JOURNAL OF BACTERIOLOGY, May 1998, p. 2636–2643 Vol. 180, No. 10
0021-9193/98/$04.00+0
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Identification and Characterization of a New Organic Hydroperoxide Resistance (*ohr*) Gene with a Novel Pattern of Oxidative Stress Regulation from *Xanthomonas campestris* pv. phaseoli

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Received 12 December 1997/Accepted 9 March 1998

We have isolated a new organic hydroperoxide resistance (*ohr*) gene from *Xanthomonas campestris* pv. phaseoli. This was done by complementation of an *Escherichia coli* alkyl hydroperoxide reductase mutant with an organic hydroperoxide-hypersensitive phenotype. *ohr* encodes a 14.5-kDa protein. Its amino acid sequence shows high homology with several proteins of unknown function. An *ohr* mutant was subsequently constructed, and it showed increased sensitivity to both growth-inhibitory and killing concentrations of organic hydroperoxides but not to either H_2O_2 or superoxide generators. No alterations in sensitivity to other oxidants or stresses were observed in the mutant. *ohr* had interesting expression patterns in response to low concentrations of oxidants. It was highly induced by organic hydroperoxides, weakly induced by H_2O_2, and not induced at all by a superoxide generator. The novel regulation pattern of *ohr* suggests the existence of a second organic hydroperoxide-inducible system that differs from the global peroxide regulator system, OxyR. Expression of *ohr* in various bacteria tested conferred increased resistance to *tert*-butyl hydroperoxide killing, but this was not so for wild-type *Xanthomonas* strains. The organic hydroperoxide hypersensitivity of *ohr* mutants could be fully complemented by expression of *ohr* or a combination of *ahpC* and *ahpF* and could be partially complemented by expression *ahpC* alone. The data suggested that Ohr was a new type of organic hydroperoxide detoxification protein.

Increased production of reactive oxygen species, including superoxides, H_2O_2, and organic hydroperoxides, is an important component of the plant defense response against microbial infection (23, 42) and is a consequence of normal aerobic metabolism (15, 16). Bacteria have evolved complex mechanisms to detoxify and repair damage caused by reactive oxygen species. For detoxification of superoxides and H_2O_2, the enzymes involved and the regulation of their genes are well studied (11, 12, 17). Much less is known regarding defense against organic peroxides.

Organic hydroperoxides are highly toxic molecules, partly due to their ability to generate free organic radicals, which can react with biological molecules and perpetuate free radical reactions (1, 19). Thus, genes involved in protection against organic peroxide toxicity are likely to play important roles in oxidative stress response and in host-pathogen interactions. Alkyl hydroperoxide reductase (AhpR) is the only major microbial enzyme that has been shown to be involved in converting organic hydroperoxides into the corresponding alcohols (3, 21, 40).

The AhpR mechanism of action is well studied. The enzyme consists of two subunits named AhpC and AhpF (4, 5, 21, 37, 38). The genes coding for these subunits are widely distributed (3, 4). Genetic analysis of several bacteria has shown that mutations in these genes lead to an organic hydroperoxide-hypersensitive phenotype, and this confirms their roles in protecting against organic hydroperoxides (2, 40, 45). Other bacterial enzymes, such as glutathione peroxidase (33), glutathione transferase (34), and peroxidases (12), can also use organic hydroperoxides as substrates with varying degrees of efficiency. However, these enzymes have not been well characterized biochemically and genetically. Also, their distribution in only certain groups of bacteria (33, 34) raises a question as to whether they play important physiological roles in the protection against organic hydroperoxide toxicity.

In this paper, we report the isolation and characterization of a possible new organic hydroperoxide detoxification enzyme with a novel regulatory pattern.

MATERIALS AND METHODS

Bacterial cultures and media. All *Xanthomonas* strains were grown aerobically at 28°C in SB medium as previously described (6, 35). To ensure the reproducibility of results, all experiments were performed on cultures at similar stages of growth. All *Escherichia coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37°C. Microaerobic growth conditions were achieved by placing the plates in an anaerobic jar with a *Campylobacter* gas pack (Oxoid) and incubating them at 28°C.

Nucleic acid extraction and analysis, cloning, and nucleotide sequencing. Restriction enzyme digestions were performed according to the manufacturers' recommendations. Molecular cloning, gel electrophoresis, and nucleic acid hybridization were performed as previously described (28). Nucleotide sequencing was done using ABI Prism dye terminator sequencing kits on an ABI 373 automated sequencer. *E. coli* and *Xanthomonas* were transformed by a chemical method (28) and by electroporation (30), respectively. Genomic DNA extraction from *Xanthomonas* spp. was done according to the method of Mongkolsuk et al. (30). Total RNA was isolated by a hot-phenol method (30). pUFR-ahpCF was generated by subcloning a DNA fragment containing *Xanthomonas campestris* pv. phaseoli *ahpCF* (24) into pUFR047.

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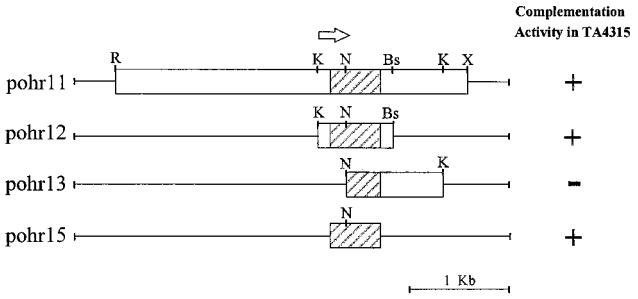

FIG. 1. Localization of *ohr*. The open boxes represent *X. campestris* pv. phaseoli genomic DNA cloned in the indicated plasmids, and the shaded boxes represent the *ohr* gene. The arrow indicates the direction of *ohr* transcription. Bs, *Bst*XI; K, *Kpn*I; N, *Not*I; R, *Eco*RI; X, *Xba*I; +, growth of TA4315 harboring the plasmid on LB-ampicillin plates containing 100 μM tBOOH; −, no growth.

Construction of an *ohr* mutant. An *ohr* mutant was created by marker exchange between a mutated copy of *ohr* and a functional genomic copy. Specifically, a *tet* gene from pSM-Tet (32) was digested with *Eco*RI and *Hin*dIII and cloned into similarly digested p18Not (10), resulting in pNot-Tet. A mutated *ohr* gene was then constructed by insertion of a *Not*I-digested *tet* gene from pNot-Tet into a unique *Not*I site located in the *ohr* coding region of pohr11 (Fig. 1). This resulted in a recombinant plasmid designated pohr-tet. *ohr* inactivation was confirmed by loss of the ability of pohr-tet to confer resistance to *tert*-butyl hydroperoxide (tBOOH) in *E. coli* TA4315 (Δ*ahpCF* [40]). The plasmid was electroporated into *X. campestris* pv. phaseoli, and transformants were selected on SB plates containing 20 μg of tetracycline/ml. Tet^r colonies appeared after 48 h of incubation at 28°C. These colonies were picked and scored for Ap^r phenotype. Those transformants which were both Tet^r and Ap^s (a double crossover resulted in marker exchange) were selected for further characterization. Marker exchanges between the mutated *ohr* and the functional copy of the gene in putative mutants were confirmed by analysis of hybridization patterns of genomic DNA digested with restriction enzymes and probed with *ohr*.

Effects of oxidants on growth and killing. Effects of low concentrations of oxidants on *Xanthomonas* growth was tested with log-phase cells. Essentially, overnight cultures of late-log-phase cells were subcultured as a 5% inoculum into fresh SB medium and allowed to grow for 1 h. Oxidants were then added at appropriate concentrations, and the growth of both induced and uninduced cultures was subsequently monitored spectrophotometrically. Quantitative analysis of the killing effects of high concentrations of oxidants on various *Xanthomonas* strains was performed as previously described (30). Qualitative analysis of levels of resistance to various reagents was done by using a killing zone method (31). Essentially log-phase cells were mixed with SB top agar and poured onto SB plates. After the top agar had solidified, 6-mm-diameter discs containing appropriate concentrations of oxidants were placed on the cell lawn, and zones of growth inhibition were measured after 24 h of incubation at 28°C.

High-level expression and purification of Ohr for antibody production. High levels of *ohr* expression for Ohr purification were achieved by using a gene fusion expression vector system in *E. coli*. One oligonucleotide primer corresponding to the 5′ region of *ohr* from the second codon (5′ TACGAATTCATGGCCTCA CCC 3′) and a second primer corresponding to the *ohr* translation termination codon (5′ TCCAAGCTTGCATTACGCCA 3′) were used to amplify *ohr* from pohr15. A PCR product of 500 bp was then digested with *Eco*RI and *Hin*dIII, gel purified, and cloned into pMAL-C2 vector (New England Biolabs Inc.). This was expected to result in the frame fusion of *ohr* with maltose binding protein. Transformants with correct inserts were screened for high levels of expression of the fusion protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. A clone designated pMalohr showed high levels of expression of the fusion protein, and it was chosen for large-scale protein purification.

A 200-ml culture of pMalohr was grown and induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 1 h. The cells were subsequently pelleted, and the pellet was resuspended in 50 mM phosphate buffer, pH 7.0. The suspension was then sonicated for 20 min, with cooling intervals. Ohr fusion protein was purified from crude lysate by using amylose affinity columns according to the manufacturer's recommendations. The purified fusion protein was then cleaved with protease factor Xa, and Ohr was repurified from SDS-polyacrylamide gels. Purified Ohr was used to raise antibody in rabbits.

Immunodetection of Ohr. Crude lysates from *Xanthomonas* were prepared according to the method of Mongkolsuk et al. (30), and the total protein concentration was determined by the dye-binding method (2). SDS-polyacrylamide gel electrophoresis, blotting, blocking, and antibody reaction analysis were performed as previously described (31) except that an anti-Ohr antibody was used as a primary antibody at 1:3,000 dilution. Antibody reactions were detected by a goat anti-rabbit antibody conjugated to alkaline phosphatase (Promega) as recommended by the manufacturer.

Expression analysis. A 40-ml mid-log-phase *X. campestris* pv. phaseoli culture was equally divided among four flasks and grown for 1 h until the optical density at 600 nm reached 0.4. H_2O_2, tBOOH, or menadione (MD) at a 100 μM final concentration was added, and the cultures were grown for 1 h longer before both induced and uninduced cultures were harvested for Western analysis of Ohr. For analysis of *ohr* RNA a similar induction protocol was performed except that the induction time was reduced to 15 min. The cells were harvested as before, and total RNA was extracted by a hot-phenol method (30).

Nuclotide sequence accession number. The nucleotide sequence accession number for *ohr* in GenBank is AF036166.

RESULTS

Isolation and localization of *ohr*. Our objective was to isolate the *Xanthomonas* genes involved in organic peroxide protection by suppressing the organic hydroperoxide-hypersensitive phenotype of an *E. coli ahpCF* mutant. An aliquot of an *X. campestris* pv. phaseoli DNA library in pUC18 was electroporated into TA4315 (Δ*ahpCF* [40]). Transformants were selected on LB-ampicillin agar containing 500 μM tBOOH and 1 mM IPTG. After 24 h of incubation, 12 colonies appeared on the plate. Plasmids were extracted from individual transformants and retransformed into TA4315, selecting for ampicillin resistance and scoring for concomitant tBOOH resistance. There were eight clones which retained tBOOH resistance. Restriction enzyme mapping and Southern analysis of plasmids purified from these colonies showed that they shared a common 1.2-kb *Kpn*I fragment that cross-hybridized (data not shown). The plasmid, designated pohr11, that contained the longest *X. campestris* pv. phaseoli DNA insert (3.8 kb) was selected for further characterization. Subcloning and deletions of pohr11 were performed to localize the gene responsible for tBOOH resistance. The results are shown in Fig. 1. The gene which was able to confer organic hydroperoxide resistance upon the *E. coli* mutant was located on the 890-bp *Kpn*I-*Bst*XI fragment (Fig. 1, pohr12).

The *Xanthomonas* DNA in pohr11 from *Kpn*I to *Bst*XI was completely sequenced (Fig. 2A). Analysis of the sequence revealed many open reading frames (ORFs) in the region that could have conferred tBOOH resistance on TA4315. An ORF (designated ORF-A) with a strong ribosome binding site 6 bp upstream of a translation initiation codon and with a coding capacity for 142 amino acids having a total predicted molecular mass of 14.5 kDa was a candidate for the tBOOH resistance gene. To confirm this, primers corresponding to DNA sequences 100 bp upstream from the translation initiation codon of ORF-A (5′ GAGAATTCCTTGGCGCGGGAT 3′) and 20 bases downstream of the stop codon of ORF-A (5′ GCATCA CGGCCTGGCCT 3′) were used to amplify *orf-A* from pohr11 in a PCR. The 560-bp PCR product was cloned into pBluescript KS, resulting in pohr15, which was transformed into TA4315. Transformants showed high levels of tBOOH resistance compared to that of TA4315 harboring the vector alone (Fig. 1). This confirmed that ORF-A was responsible for the organic hydroperoxide resistance phenotype, and it was therefore designated *ohr*.

The coding potential for *ohr* was confirmed by determination of pohr15-encoded proteins by using a coupled in vitro transcription-translation *E. coli* system (Promega). The results (Fig. 2B) showed that pohr15 encoded a polypeptide of 14.5 kDa, identical to the calculated molecular mass of Ohr.

Sequence analysis. The predicted Ohr amino acid sequence had the highest homology (63%) with an unknown protein from *Acinetobacter calcoaceticus* (36). Moderate homology (46%) with two unknown proteins (YklA and YkzA) from *Bacillus subtilis* was observed. There was lower homology with OsmC (an osmotic inducible protein) from *E. coli* (31% [18]) and an ORF of unknown function from *Mycoplasma genitalium*

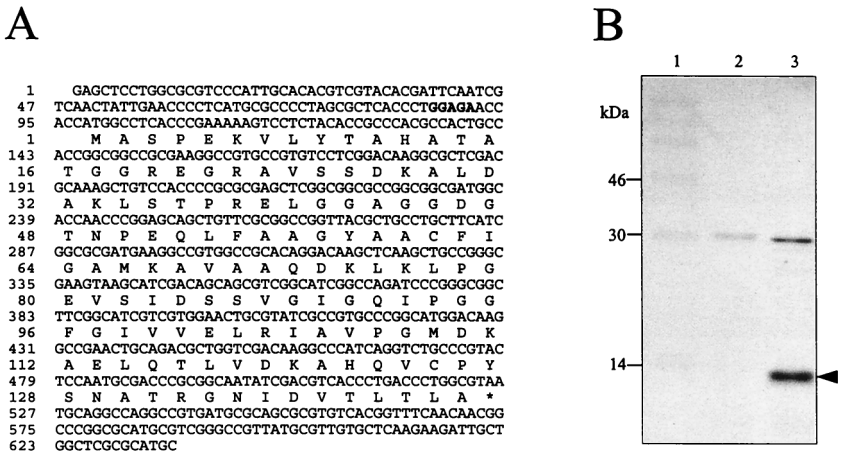

FIG. 2. Nucleotide sequence (A) and in vitro translation products (B) of *ohr*. (A) Nucleotide sequence and a predicted translation product of *ohr*. A putative ribosome binding site for *ohr* is shown in boldface. (B) In vitro translation of plasmid-encoded proteins with *E. coli* S-30 extracts. A vector control or pohr15 was added to S-30 coupled in vitro transcription-translation lysates as described in Materials and Methods. The radioactively labeled translation products are shown. Lane 1, protein molecular mass markers; lane 2, pUC18; lane 3, pohr15. The arrow indicates in vitro translation products of *ohr*. The second band at around 30 kDa is the product of the ampicillin resistance gene.

(31% [14]). The multiple alignment of these ORFs is shown in Fig. 3. It was striking that all four proteins were similar in size and had two highly conserved redox-sensitive cysteine residues, suggesting they could be important in the structure and functions of Ohr.

Structural organization and distribution of *ohr*. The copy number of *ohr* in *X. campestris* pv. phaseoli was determined. *X. campestris* pv. phaseoli genomic DNA was individually digested with five restriction enzymes, separated, blotted, and subsequently probed with the coding region of *ohr*. The results showed hybridization patterns consistent with *ohr* being a single-copy gene (data not shown).

To determine the distribution of *ohr* in *Xanthomonas* and various other bacteria, both Southern and Western analysis were performed. Western blots prepared from total-protein lysates from six *Xanthomonas* species each showed a 14.5-kDa protein that specifically cross-reacted with an anti-Ohr antibody (Fig. 4A). In addition, genomic DNA from five *Xanthomonas* species was digested with a restriction enzyme, blotted, and probed with *ohr*. Under high-stringency washing conditions, positive hybridization signals were detected in all strains tested (Fig. 4B). By contrast, Southern blots prepared from genomic DNAs from *Pseudomonas aeruginosa* and *Burkholderia cepacia* did not cross-hybridize with *ohr* probes, even under low-stringency washing conditions. Furthermore, Western analysis of cell lysates from these bacteria did not show any proteins of a size similar to that of Ohr that specifically cross-reacted with the anti-Ohr antibody (data not shown).

Construction and physical characterization of a *Xanthomonas ohr* mutant. The role of *ohr* in protecting against organic hydroperoxide toxicity in *Xanthomonas* was evaluated by construction of an *ohr* inactivation mutant, as described in Materials and Methods. Essentially, a gene conferring Tet^r was inserted into the coding region of *ohr* and the recombinant

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OHR-XP      1              MASPEKVLYTAHATATGGR-EGRAVSSDKALDAKLSTPRELG-GAGGDG
ORF2-AC     1               M.L.Q.V.....K.....-D...T...NI..VQ.TV.K.M.-.M.--.
YKLA-BS     1                 MSQP.F..TVS.V...-..KVI...RV.ELDVAM.GTPRAKKLEKA
YKZA-BS     1                   MA.F..KV..R...-A.HIT.D.GV..FDIVM.NAK--KE.QT.
OSMC-EC     1                MTIHKKGQ..WEGDIK.GK.TVSTESGV.NQQPYGFNTR--FE.EK.
OSM-MY      1  MLFNIFTKILSILINMALI.KTV.QTET..-..SVKTL.-GFQT...F.KPD--LSVQTE
consensus                            ΔΔ   Δ ΔΔ*  *   Δ Δ  Δ      Δ       Δ  Δ

OHR-XP     48 TNPEQLFAAGYAAGFIGAMKAVAAQDKLKLPGEVSIDSSVGIGQIPGGFGIV---VELRI
ORF2-AC    46 ..........S..L....F..TR..FNI.KDAYVEGD....P..N....E---.K.HV
YKLA-BS    46 ..............DS.LQL..RTERV.VET..TANV.LLKDEADQ.YKLG---.T.QV
YKZA-BS    42 ..............G..LEH..KEQNIEIDS.IEGQV.LMKDESD...K.G---.T.VV
OSMC-EC    46 ....E.IG.AH...SM.LSLMLG-EAGFT.TSIDTTAD.SLDKVDA..A.TKIALKSEV
OSM-MY     57 N.......SA..S..SQ.VIV.MQ.HQFSFSKKPVVSVK.ELH.EN.L.H.K---AGVEL
consensus     Δ***Δ*ΔΔΔΔΔΔ**  *   ΔΔ                Δ       ΔΔ Δ    Δ Δ Δ

OHR-XP    105 AVPGMDKAELQTLVDKAHQVGPYSNATRGNIDVTLTL-----A
ORF2-AC   103 HL....TD.AKK...A..I.........N....DFEIVTDA--
YKLA-BS   103 KGE.VSAS..EA..K...G.....K..S.......EVAE----
YKZA-BS    99 NTKDL.REKA.E..NA..EF.....K.....V..K.E.--K---
OSMC-EC   105 ....I.ASTFDGIIQ..KAG..V.QVLKAE.TLD---------
OSM-MY    114 TTNSN.QEVGKK.IQ...EM..F.RLI.NENFLG...NGIKL-
consensus         Δ Δ      ΔΔ Δ*Δ  **Δ* ΔΔΔ ΔΔΔΔ Δ
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FIG. 3. Multiple alignment of *X. campestris* pv. phaseoli Ohr (OHR-XP [AF036166]), ORF2 of *Acinetobacter* (ORF2-AC [Y09102]), proteins from *B. subtilis* (YKLA-Bs [AJ002571] and YKZA-Bs [AJ002571]), OsmC from *E. coli* (OSMC-EC [X57433]), and OsmC-like protein from *M. genitalium* (OSM-MY [U39732]). Amino acid sequences were aligned by the Clustal W program (43). Periods represent amino acids identical to those found in Ohr, asterisks indicate identical amino acids in all six sequences, and triangles represent matches of at least four amino acids in the sequences. Hyphens indicate gaps in the sequences. Conserved cysteine residues are shaded.

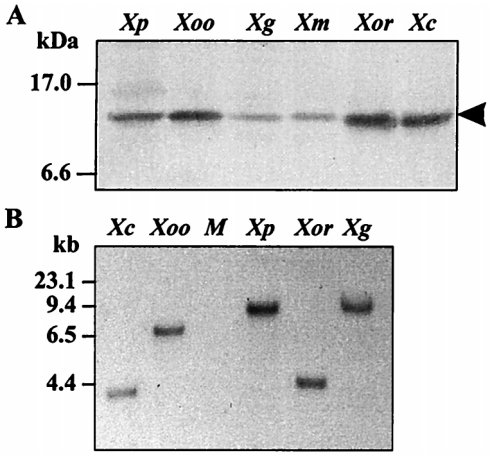

FIG. 4. Detection of Ohr and *ohr* in various *Xanthomonas* species. (A) Western analysis of Ohr in protein lysates from *X. campestris* pv. phaseoli (*Xp*), *Xanthomonas oryzae* pv. oryzae (*Xoo*), *Xanthomonas campestris* pv. glycine (*Xg*), *Xanthomonas campestris* pv. malvacerum (*Xm*), *Xanthomonas oryzae* pv. orizicolar (*Xor*), and *Xanthomonas campestris* pv. campestris (*Xc*). Fifty micrograms of protein was loaded into each lane. After electrophoresis, the gel was blotted onto a piece of polyvinylidene difluoride membrane that was reacted with an anti-Ohr antibody. Protein molecular mass markers are shown on the left. The arrow indicates the position of Ohr protein. (B) Southern analysis of total genomic DNA digested with *Eco*RI and probed with the coding region of *ohr*. Hybridization and high-stringency washing conditions were as previously described (30). Lane M contains DNA molecular size markers; the sizes are shown on the left.

plasmid was electroporated into *X. campestris* pv. phaseoli with selection for Tet^r transformants that also had an Ap^s phenotype. Subsequently, the levels of resistance to tBOOH of these 20 Tet^r Ap^s *X. campestris* pv. phaseoli transformants were tested by the killing zone method. The results showed that all putative *ohr* mutants were less resistant to tBOOH killing than parental *X. campestris* pv. phaseoli (data not shown).

An *ohr* mutant designated *Xp*18 was selected for detailed structural analysis. The results of Southern analysis of *Xp*18 DNA digested with restriction enzymes and probed with *ohr* are shown in Fig. 5. Hybridization of the *ohr* probe with *X. campestris* pv. phaseoli or *Xp*18 DNA digested with either *Bst*XI or *Cla*I showed that the positively hybridized bands in *Xp*18 were around 3 kb larger than a corresponding band detected in similarly digested *X. campestris* pv. phaseoli DNA (Fig. 5A). The size increase corresponded to the size of the inserted *tet* gene. There were no additional positively hybridized signals in digested *Xp*18 DNA. These results indicated that the mutated *ohr* had replaced the functional gene. Inactivation of *ohr* in *Xp*18 was also confirmed at the protein level by Western blot analysis with an anti-Ohr antibody. The results (Fig. 5B) showed that the Ohr protein was absent from *Xp*18.

Physiological and biochemical characterization of the *ohr* mutant. The availability of the *ohr* mutant allowed investigation of the physiological role of *ohr* in the *Xanthomonas* oxidative stress response. The effects of both oxidative and nonoxidative stress on the growth and survival of the mutant were evaluated.

In most bacteria, the mutation of genes involved in oxidative stress protection leads to a reduced aerobic growth rate and a lower plating efficiency (26). We tested these parameters first on the *ohr* mutant. The mutant showed an aerobic growth rate and plating efficiency similar to those of a wild-type strain on either rich or minimal media (data not shown). Next, the effect of low oxidant concentrations on the growth rates of *X. campestris* pv. phaseoli and *Xp*18 was investigated. No differences between the growth rates of these strains were observed in the presence of several concentrations of either H_2O_2 or MD (data not shown). However, a low concentration of tBOOH had a significant growth-inhibitory effect on the mutant. In the presence of 600 μM tBOOH, *Xp*18 had a doubling time exceeding 300 min, contrasting with a 140-min doubling time for *X. campestris* pv. phaseoli (Fig. 6A). Cumene hydroperoxide (CHP) produced a similar growth-inhibitory effect on the mutant (data not shown). We then tested the sensitivity of the mutant to killing concentrations of tBOOH, H_2O_2, and MD. The quantitative results are shown in Fig. 6. The mutant was more sensitive (over 100-fold) to tBOOH killing than the wild type, but there were no differences in sensitivity to either H_2O_2 or MD killing. Qualitative analysis of mutant and wild-type levels of resistance to killing concentrations of other oxidants, such as diamide, *N*-ethylmaleimide, paraquat, and chemical mutagens (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and methyl methanesulfonate) were performed by the killing zone method. No differences in sensitivity to these agents were detected. Similarly, levels of resistance to nonoxidative stress killing agents, such as heat or pH, were identical for the two strains (data not shown).

Mutation in genes involved in stress protection can often lead to compensatory increases in the expression of other functionally related genes (3, 39). Thus, basal levels of enzymes involved in peroxide detoxification (AhpC, catalase, glutathione transferase, and peroxidase) or in oxidative stress protection (glucose-6-phosphate dehydrogenase and superoxide dismutase) were determined in *Xp*18. The results showed that levels of these enzymes were not significantly different between the wild type and the mutant (data not shown).

***ohr* expression in response to stress.** Characterization of the *ohr* mutant suggested that *ohr* played an important role in the protection of *X. campestris* pv. phaseoli from organic peroxide toxicity. In *Xanthomonas*, as in other bacteria, exposure to low levels of oxidants leads to a severalfold increase in expression of peroxide stress protective enzymes, such as catalase and AhpR (7, 31). This inducible response likely plays an important role in protecting the bacterium against stress. This knowledge prompted an investigation into the regulation of *ohr* in response to various oxidants. The steady-state levels of Ohr in response to oxidant treatments was determined by Western analysis. The results in Fig. 7A show a fourfold increase in the amount of Ohr after treatment with inducing concentrations of tBOOH. By contrast, only a marginal increase in Ohr was

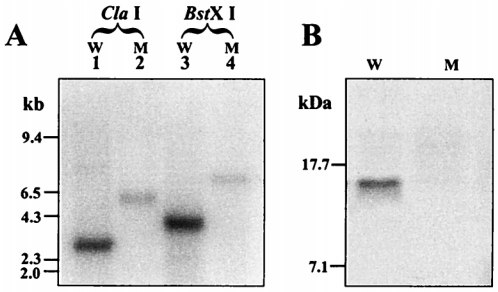

FIG. 5. Characterization of an *ohr* mutant at DNA (A) and protein (B) levels. (A) *X. campestris* pv. phaseoli (W; lanes 1 and 3) and *X. campestris* pv. phaseoli *ohr* mutant (M; lanes 2 and 4) genomic DNAs were digested with *Cla*I (lanes 1 and 2) and *Bst*XI (lanes 3 and 4) restriction enzymes and Southern blotted, and the membranes were probed with *ohr*. DNA molecular size markers are shown on the left. (B) Western analysis of *X. campestris* pv. phaseoli (W) and an *X. campestris* pv. phaseoli *ohr* mutant (M). The experiment was performed as described in the legend to Fig. 4A and Materials and Methods. Molecular mass markers are shown on the left.

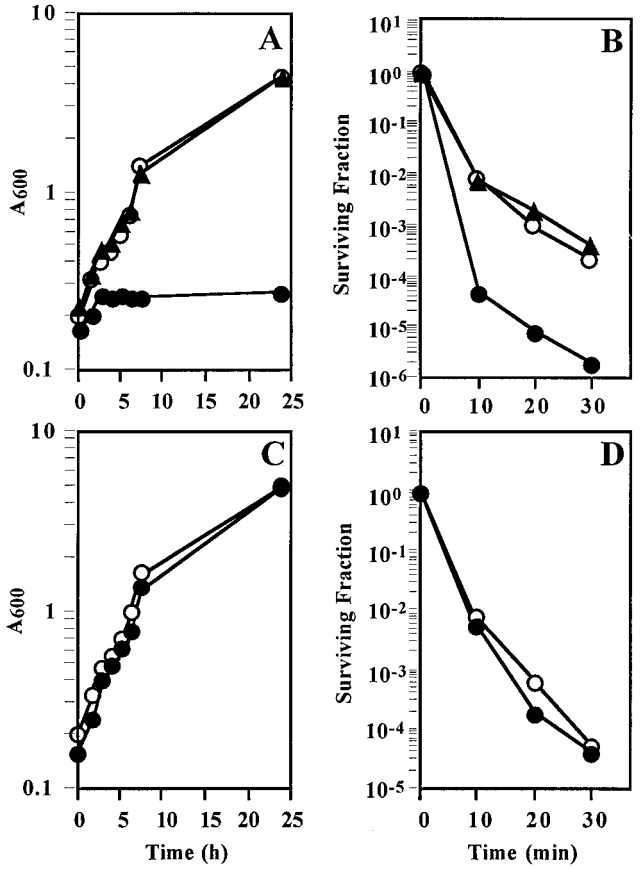

FIG. 6. Effects of peroxides on growth and survival of *X. campestris* pv. phaseoli and an *X. campestris* pv. phaseoli *ohr* mutant. The effects of low concentrations of either tBOOH (600 μM) (A) or H_2O_2 (200 μM) (C) on the growth, or of high concentrations of tBOOH (150 mM) (B) or H_2O_2 (30 mM) (D) on the survival, of *X. campestris* pv. phaseoli (○), *Xp*18 (●), and *Xp*18 containing pUFR-ohr (▲) were measured as described in Materials and Methods. The surviving fraction is defined as the number of living cells prior to a treatment divided by the number of living cells after a treatment. The experiments were done at least three times, and representative results are shown.

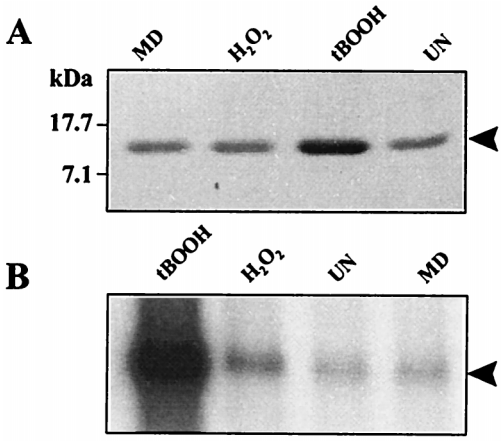

FIG. 7. Expression of *ohr* in response to various oxidants. (A) Western analysis of Ohr levels in *X. campestris* pv. phaseoli uninduced (UN) and induced with 100 μM MD, H_2O_2, or tBOOH was performed as described in Materials and Methods and in the legend to Fig. 4A. (B) Northern blot of total RNA isolated from *X. campestris* pv. phaseoli uninduced (UN) or induced with 100 μM of tBOOH, H_2O_2, or MD. Growth and induction conditions were the same as for panel A. Electrophoresis, blotting, hybridization, and washing were performed as previously described. The membrane was probed with radioactively labeled *ohr* probes. Ten micrograms of total RNA was loaded in each lane.

detected after exposure to H_2O_2 and none was detected after exposure to MD. To confirm that increased Ohr levels were due to increased *ohr* transcription, the amount of *ohr* mRNA was measured in a Northern experiment. Total RNA isolated from uninduced and tBOOH-, H_2O_2-, and MD-induced cultures was hybridized to *ohr* probes. The results are shown in Fig. 7B. Densitometer analysis of hybridization signals indicated that there was a greatly increased (15-fold) amount of *ohr* mRNA in response to tBOOH treatment. By contrast, there was only a minor increase (2.5-fold) after H_2O_2 treatments and no increase after MD treatment. The difference in *ohr* induction by tBOOH shown by protein and mRNA levels was likely due to the high stability of Ohr protein and its accumulation in the induced *X. campestris* pv. phaseoli.

Increased expression of *ohr* in *Xanthomonas* species. We have observed that in *Xanthomonas* increased expression of genes coding for oxidative stress protective enzymes can confer additional resistance to oxidants (24, 30). The levels of resistance of *X. campestris* pv. phaseoli harboring either pUFR-ohr or vector plasmids (9) to H_2O_2, tBOOH, and MD killing were determined. The results (Table 1) indicated that *X. campestris* pv. phaseoli and other *Xanthomonas* harboring the vector pUFR047 or pUFR-ohr had similar levels of resistance to killing concentrations of all three oxidants. Western analysis with an anti-Ohr antibody confirmed that lack of increased protection from tBOOH killing in strains harboring pUFR-ohr was not due to aberrant expression of *ohr* by the plasmid. In addition, the possibility that pUFR-ohr contained a defective copy of the gene was ruled out by the plasmid's ability to complement an *X. campestris* pv. phaseoli *ohr* tBOOH-hypersensitive mutant to a wild-type level of resistance (Table 2).

Heterologous expression of pUFR-ohr in various bacteria. Unexpectedly, increased expression of *ohr* from an expression vector did not confer additional tBOOH resistance on various *Xanthomonas* strains tested (Table 1). This effect of *ohr* expression on tBOOH resistance was tested in various other bacteria. The broad-host-range expression vector (9) containing *ohr* (pUFR-ohr) was electroporated into various bacteria. The tBOOH resistance of transformants harboring pUFR-ohr was then compared to that of cells harboring the

TABLE 1. Summary of levels of resistance of various bacteria to oxidants[a]

Species and strain	Zone of inhibition (mm)		
	CHP (200 mM)	tBOOH (500 mM)	H_2O_2 (500 mM)
X. campestris pv. phaseoli/pUFR047[b]	12	10	15
X. campestris pv. phaseoli/pUFR-ohr	12	11	15
X. campestris pv. campestris/pUFR047	12	15	12
X. campestris pv. campestris/pUFR-ohr	11	14	11
X. oryzae pv. oryzae/pUFR047	20	31	11
X. oryzae pv. oryzae/pUFR-ohr	20	32	11
E. coli TA4315/pUFR047	28	30	12
E. coli TA4315/pUFR-ohr	12	15	12
P. aeruginosa/pUFR047	14	34	12
P. aeruginosa/pUFR-ohr	11	27	12
A. tumefaciens/pUFR047	24	23	20
A. tumefaciens/pUFR-ohr	19	20	21

[a] Experiments were performed as described in Materials and Methods.
[b] pUFR047 is a broad-host-range expression vector (9).

TABLE 2. Summary of levels of resistance of wild-type *X. campestris* pv. phaseoli and an *ohr* mutant to oxidants[a]

Strain	Zone of inhibition (mm)			
	CHP (200 mM)	tBOOH (500 mM)	H_2O_2 (500 mM)	MD (500 mM)
X. campestris pv. phaseoli/pUFR047[c]	12	10	15	10
*Xp*18 (*ohr* mutant)/pUFR047	20	18	16	11
*Xp*18/pUFR-ohr	12	11	15	ND[b]
*Xp*18/pUFR-ahpCF	15	14	16	ND
*Xp*18/pahpC	17	16	15	ND
*Xp*18/pUFR-kat	20	19	12	ND

[a] Experiments were performed as described in Materials and Methods.
[b] ND, not done.
[c] pUFR047 is a broad-host-range expression vector (9).

vector alone by the killing zone method. The results shown in Table 1 clearly showed that expression of *ohr* in *E. coli* K-12, *P. aeruginosa*, and *Agrobacterium tumefaciens* conferred increased resistance to tBOOH killing.

Complementation analysis of the *ohr* mutant. We were interested in seeing whether increased expression of the genes involved in peroxide stress protection (those for catalase [*kat*] and alkyl hydroperoxide reductase [*ahpCF*]) or *ohr* could compensate for the mutant's hypersensitivity to tBOOH killing. For this purpose, plasmids containing *katX* (pUFR-kat [30]), *ahp* C (pahpC [24]), combined *ahp* C and F (pUFR-ahpCF), or *ohr* (pUFR-ohr) were electroporated into mutant *Xp*18 and levels of resistance to alkyl hydroperoxides were qualitatively determined by the killing zone method. The results are shown in Table 2. As expected, pUFR-ohr, complemented the mutant's increased sensitivity to alkyl hydroperoxide. In addition, increased expression of *ahpC* alone partially compensated for hypersensitivity to tBOOH killing, but the resistance achieved was still below that of the parental *X. campestris* pv. phaseoli strain. On the other hand, *Xp*18 harboring pahpCF showed resistance to tBOOH killing similar to that of wild-type *X. campestris* pv. phaseoli. Increased expression of *katX* provided no protective effect against tBOOH killing.

DISCUSSION

From *X. campestris* pv. phaseoli we have isolated a new gene (*ohr*) that is involved in organic hydroperoxide protection. An *ohr* mutant showed no significant growth defects under normal conditions, indicating that the gene was not essential. Important evidence from analysis of its mutant and heterologous expression suggests that Ohr plays a novel role in organic hydroperoxide metabolism. First, in many bacteria, the mutation of genes involved in oxidative stress protection always results in increased sensitivity to oxidative stress (3, 11, 12). Similarly, *ohr* mutants showed increased sensitivity to organic hydroperoxides. It is worth noting that the sensitivity of the *ohr* mutant was specific to organic hydroperoxides, unlike the mutants of other known peroxide protective (11, 12) and nonspecific DNA binding (29) genes, which often conferred increased sensitivity to several oxidants. The ability of *ohr* to complement the organic hydroperoxide hypersensitivity of the *Xp*18 mutant confirmed that the phenotype was due to a mutation in *ohr*. The biochemical action of Ohr is not known; its ability to increase tBOOH resistance in several unrelated bacterial species suggested that it might function directly in detoxification of organic hydroperoxides. However, we have not ruled out the possibility that Ohr is involved in the transport processes of organic molecules.

The highest degree of homology (63%) was found between Ohr and a protein of unknown function from *Acinetobacter* sp. (36) which is known to have metabolic pathways for *n*-alkane oxidation. In this bacterium, the first step in the oxidation pathway involves an attack by dioxygenase, which leads to the formation of *n*-alkyl hydroperoxides. These unstable intermediates are subsequently metabolized to various end products (13, 27). Thus, Ohr-like proteins in these bacteria could be involved either directly in the alkane metabolism pathway or indirectly, by providing protection against the toxic effects of alkyl hydroperoxides in a reaction similar to that of catalysis by AhpR. Some degree of homology (31%) with an unknown ORF from *Mycoplasma* was detected. Analysis of *M. genitalium* and *Mycoplasma pneumoniae* genome sequences surprisingly revealed a lack of known genes involved in peroxide metabolism (14, 20). This raises the question of how these bacteria protect themselves from peroxide toxicity. It is feasible that an Ohr-like protein in these bacteria could functionally substitute for AhpR and peroxidases in dealing with organic peroxides. The putative Ohr-like proteins had similar lengths and amino acid sequence motifs. This implied that they belonged to a new family of proteins involved in organic hydroperoxide metabolism.

Ohr or Ohr-like proteins as well as AhpR were found in *Xanthomonas*, *B. subtilis*, and *E. coli*. Both gene products seem to be involved in organic hydroperoxide detoxification. This notion was supported by the observation that expression of combined *ahpC* and *ahpF* complemented the tBOOH hypersensitivity of the *Xanthomonas ohr* mutant to wild-type levels. Similarly, *ohr* complemented the phenotype of an *ahpC-ahpF E. coli* mutant. Thus, there appeared to be a functional complementation between Ohr and AhpC.

Examination of the regulation of *ohr* expression in *Xanthomonas* indicated that there are at least two regulatory systems for organic hydroperoxide-inducible genes. We have shown that in one system in *Xanthomonas*, genes under *oxyR* regulation (i.e., the catalase gene and *ahpC*) can be induced by treatment with low concentrations of H_2O_2, organic hydroperoxides, or superoxide generators (7, 25, 31). The responses of *ohr* to peroxides and superoxide generators clearly differ, since they can be highly induced only by organic peroxides and only weakly by H_2O_2. In fact, the weak induction of *ohr* by H_2O_2 may be an indirect result of H_2O_2 reaction with membrane lipids that resulted in organic hydroperoxide production (22, 44). Thus, regulation of *ohr* responds to a narrower but overlapping range of inducing stresses than does the global peroxide regulator OxyR (8, 41).

Another difference between Ohr and AhpC could be related to cellular localization. Ohr has 31% homology with OsmC, a well-characterized periplasmic protein (18), while AhpC is a cytoplasmic protein. This might explain the unexpected observation that increased *ohr* expression in wild-type *Xanthomonas* did not confer resistance to higher levels of tBOOH. In wild-type *Xanthomonas*, transport of Ohr to the periplasmic space could be a rate-limiting step. If so, increased expression of *ohr* from an expression vector might not result in increased tBOOH resistance. We do not know whether OsmC is involved in organic peroxide metabolism. We are investigating this possibility.

The absence of Ohr-like protein sequences from many bacterial genome sequences in the database (e.g., those of *Haemophilus influenzae* and *Helicobacter pylori*) suggests that Ohr may perform special tasks in organic hydroperoxide detoxification for a subset of bacterial species. Indeed, these observa-

tions raise the possibility that Ohr and AhpC in *Xanthomonas* may have similar enzymatic functions but differ in regulation and cellular localization. The differences imply disparate physiological roles in spite of similar biochemical actions in organic hydroperoxide detoxification. Until the physiological substrates of Ohr and AhpC are known, a more definitive evaluation of their roles in organic peroxide detoxification cannot be made.

ACKNOWLEDGMENTS

We thank Tim Flegel for critically reviewing the manuscript, G. Storz for providing *E. coli* strains, and J. Helmann for useful suggestions. S. Kasantsri and W. Whangsuk assisted in performing several experiments.

The work was supported by grants from Chulabhorn Research Institute, a Thai-Belgian ABOS grant, and the Thai Research Fund grant BRG 10-40. S.C. and W.P. were partially supported by graduate student training grants from NSTDA.

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