purified OhrR and labeled probe. UD and UP indicate reactions containing 2 μg of unrelated DNA (pBBR1MCS-4 plasmid) and 1 μg of unlabeled promoter, respectively. C, reactions in which CuOOH (1.0 mM) was added to the binding reaction. If not indicated, the amount of purified OhrR in the binding reaction was 0.3 μM. B, bound probe.

close proximity of the divergently transcribed *ohr* and *ohrR* promoters suggested that they might share regulatory sites. Examination of the *ohr-ohrR* intergenic region revealed the presence of the AT-rich inverted repeat sequence, 5′-gcgTAC AATTnAATTGTAcgc-3′ (uppercase letters indicate part of the conserved OhrR box), that was similar to the putative OhrR box sequence thought to be involved in the binding of OhrR to target promoters in *B. subtilis* and *X. campestris* (10, 32) (Fig. 5B). The inverted repeat was situated 19 bp upstream of the *ohrR* −35 promoter element and overlapped the region between the *ohr* −35 and −10 promoter elements (Fig. 5A), suggesting that a single OhrR box could be involved in the regulation of both the *ohr* and *ohrR* promoters.

In order to probe the function of the putative OhrR binding site, a number of promoter-*lacZ* transcriptional fusion plasmids were constructed that contained various amounts of sequence upstream of the *ohr* and *ohrR* promoters (Fig. 6A). The ability of each fusion to be induced by organic hydroperoxide treatments was tested in vivo. The results shown in Fig. 6B and C indicate that the OhrR box is necessary for normal organic hydroperoxide inducible regulation of both promoters. Deletion of the sequence upstream of position −55 (p921) in the *ohr* promoter had no appreciable effect on promoter function relative to the full-length control promoter (pP_{ohr}) (Fig. 6B). Deletion of the sequence upstream of −22, in p1236, that removed the upstream half of the putative OhrR binding box along with the −35 promoter element resulted in inactivation of the promoter (Fig. 6B). Thus, the *ohr* promoter resides in the region within 55 bp of the *ohr* transcription start containing the OhrR box and the −10 and −35 promoter elements.

A similar analysis of the *ohrR* promoter showed that a fusion (p974) containing 80 bp upstream of the *ohrR* transcription start and spanning the OhrR-box, as well as the −10 and −35 promoter elements, was regulated normally. Deletion of all or part of the Ohr box, in fusion plasmids p920 (deleted to position −36), and p975 (deleted to position −61), respectively, yielded expression patterns that were similar to that of the full-length *ohrR* promoter in an *ohrR* mutant, i.e., high-level constitutive expression that was unaffected by organic hydroperoxide (Fig. 4C and 6C). Taken together, the data indicate that the *ohrR* promoter was located within 80 bp of the *ohrR* transcription start and that the OhrR box was required for organic hydroperoxide dependent regulation. One interesting finding was the fact that overexpression of OhrR from plasmid pOhrR restored organic hydroperoxide-dependent regulation to the *lacZ* fusion plasmid p975 (Fig. 6C). Since this fusion contained only the proximal half of the OhrR box, the result suggested that OhrR could still bind to this site, albeit with a lower affinity than to the full OhrR box.

Binding of OhrR to the *ohr-ohrR* intergenic region. The direct interaction of OhrR with the *ohr* and *ohrR* promoters was tested by using purified *A. tumefaciens* OhrR and a 363-bp DNA fragment spanning the *ohr-ohrR* intergenic region, that contained the putative OhrR binding box, using gel mobility shift assays. OhrR specifically bound to the intergenic region

since binding was blocked by the addition of excess unlabeled probe fragment (UP) but not by nonspecific competitor DNA, pBBR1MSC-4 (UD) (Fig. 7A). The genetic and physiological analyses reported in the present study indicate that the likely role of OhrR is as a sensor of organic hydroperoxide. More direct evidence of this was obtained when the organic hydroperoxide CuOOH was added to the gel mobility shift reactions containing purified OhrR and the 363-bp intergenic region probe (Fig. 7A). The addition of CuOOH to the binding reaction leads to the loss of OhrR binding to its target site (Fig. 7A). This is consistent with the proposed mechanism of OhrR sensing of organic hydroperoxide in which oxidation of a sensing Cys residue(s) leads to inactivation of the repressor that, in turn, allows RNA polymerase to bind to the promoter and activate transcription (11, 19, 25). In light of both the in vivo and in vitro data, it is clear that *A. tumefaciens* OhrR has evolved to sense and respond to organic hydroperoxide.

Similar gel mobility shift experiments were performed with deleted OhrR promoter fragments spanning either all (p920) or part (p975) of the OhrR box. Consistent with the *lacZ* fusion results, no binding of OhrR to fragment p920 was detected. However, fragment p975, containing half of the OhrR box, was still bound by OhrR (Fig. 7B). This was in good agreement with the *lacZ*-fusion results with p975, where hydroperoxide inducibility of this promoter deletion was restored when OhrR was expressed at high levels from plasmid pOhrR (Fig. 6C). This implies that OhrR binds to the target half-site in the proper configuration and retains its function.

Finally, precise localization of the OhrR binding site within the *ohr-ohrR* intergenic region was accomplished by DNase I footprinting (Fig. 8). OhrR protected a region a 49-bp region from positions −6 to −54 relative to the *ohr* transcription start. The extent of protection was typical of previously mapped OhrR binding sites in *B. subtilis* and *X. campestris* (10, 20) and indicates that OhrR binding represses expression of both genes by covering the −10 and −35 elements of the *ohr* promoter, as well as the −35 region of the *ohrR* promoter. Given the data presented here, it seems reasonable to assume that maximal repression requires the binding of multiple OhrRs within this region. The binding of a single OhrR dimer to the high-affinity consensus *ohr*-box could function as a nucleation site for the cooperative binding of additional dimmers that would further stabilize the complex. Such a scenario might allow for the fine-tuning of *ohr* expression under conditions in which organic hydroperoxide levels are low and full derepression of *ohr* is not required.

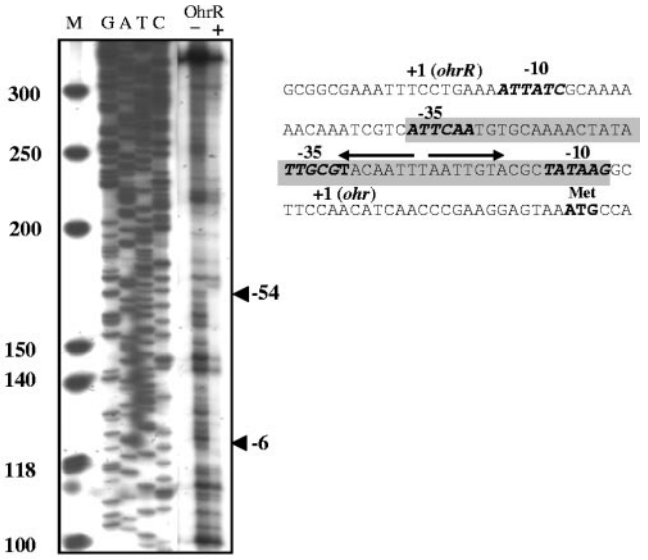

FIG. 8. DNase I protection assay of OhrR binding to the *ohr-ohrR* intergenic region. The results of a DNase I footprinting assay using purified OhrR and a ^{32}P-labeled probe spanning the *ohr-ohrR* intergenic region are presented. The minus sign (−) represents the probe fragment treated with DNase I in the absence of OhrR. The plus sign (+) represents the probe fragment treated with DNase I in the presence of OhrR. Arrowheads and numbers indicate the limits of the protected sites and their corresponding position relative to the *ohr* transcription start (+1). The sequence of the *ohr-ohrR* intergenic region is also shown in which the OhrR protected region is shaded. Divergent arrows indicate the putative OhrR box. The −10 and −35 regions of *ohr* and *ohrR* promoters are shown in boldface.

Evaluation of the physiological and biochemical role of putative genes encoding organic hydroperoxide scavenging enzymes other than *ohr*. The objective of the investigation was to evaluate the roles of various genes encoding putative organic hydroperoxide-metabolizing enzymes in protecting *A. tumefaciens* from organic hydroperoxide exposure. The physiological analyses of the *ohrR*/*ohr* system mutants clearly indicate that this system is the major organic hydroperoxide defense system in *Agrobacterium*; however, it should be noted that the *ohr* mutant still retained a significant capacity to degrade CuOOH, suggesting that other enzymes are also involved in the process. BLAST algorithm (2) searches of the annotated genome of *A. tumefaciens* (35) identified at least six predicted ORFs that had a high degree of sequence similarity to enzymes that have been shown to be involved in organic hydroperoxide metabolism in other organisms. These ORFs could be grouped into either the peroxiredoxin (TSA/AhpC) or Ohr families (3, 13, 16, 21). The ORFs belonging to the peroxiredoxin family were *prx1* (peroxiredoxin, Atu1480), *prx2* (Atu0779), *prx3* (Atu2399), *bcp1* (bacterioferritin comigratory protein, Atu1830), and *bcp2* (Atu3655). The deduced amino acid sequence of *prx1* is 20% identical to that of *E. coli ahpC*, whereas *prx2* and *prx3* are more similar to the atypical 2-cysteine peroxiredoxin, *prxS*, of *Rhizobium etli* (8) with sequence identities of 32 and 75%, respectively. This is in contrast to the 9% sequence identity between *prx1* and *prxS*. *A. tumefaciens bcp1* and *bcp2* are 40 and 26% identical, respectively, to *E. coli bcp*, while the deduced amino acid sequence of *A. tumefaciens ohr* (Atu0847), a member of the Ohr family of thiol peroxidases, is 51% identical to that of *X. campestris ohr* (21).

As an initial step toward understanding the physiological function of these genes in protecting *A. tumefaciens* from lethal doses of organic hydroperoxides, mutants lacking a functional copy of either; *prx1*, *prx2*, *prx3*, *bcp1*, or *bcp2* were constructed by insertional inactivation using the pKNOCK system (1). The resistance levels of these mutants to organic hydroperoxides were determined by using both growth inhibition zone and a more sensitive plate sensitivity assays and compared to those of the wild-type strain NTL4 and the *ohr* mutant. Only the *ohr* mutant showed increased sensitivity toward CuOOH and tBOOH, whereas none of the single peroxiredoxin mutants showed any change in resistance relative to wild-type NTL4

(data not shown). It was possible that *prx1*, *prx2*, *prx3*, *bcp1*, and *bcp2* played minor roles in organic hydroperoxide resistance such that expression of the Ohr system masked the effects of mutations in these genes. Thus, double mutants were constructed in which *ohr* was inactivated along with either *prx1*, *prx2*, *prx3*, *bcp1*, or *bcp2*. Each of the double mutants showed resistance levels to tBOOH and CuOOH that were similar to those of the *ohr* mutant (data not shown).

Another approach used to evaluate the in vivo function of the putative organic hydroperoxide protective genes was to test whether high-level expression of plasmid-borne *prx1*, *prx2*, *prx3*, *bcp1*, or *bcp2* affected the organic hydroperoxide-sensitive phenotype of an *ohr* mutant background. Each of the genes was cloned into pBBR1MSC-4 to create pPrx1, pPrx2, pPrx3, pBcp1 pBcp2, and pOhr (see Materials and Methods). Each plasmid was introduced into an *A. tumefaciens ohr* mutant, and the organic hydroperoxide resistance levels were determined. As expected, pOhr restored the CuOOH resistance level of an *ohr* mutant to that of wild type (data not shown). In contrast, expression of plasmid-borne *prx1*, *prx2*, *prx3*, *bcp1*, or *bcp2* did not alter the CuOOH resistance level of the *ohr* mutant strain (data not shown). Hence, *prx1*, *prx2*, *prx3*, *bcp1*, or *bcp2*, individually, are unlikely to play important roles in the protection of *A. tumefaciens* from organic hydroperoxide toxicity under the conditions tested.

It is possible that some of these gene products could contribute to organic hydroperoxide degradation; however, their contributions might not be sufficient to confer significant resistance to the lethal concentrations of organic hydroperoxide used in the study. In order to detect more subtle changes in the capacity to detoxify organic hydroperoxides, the effects of either gene inactivation or overexpression, on a strain's ability to degrade organic hydroperoxide, were determined. The *ohr* single and *ohr prx1* and *ohr bcp1* double mutants were incubated with CuOOH and the rate of hydroperoxide degradation was determined. These genes were initially chosen for further analysis due to the fact that homologs of both *prx1* and *bcp1* had been shown to be involved in organic hydroperoxide resistance in other bacteria (13, 36). As previously stated, wild-type strain NTL4 rapidly metabolized CuOOH, whereas only the *ohr* mutant showed a significant reduction in the ability to metabolize CuOOH that could be complemented by the introduction of plasmid-borne *ohr* in pOhr (Fig. 1). The double mutants, i.e., the *ohr prx1* and *ohr bcp1* mutants, had rates of CuOOH degradation that were similar to that of the *ohr* single mutant (Fig. 1).

The CuOOH degradation assay was also used to assess the effects of overexpression of genes, putatively involved in organic hydroperoxide metabolism, on an *ohr* mutant's ability to degrade CuOOH. The expression plasmids were transformed into *ohr* mutant, and the transformant's ability to degrade CuOOH was determined. Overexpression of *prx1*, *prx2*, *prx3*, *bcp1*, or *bcp2* in an *ohr* mutant did not significantly alter the rate of CuOOH degradation (data not shown).

It should be noted that the *ohr* mutant still retained a significant capacity to degrade CuOOH, suggesting that other enzymes are also involved in the process. Obvious candidates for this role were the peroxiredoxin homologs encoded by *prx1*, *prx2*, *prx3*, *bcp1*, and *bcp2*. However, inactivation of each of these genes had no effect on the ability of the bacterium to either resist lethal exposure to CuOOH or to degrade CuOOH present in the culture medium. Although participation of these genes in organic hydroperoxide metabolism cannot be ruled out, it is likely that other, as yet unidentified, enzymes are responsible for the residual CuOOH degradation observed in the *ohr* mutant

The genetic and physiological data clearly indicate that *ohr* is the major protective system against organic hydroperoxide stress. The finding that *prx1*, *prx2*, *prx3*, *bcp1*, and *bcp2* did not participate in organic hydroperoxide resistance was surprising. This was especially true for *A. tumefaciens prx1* that encodes an AhpC (alkyl hydroperoxide reductase) homologue. AhpC is a structurally and functionally conserved hydroperoxide-metabolizing enzyme that has been shown to be involved in organic hydroperoxide resistance in other bacteria (26). It remains to be seen whether some of these genes might have functions under specific conditions. Alternatively, it is possible that the genes may have overlapping functions such that phenotypic effects would only be seen in multiple mutants.

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