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Novel Roles of *ohrR-ohr* in *Xanthomonas* Sensing, Metabolism, and Physiological Adaptive Response to Lipid Hydroperoxide

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Lipid hydroperoxides are highly toxic to biological systems. Here, the *Xanthomonas campestris* pv. phaseoli sensing and protective systems against linoleic hydroperoxide (LOOH) were investigated by examining the phenotypes, biochemical and regulatory characteristics of various *Xanthomonas* mutants in known peroxide resistance pathways. Analysis of LOOH resistance levels indicates that both alkyl hydroperoxide reductase (AhpC) and organic hydroperoxide resistance enzyme (Ohr) have important and nonredundant roles in the process. Nonetheless, inactivation of *ohr* leads to a marked reduction in LOOH resistance levels. The regulatory characteristics of an *ohr* mutant add further support to its primary role in LOOH protection. Northern analysis shows that LOOH had differential effects on induction of *ahpC* and *ohr* expression with the latter being more sensitive to the inducer. Analysis of the *ahpC* and *ohr* promoters confirmed that the LOOH-dependent induction of these promoters is mediated by the transcription regulators OxyR and OhrR, respectively. Using the in vivo promoter assays and the in vitro gel mobility shift assay, we show that LOOH directly oxidized OhrR at the sensing residue Cys-22 leading to its inactivation. In addition, physiological analysis shows that pretreatment of *X. campestris* pv. phaseoli with a sublethal dose of LOOH induced high levels of resistance to subsequent exposure to lethal concentrations of LOOH. This novel LOOH-induced adaptive response requires a functional *ohrR-ohr* operon. These data illustrate an important novel physiological role for the *ohrR-ohr* system in sensing and inactivating lipid hydroperoxides.

During normal growth *Xanthomonas* spp. are exposed to harmful reactive oxygen species (ROS) including H_2O_2, organic peroxide, and superoxide anions generated from other soil organisms and as a part of active plant defense responses. Lipid hydroperoxides are important components of the ROS produced during the plant defense response (8), and are both highly reactive and toxic to bacterial cells. Plant lipoxygenases catalyze the formation of fatty acid hydroperoxides through the reaction of fatty acid precursors such as linoleic or linolenic acids with molecular oxygen (3, 8). The expression of these enzymes has been shown to be induced in response to microbial invasion and has been linked to the plant microbial defense response (10). Consequently, in order to survive and proliferate during infection, invading bacteria must detoxify lipid hydroperoxides.

To date, very little is known regarding how bacteria protect themselves from fatty acid hydroperoxides. The best-characterized bacterial system for the detoxification of organic hydroperoxides is the alkyl hydroperoxide reductase (AhpC). AhpC catalyzes the reduction of organic peroxides to their corresponding alcohols (24). In many bacteria, inactivation of *ahpC* results in increased sensitivity to organic peroxides and pleiotropic alterations in the oxidative stress response (2, 20, 27, 31, 32). A second system for organic hydroperoxide protection, designated *ohr*, has been discovered in *Xanthomonas* (19). *ohr* confers resistance to organic hydroperoxides, and inactivation of the gene leads to increased sensitivity to organic peroxides (19). *ohr* homologues are widely distributed in both gram-positive and gram-negative bacteria (1, 6, 12, 22, 25, 28). The structure and biochemical mechanism of Ohr have been elucidated (12). Ohr is a thiol peroxidase that catalyses the reduction of an organic hydroperoxide to its corresponding organic alcohol (4). AhpC and Ohr appear to have similar biochemical properties and possibly overlapping physiological functions. The genes are independently regulated. *ahpC* is regulated by OxyR (14, 32), whereas *ohr* is controlled by the transcription repressor OhrR (16). In *Xanthomonas*, AhpC and Ohr were shown to have slightly different organic peroxide substrate preferences (30). Recently, the thiol peroxidases, bactoferritin comigratory protein (BCP), and glutathione peroxidases (Gpx-like) have been reported to contribute to the protection of bacteria from organic peroxide (7, 9). However, the corresponding genes are either found only in a few bacteria and are not well characterized or they have highly specialized physiological roles. Thus, their general role in the protection of bacteria from organic peroxide has yet to be elucidated.

Here, we examined the physiological and biochemical roles of AhpC and Ohr in the protection against lipid hydroperoxide toxicity. The results of the study demonstrate the importance of the *ohrR-ohr* system in the ability to tolerate lipid hydroperoxides and revealed a novel bacterial adaptive response to lipid hydroperoxide exposure. (Parts of this work are from the dis-

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Different sensitivity to LOOH in *ahpC* and *ohr* mutants. Many organic hydroperoxide-metabolizing systems have been studied in bacteria; however, these studies have not addressed the integral roles of gene regulation and bacterial physiology in these defense systems (7, 9, 12, 17, 24, 27, 30). Thus, a growth inhibition zone assay (19) was used to measure the sensitivity to LOOH (prepared as described by Evans et al. [5]) of wild-type *Xanthomonas campestris* pv. phaseoli and various *Xanthomonas* strains. Wild-type *X. campestris* pv. phaseoli was highly resistant to LOOH and exhibited no zone of growth inhibition when exposed to 50 mM LOOH. However, mutants in which the *ahpC* (17) and *ohr* (19) genes were inactivated gave zones of growth inhibition of 6 and 12 mm, respectively. In the double mutant, a zone of inhibition of 16.5 mm was observed. At present, the mechanism responsible for uptake of LOOH is not known. At high concentrations of LOOH diffusion is thought to contribute to the uptake process while at low concentrations of LOOH, the energy-dependent fatty acid uptake system could be involved (21).

We extended these studies to determine the ability of *ahpC1*, *ohr*, and *ahpC ohr* mutants to metabolize LOOH using the Fox assay as described by Ochsner et al. (22) and Shea and Mulks (28). Exponential-phase cultures (optical density at 600 nm [OD_{600}] of 0.5) of the parental strain, *ahpC1*, *ohr*, and *ahpC1 ohr* mutants were incubated with 200 μM LOOH, and the amount of LOOH remaining after a 30-min incubation was determined. The results mirrored the resistance studies in that both *ahpC1* and *ohr* single mutants displayed a decreased ability to metabolize LOOH, with the *ohr* mutant showing the higher degree of impairment, while an *ahpC1 ohr* double mutant was less able to metabolize LOOH than either of the single mutants (data not shown). The ability of the *ahpC1 ohr* double mutant to metabolize LOOH could be restored to levels that were equal to or greater than that of wild type by the overexpression of plasmid-borne *ahpC* and *ohr*, respectively (data not shown), indicating that both enzymes could use LOOH as a substrate. The data suggest that the two systems act through independent pathways with *ohr* being the major protective system and *ahpC* playing a secondary backup role in protecting *Xanthomonas* from LOOH. A possible explanation for this observation could be due to a difference in the cellular locations of the two enzymes. Ohr is structurally related to OsmC, a putative thiol peroxidase that is localized in the periplasmic space (12, 13), and initial studies in our laboratory have shown that Ohr is found in both the periplasm and the cytoplasm (S. Mongkolsuk et al., unpublished observation). By contrast, AhpC is likely to be a cytoplasmic protein (24). Thus, periplasmic Ohr could detoxify LOOH before it entered the cytoplasm, thereby limiting damage to intracellular macromolecules.

LOOH induced the expression of *ahpC* and *ohr*. The LOOH-dependent regulation of *ahpC* and *ohr* is of particular interest, due to the fact that the genes are regulated by different global peroxide-sensing transcriptional regulatory systems and display different patterns of oxidant-induced expression (14, 29). Thus, the effect of treatment with LOOH or the synthetic organic hydroperoxide, *tert*-butyl hydroperoxide (tBOOH), on the expression of these genes in *X. campestris* pv. phaseoli was investigated using Northern blot hybridization analysis. It was found that LOOH was a strong inducer of *ohr* expression. *ohr* was induced by exposure to 10 μM LOOH whereas a similar treatment with 10 μM tBOOH did not induce expression of the gene (Fig. 1A). As inducing concentrations of LOOH increased, there was a parallel increase in the magnitude of induction of *ohr* expression that reached a maximum level of 80-fold (as determined by densitometry), relative to the level in uninduced cells, following treatment with 100 μM LOOH (Fig. 1A). *ohr* expression was also induced by tBOOH, but to a lesser degree (Fig. 1A). Treatment with 100 μM tBOOH induced *ohr* expression by less than 10-fold. When *ahpC* expression was examined, the situation was reversed. As was the case with *ohr*, both peroxides were able to induce *ahpC* expression.

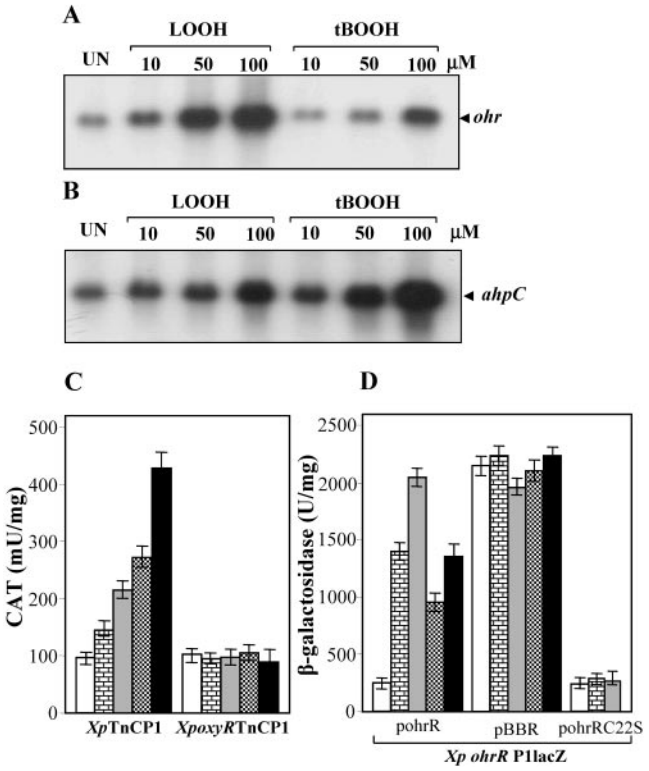

FIG. 1. Induction of *ahpC* and *ohr* expression in *X. campestris* pv. phaseoli by LOOH and tBOOH. Total RNA (10 μg) prepared from *X. campestris* pv. phaseoli cultures induced with 10, 50, 100 μM LOOH; 10, 50, 100 μM tBOOH; and uninduced cells (UN) were loaded into each lane and hybridized with either ^{32}P-labeled *ohr* (A) or *ahpC* (B) probes as previously described (30). The numbers above each lane in both panels A and B indicate the concentrations of peroxide added to the cultures. (C) Induction of the *ahpC* promoter fused to *cat* was monitored by determination of chloramphenicol acetyltransferase (CAT) activity (26) in *X. campestris* pv. phaseoli TnCP1 (*Xp* TnCP1) and an *oxyR* mutant containing TnCP1 (*Xp oxyR* TnCP1). (D) Induction of the *ohrR* P1 promoter fused to *lacZ* was monitored by determining β-galactosidase activity (15) in an *ohrR* mutant containing a P1 *lacZ* fusion (*Xp ohrR* P1lacZ) harboring pohrR, pBBR1MCS-5 (11) (pBBR), and pohrRC22S. For experiments in both panels C and D, exponential-phase cultures were untreated (open bars) or treated with LOOH (100 μM, brick bars, and 200 μM, gray bars), or tBOOH (100 μM, checkered bars, and 200 μM, black bars) for 30 min. Crude lysate preparation and enzymatic assays were performed as previously described (23). The CAT- or β-galactosidase-specific activities from induced cultures and uninduced cultures are shown.

However, tBOOH was the more effective of the two. Treatment with 100 μM tBOOH produced an 80-fold induction in *ahpC* expression levels compared to a 30-fold increase in the *ahpC* levels following treatment with 100 μM LOOH (Fig. 1B). The data clearly showed that the regulation of *ohr* responded more sensitively to the complex organic hydroperoxide, LOOH, than to the simple organic hydroperoxide molecule, tBOOH. By contrast, induction of *ahpC* expression was more sensitive to tBOOH than to LOOH treatments.

We extended these observations by determining the effect of LOOH and tBOOH on the transcription of *ahpC* and *ohrR* by monitoring the promoter activities of these genes using strains containing transcriptional fusions of the *ahpC* promoter with chloramphenicol acetyltransferase (*cat*) (*Xp* TnCP1 [14]) and the *ohrR* P1 promoter with β-galactosidase (*Xp ohrR* P1lacZ [23]) that were constructed by insertion of the reporter gene cassette within the chromosomal copy of *ahpC* or *ohrR*. The results reinforced those of the Northern blot analyses in demonstrating that the *ahpC* promoter was more efficiently induced by tBOOH. Treatment of *Xp* TnCP1 with 200 μM tBOOH resulted in a 4.5-fold increase in *ahpC* promoter activity, relative to an uninduced culture, compared with only a 2.2-fold increase in the presence of 200 μM LOOH (Fig. 1C). Furthermore, induction of the *ahpC* promoter by either organic peroxide depended on the presence of functional OxyR since no induction of the *ahpC* promoter was observed in an *oxyR* mutant background (*Xp oxyR* TnCP1) (Fig. 1C). Analysis of the hydroperoxide dependent induction of *ohrR* P1 promoter activity was complicated by the fact that the *lacZ* reporter gene insertion in this strain inactivates *ohrR*, encoding the *ohr* repressor (23). Thus, it was necessary to first complement this strain with a plasmid-borne copy of *ohrR* (pohrR) (18). As expected, LOOH was more efficient at inducing *ohrR* P1 promoter activity than tBOOH. Treatment of the strain containing the *ohrR* P1*lacZ* fusion (*Xp ohrR* P1lacZ harboring pohrR) with 100 and 200 μM LOOH induced P1 promoter activity by 6.8- and 9.7-fold, respectively, while treatment with the same concentrations of tBOOH resulted in respective increases in P1 promoter activity of 4.5- and 6.4-fold (Fig. 1D). The induction of the P1 promoter was found to be dependent on the presence of functional OhrR since the uncomplemented *ohrR* mutant strain (pBBR) did not show hydroperoxide-specific induction of the P1 promoter (Fig. 1D). The in vivo promoter fusion data supported the Northern blot results and confirmed that the observed increases in the levels of *ahpC* and *ohr* mRNA, in response to organic hydroperoxide treatments, were due to increased rates of *ahpC* and *ohr* transcription. Furthermore, the data show that in the presence of LOOH and tBOOH, the *ahpC* and *ohrR* promoters are induced by separate peroxide sensing regulatory systems. It appears that both OxyR and OhrR can sense changes in lipid hydroperoxide levels with the latter being more sensitive to the presence of the more complex hydroperoxide, LOOH, while OxyR is more sensitive to the simple organic hydroperoxide molecule, tBOOH.

In *Xanthomonas* the mechanism of organic hydroperoxide-dependent derepression of *ohr* transcription is thought to proceed via the oxidation of the highly conserved peroxide sensing cysteine residue, Cys-22, of OhrR (23). In order to test whether Cys-22 is required for LOOH inactivation of OhrR, a plasmid carrying a copy of the mutant *ohrR* (pohrRC22S), in which Cys-22 has been changed to serine (C22S), was transformed into *Xp ohrR* P1lacZ and the ability of LOOH to induce the P1 promoter in this strain was evaluated. The results showed that LOOH-dependent induction of the P1 promoter was abolished in *Xp ohrR* P1lacZ harboring pohrRC22S (Fig. 1D). This indicates that residue Cys-22 of OhrR is essential for LOOH-dependent derepression of the P1 promoter. This favors the idea that in vivo, LOOH or its metabolites mediate the oxidation of residue Cys-22 thus inactivating OhrR.

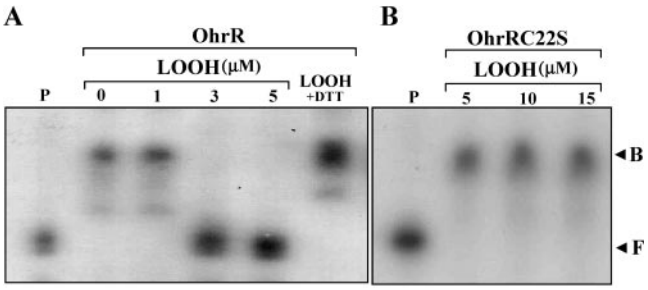

FIG. 2. The effect of LOOH and DTT on OhrR binding to the *ohrR-ohr* promoter. The results of DNA mobility shift experiments, testing oxidation of OhrR by LOOH. The DNA binding reaction contained ^{32}P-labeled P1, the *ohrR-ohr* promoter fragment (18), 3 fmol of purified OhrR or purified OhrRC22S (23). The binding reactions and electrophoresis were performed as previously described (23). In panel A, the binding reactions containing the P1 promoter fragment and OhrR were treated with either various concentrations of LOOH or 5 μM LOOH followed by 10 mM DTT treatment (LOOH+DTT) as previously described (23). In panel B the binding of P1 fragment to 3 fmol of purified OhrR C22S before 5, 10, and 15 μM LOOH were added to the binding reactions. The numbers above each lane indicate the concentration of LOOH added. P indicates free probe. The positions of bound (B) and free (F) probes are indicated.

LOOH oxidizes and inactivates OhrR binding to the promoter. In vivo experiments suggested that LOOH or its metabolites probably oxidized OhrR at Cys-22, but the experiment could not provide a definitive answer regarding the mechanism of LOOH sensing. Thus, gel mobility shift experiments were performed to further characterize the LOOH-sensing mechanism of OhrR. First purified OhrR and OhrRC22S (23) were incubated with a radioactively labeled 170-bp P1 promoter fragment in the presence and absence of LOOH. In the absence of LOOH, OhrR strongly bound to the P1 promoter fragment as shown by the slower-migrating P1 promoter fragment OhrR complex (Fig. 2A). Addition of 3 μM LOOH to the binding reaction completely negated OhrR binding to the P1 promoter fragment. The concentration of LOOH required to completely inhibit the binding of OhrR to the P1 promoter was 100-fold lower than that previously determined for tBOOH (18). Next, we tested whether the inactivation of OhrR by LOOH was due to direct oxidation of the protein by assessing whether the process could be reversed by treatment with a reducing agent (dithiothreitol [DTT]) and determining the effect of LOOH on a nonsensing mutant protein OhrRC22S. The results show that 10 mM DTT reversed the inhibitory effects of LOOH on the binding of OhrR to the P1 promoter (Fig. 2A). In addition, the mutant OhrRC22S had no binding defect as shown by its ability to efficiently bind to the promoter fragment at a similar concentration as wild-type OhrR (Fig. 2B). However, treatment of OhrRC22S with in-

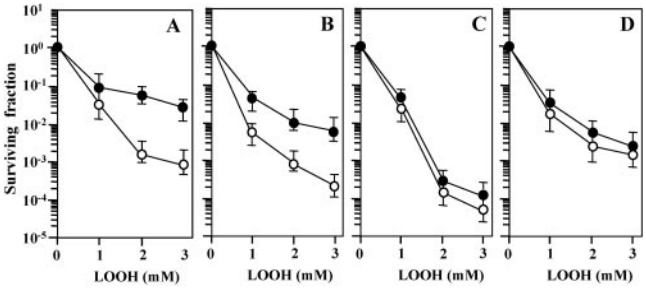

FIG. 3. Induced adaptive protection responses to LOOH in *X. campestris* pv. phaseoli, *ohr* and *ohrR* mutants. The results of LOOH-induced adaptive protection response experiments testing the effect of a 30-min preexposure to 50 μM LOOH on the survival of *X. campestris* pv. phaseoli exponential-phase cultures to subsequent exposure to 0, 1, 2 or 3 mM LOOH for 30 min. Plots of the surviving fraction of cells in cultures that did (●) or did not (○) receive a pretreatment are shown. (A) *Xp*, *X. campestris* pv. phaseoli; (B) *ahpC1* mutant; (C) *ohr* mutant; (D) *ohrR* mutant. Each value presented is the mean and standard deviation of four replicates.

creasing concentrations of LOOH had no effect on the mutant protein's ability to bind to the P1 promoter (Fig. 2B). The results support the idea that LOOH inactivated OhrR through the direct oxidation of the sensing Cys-22 residue. The available in vitro DNA binding data from this and previous studies (18) support the in vivo promoter analyses in showing that OhrR is 80-fold more responsive to the complex hydroperoxide, LOOH, than to the simple hydroperoxide, tBOOH. This favors the idea that OhrR may have evolved to preferentially sense complex organic hydroperoxides such as lipid hydroperoxides via oxidation of the highly conserved peroxide sensing residue Cys-22. The in vivo and in vitro regulatory characteristics of the *ohrR-ohr* operon support its role as the major system for the sensing of and protection from lipid hydroperoxides such as LOOH.

The novel physiological LOOH adaptive response required functional *ohrR* and *ohr*. The adaptive response is an important strategy for microbial survival under stressful conditions; however, an adaptive response to lipid peroxide has not been reported previously. Experiments were done to test if *Xanthomonas* has the capacity to mount an adaptive response to LOOH and whether *ahpC* and *ohr* are involved in the process. *Xanthomonas* cultures that had been pretreated with 50 μM LOOH were exposed to lethal concentrations (1, 2, 3 mM) of LOOH for 30 min and the fraction of surviving cells was determined. The results in Fig. 3A show that LOOH induced cells were 50-fold more resistant to LOOH killing than uninduced cells. This is the first demonstration of a bacterial adaptive response to a lipid hydroperoxide. Similar experiments were then performed using *ohr* and *ahpC* mutants in order to determine the roles of *ohr* and *ahpC* in the LOOH adaptive response. Pretreatment of an *ahpC1* mutant with LOOH induced high-level resistance to subsequent LOOH killing (Fig. 3B). By contrast, a similar preexposure of the *ohr* mutant to LOOH failed to induce increased protection, relative to uninduced cells, against subsequent LOOH killing treatments (Fig. 3C). Clearly, *ohr*, but not *ahpC*, is required for the LOOH adaptive response. We extended the investigation by determining whether proper regulation of *ohr* or simply the presence of functional *ohr* was required for the LOOH adaptive response. In the previous section, we showed that the transcription repressor, OhrR, was involved in LOOH-dependent induction of *ohr*. We therefore tested whether OhrR was also the regulator involved in the LOOH adaptive response. The LOOH adaptive response experiment was repeated using the *ohrR* mutant. As expected, pretreatment of the *ohrR* mutant with LOOH did not induce adaptive protection against subsequent LOOH killing (Fig. 3D) indicating that proper regulation of the operon is required for the LOOH-induced adaptive response. Loss of the induced adaptive protection in *ohrR and ohr*, mutants, but not in *ahpC1* mutant is consistent with the data from the physiological, and gene regulation analyses indicating that the *ohrR-ohr* system plays the major role in protecting *Xanthomonas campestris* pv. phaseoli from LOOH.

An important physiological question is whether *Xanthomonas* is likely to be exposed to LOOH in its natural environment. *Xanthomonas* spp. are important bacterial phytopathogens. During plant microbe interactions, bacteria are likely to be exposed to lipid hydroperoxide produced by plants as part of an active defense response against microbial invasion. It has been shown that increased lipoxygenase, an enzyme involved in lipid hydroperoxide synthesis, is associated with the plant defense response and fatty acid precursors such as linoleic or linolenic acids are abundant in plants (3, 8). Thus, *Xanthomonas* is likely to encounter LOOH during its interaction with host plants. Interestingly, *ohr* homologues have been found in all genomes of bacterial plant pathogens thus far sequenced (Mongkolsuk et al., unpublished observation). This conservation of *ohr* implies its important physiological role in the protection against lipid hydroperoxide exposure during plant-microbe interactions.

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