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Characterization of Transcription Organization and Analysis of Unique Expression Patterns of an Alkyl Hydroperoxide Reductase C Gene (*ahpC*) and the Peroxide Regulator Operon *ahpF-oxyR-orfX* from *Xanthomonas campestris* pv. phaseoli

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We have analyzed the transcription organization of *ahpC*, *ahpF*, *oxyR*, and *orfX* from *Xanthomonas campestris* pv. phaseoli. *ahpC* was transcribed as a monocistronic 0.6-kb mRNA, while *ahpF-oxyR-orfX* were transcribed as a polycistronic approximately 3.0-kb-long mRNA. The novel transcription organization of these genes has not observed in other bacteria. Western analysis showed that oxidants (peroxides and superoxide anions), a thiol reagent (*N*-ethylmaleimide), and $CdCl_2$ caused large increases in the steady-state level of AhpC. Growth at alkaline pH also moderately induced AhpC accumulation. Thermal and osmotic stresses did not alter the levels of AhpC. Northern blotting results confirmed that oxidant- and $CdCl_2$-induced AhpC accumulation was due to increased levels of *ahpC* transcripts. Analysis of *oxyR* expression revealed a unique pattern. Unlike other bacterial systems, peroxides and a superoxide generator induced accumulation of OxyR. Northern blotting results confirmed that these oxidants induced expression of *oxyR* operon. This novel regulatory pattern could be generally important. The transcription organization and patterns of chemicals and stress induction of *ahpC* and *oxyR* differed from those of other bacteria and are likely to be important for *X. campestris* pv. phaseoli survival during exposure to oxidants.

During plant-microbe interactions, the initial plant defense response involves increased production of reactive oxygen species, including H_2O_2, organic peroxides, and superoxides. They function as bacteriocidal agents and as secondary signal molecules to further activate plant defense responses (22, 38). To survive and proliferate, bacterial pathogens must overcome reactive oxygen species.

Microbial defense against oxidative stress required well-orchestrated enzyme reactions involving both detoxification of the stress and repair processes. Catalases have important protective roles against H_2O_2 toxicity (11, 14). So far, the best-characterized bacterial scavenging enzyme against organic hydroperoxides is alkyl hydroperoxide reductase (AhpR), which consists of two components, a 22-kDa protein, AhpC, and a 57-kDa flavoprotein, AhpF (19, 36). Both components are required for NADH- or NADPH-dependent reduction of organic peroxides to corresponding alcohols (31, 32). The genes coding for these enzymes have been isolated from several micobes, but extensive analyses of their regulation have been done in few cases (1–3, 5, 13, 32, 40). In these cases, the inducing conditions of *ahpC* vary a great deal for different bacterial species. Increased expression of *ahpC* has been shown to confer resistance to organic hydroperoxides and in some cases can compensate for the lack of catalase enzyme (34, 41). Thus, conditions which affect *ahpC* expression are likely to play important physiological roles in the peroxide stress response.

Oxidative stress response is regulated by several redox-sensitive transcription regulators, such as OxyR (for the peroxide regulon [6, 10, 11, 35, 37]) and SoxRS (for the superoxide regulon [11, 17]). In general, alterations in the cellular redox state lead to changes in the redox status of these proteins that result in activation of genes under their regulation (20, 21, 35, 37). This permits a concerted response to the stress. The regulation of the regulatory genes themselves is important; minor alterations in their patterns of expression would have profound effects on the bacteria. *oxyR* expression is autoregulated, in addition to having a posttranscriptional regulation step (20, 21). *oxyR* homologs have been identified in several bacteria. Details of the structural function and analyses of expression have mostly been done for enteric bacteria (12, 36). Interestingly, many *Mycobacterium tuberculosis* strains are natural *oxyR* mutants with altered expression in peroxide stress protection genes and a drug resistance phenotype (12, 34, 41). OxyR regulates a number of genes involved in peroxide protection and detoxification, i.e., those coding for catalase, AhpR, and Dps (11, 14). OxyR can act either as a transcription repressor or transcription activator, depending on the target promoters and the redox state of the protein (20, 21).

Many aspects of oxidative stress response in *Xanthomonas* are different from those of other bacteria (7, 8, 25). To investigate the regulation of the peroxide stress response and genes involved in protection against alkyl hydroperoxides, we have isolated *ahpC*, *ahpF*, and *oxyR* from *Xanthomonas campestris* pv. phaseoli (24). We have shown that expression of *ahpC* alone provides partial protection against organic hydroperoxides (ROOH) but not H_2O_2 toxicity (24). The aim of this study was to ascertain the transcription organization and expression patterns of *ahpC*, *ahpF*, *oxyR*, and *orfX* in *Xanthomonas.*

MATERIALS AND METHODS

Bacterial strains and induction of *X. campestris* pv. phaseoli cultures. All *Xanthomonas* strains were grown aerobically at 28°C on SB medium (30). To ensure the reproducibility of the experiments, it was important to use bacteria at

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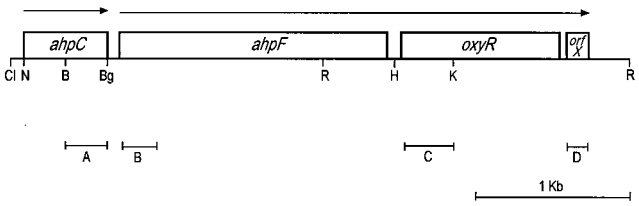

FIG. 1. Organization of *ahpC* and *ahpF-oxyR-orfX* in *X. campestris* pv. phaseoli. The arrows indicate the direction and length of transcripts. Regions marked A, B, C, and D, were used as gene-specific probes in Northern and Southern blotting experiments. B, *Bst*XI; Bg, *Bgl*II; Cl, *Cla*I; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; R, *Eco*RI.

similar stages of growth. Overnight (15-h) late-log-phase cultures were subcultured into fresh SB medium at equal density and grown for 1 h. Various inducers were then added (7, 8). Unless otherwise stated, the induced cultures were grown for half an hour before cells were harvested and used in lysate preparation for Western blot analysis. For Northern analysis, total RNA was extracted from cultures after 10 min of incubation with inducers. Bacterial growth was monitored spectrophotometrically at A_{600}.

Construction of an OxyR expression plasmid and antibody production. OxyR for antibody production was purified from an overexpression plasmid. Essentially, oligonucleotide primers corresponding to the N terminus (5′ TAG GAT CCG AAT CTG CGT GAC 3′) and C terminus (5′ ACC ACA GCC AAA GCG TAC GCA A 3′) of OxyR were used in a PCR with pOXX (24) as a DNA template. The 950-bp PCR product was digested with *Bam*HI and cloned into pBluescript KS digested with *Bam*HI and *Eco*RV (26). The resultant plasmid, pKS-OXX, was digested with *Bam*HI and *Hin*dIII, and 0.9-kb fragments containing *oxyR* were cloned into a His-tagged gene fusion vector, pQE 31 (Qiagen, Inc.). The resultant plasmid, pQE-OXX, overproduced His-tagged OxyR fusion protein at high levels. The fusion protein was purified from a 1-liter culture with Ni-nitrilotriacetic acid resin as suggested by the manufacturer. Purified *X. campestris* pv. phaseoli OxyR was injected into rabbits for antibody production.

Lysate preparation and Western blot analyses. Cell lysates were prepared from fresh cell pellets by being resuspended in 50 mM potassium phosphate buffer (pH 7.0), followed by sonication for 2 min with cooling intervals. Cell debris was removed by centrifugation at 10,000 × *g* for 15 min, and clear lysates were either used immediately or stored frozen (7). Protein concentration was measured by dye binding method (4). For analysis of AhpC induction by Western blotting, 30 μg of protein from various samples was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [15% polyacrylamide]) gels and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and reacted with either anti-*Escherichia coli* AhpC antibody (a gift from G. Storz) or anti-*X. campestris* pv. phaseoli OxyR antibody. They were subsequently developed with a goat anti-rabbit antibody conjugated to alkaline phosphatase. For analysis of the dimeric form of AhpC, essentially the same protocol for gel electrophoresis and immunodetection was used except, that dithiothreitol was omitted from the loading dye. The results of AhpC and OxyR Western analysis were quantitated with a Bio-Rad GS-700 densitometer.

Northern analyses. Total RNA isolation from *Xanthomonas* cultures, agarose formaldehyde gel electrophoresis, blotting, and hybridization conditions were as previously described (28). The coding regions of *ahpC* (a 367-bp *Bst*XI-*Bgl*II fragment), *ahpF* (a 575-bp *Pst*I fragment), *oxyR* (a 950-bp *Bam*HI-*Hin*dIII fragment from pQE-OX), or *orfX* (a 180-bp *Bss*HII-*Sph*I fragment) were used for radioactive probes (Fig. 1).

RESULTS AND DISCUSSION

Transcription organization of *ahpC*, *ahpF*, *oxyR*, and *orfX*. The nucleotide sequences of pAhp4-1 and pOX show that *ahpC*, *ahpF*, *oxyR*, and *orfX* are arranged in a head-to-tail fashion (24). This unusual genome organization is highly conserved in *Xanthomonas* spp. and is likely to be important in the regulation of these genes. To elucidate the transcription organization of these genes, Northern blot analysis was performed with the coding regions of different genes as probes. The results are shown in Fig. 2. The *ahpC* probe hybridized to the 0.6-kb mRNA (Fig. 2, lane 1). This was consistent with the gene being transcribed as a monocistronic mRNA. In contrast, the *ahpF*, *oxyR*, and *orfX* probes each hybridized to mRNA approximately 3.0 kb in length (Fig. 2, lanes 2 to 4). The results supported the idea that these genes were arranged in an operon and transcribed as a polycistronic mRNA. In addition, no hybridization signals with the 3.0-kb *ahpF-oxyR-orfX* mRNA were detected when a region located 3′ outside the *orfX* coding region was used as a Northern probe (data not shown). Similarly, no hybridization with the 0.6-kb *ahpC* mRNA was detected when a region located 5′ outside *ahpC* was used as a probe. These controls confirmed the proposed transcription organization.

In *E. coli* and *Bacillus subtilis*, *ahpC* and *ahpF* are coregulated, and in the latter case, the genes are also arranged in an operon (1, 3, 36). In contrast, *Xanthomonas ahpC* was transcribed as a monocistronic mRNA. In other bacteria, *ahpC* is regulated by OxyR (13, 37). Thus, the separation of transcription regulation of genes coding for subunits of alkyl hydroperoxide reductase (*ahpC* and *ahpF*) from coregulation of *ahpF* with *oxyR* suggests novel complex interactions between these three genes. The physiological significance of close regulation of *ahpC*, *ahpF*, and *oxyR* in *Xanthomonas* is currently under investigation.

Expression analysis of *ahpC* in response to stress. It is generally accepted that physiological and/or environmental conditions which act as inducing signals for high expression of *ahpC* are likely to be important in bacterial oxidative stress response. We performed Western analyses to monitor the *ahpC* expression of *X. campestris* pv. phaseoli in response to various stress conditions.

First, we investigated the effects of superoxide anions and various peroxides on *ahpC* expression in *X. campestris* pv. phaseoli. AhpR can use a wide range of peroxide substrates (19, 31, 32), and this suggests that its expression may be regulated by many inducers. At appropriate concentrations, all peroxides (H_2O_2, tBOOH, and CuOOH) and superoxide generators (menadione [MD] and paraquat) tested were equally potent inducers of *ahpC*, causing four- to fivefold increases in steady-state levels of AhpC (Fig. 3A). In addition, we had tested the *ahpC* response to oxidative stress in four other *Xanthomonas* spp. and obtained a similar pattern of response to *X. campestris* pv. phaseoli (data not shown).

Expression analysis of *ahpC* showed interesting patterns. The induction of *ahpC* by peroxides was similar to that reported for other bacteria, except for *Staphylococcus aureus*, which has an oxidative stress-noninducible phenotype (2). The superoxide generators were potent inducers of *ahpC*. It is not possible to differentiate whether the superoxide anions themselves or their conversion via superoxide dismutase or sponta-

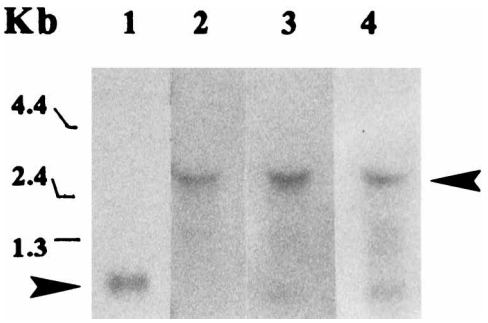

FIG. 2. Transcription organization of *ahpC* and the *ahpF-oxyR-orfX* operon. RNA isolation, electrophoresis, and hybridization were performed as previously described (11, 12). RNA was loaded at 5 μg in lane 1 and 25 μg in lanes 2, 3, and 4. The coding regions of radioactively labelled *ahpC* (lane 1), *ahpF* (lane 2), *oxyR* (lane 3), and *orfX* (lane 4) were used as probes (Fig. 1) for hybridization with Northern blots. The arrowhead to the left indicates monocistronic 600-bp *ahpC* mRNA, and the arrowhead to the right indicates the approximately 3.0-kb polycistronic *ahpF-oxyR-orfX* mRNA. Positions of RNA molecular weight markers (Bethesda Research Laboratories) are shown to the left.

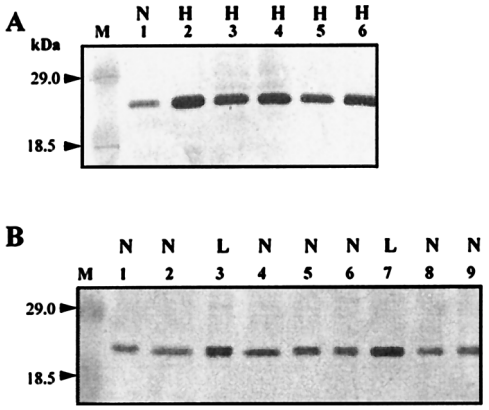

FIG. 3. Western analysis of AhpC levels in response to oxidants (A) and various stresses (B). (A) Total protein samples (30 μg) were separated on an SDS-PAGE gel, blotted, reacted with anti-*E. coli* AhpC antibody, and detected as described in Materials and Methods. Results are shown for uninduced cells (lane 1) and cells induced with 150 μM H_2O_2 (lane 2), 100 μM MD (lane 3), 100 μM paraquat (lane 4), 100 μM tBOOH (lane 5), and 100 μM cumene hydroperoxide (lane 6). (B) Sampling, gel electrophoresis, blotting, and immunodetection were performed as described for panel A. Lanes: 1, uninduced cells; 2 to 9, cells induced with SB medium at pH 5.0 (lane 2), SB medium at pH 8.5 (lane 3), 1.5 M NaCl (lane 4), 200 μM dipyridyl (lane 5), 200 μM diamide (lane 6), 200 μM NEM (lane 7), heat shock at 37°C (lane 8), and cold shock at 15°C (lane 9). N, noninduced; L, moderately induced (two- to threefold over the uninduced level); H, highly induced (increased four- to sixfold over the uninduced level).

neous dismutation to H_2O_2 is responsible for *ahpC* induction (15–17). Thus, the superoxide induction could be mediated via either superoxide (i.e., SoxRS) or peroxide (i.e., OxyR) sensor and activator systems. Alternatively, both systems could act synergistically to activate *ahpC*. In *E. coli* cells, which have both *oxyR* and *soxRS*, superoxide generators also induce *ahpC* and *ahpF* (16). In this system, OxyR and not SoxRS is responsible for the induction of *ahpC* and *ahpF* by superoxide generators. This favors the hypothesis that superoxide anions are being converted to H_2O_2 that in turn activates OxyR. However, the lack of *oxyR* and *soxR* mutants in *Xanthomonas* and closely related bacteria prevents a definitive answer regarding the mechanism of superoxide induction of *ahpC*. Only recently a *soxR* homolog from nonenteric bacteria was isolated from *Pseudomonas aeruginosa*, but its physiological roles have not been analyzed (23). The patterns of catalase (*katX* [28]), *ahpC*, and *oxyR* expression in response to oxidants in *X. campestris* pv. phaseoli showed many similarities and differences. All three genes were highly induced by superoxide generators, whereas peroxides were weak inducers of *katX* (8, 28) but potent inducers of *ahpC* and *oxyR*. Differences between the oxidant induction patterns of these genes imply that more than one mechanism may regulate these genes. A question remains of whether OxyR is acting as the sensor for different inducers and as a transcription regulator of *ahpC* in *X. campestris* pv. phaseoli. We are currently investigating the roles of OxyR in *ahpC* regulation.

Second, we examined the effects of various compounds that are known to induce oxidative stress on AhpC levels. The effects of the thiol reagents *N*-ethylmaleimide (NEM) and diamide were tested. In response to inducing concentrations of NEM, an accumulation of AhpC was observed (Fig. 3B, lane 7). Surprisingly, diamide had no effects on *ahpC* expression (Fig. 3B lane 6). This suggested that the two SH-depleting agents may operate by different mechanisms. The induction of *ahpC* by NEM is likely to be mediated by its ability to induce oxidative stress. Additionally, NEM can also directly inactivate AhpC and lead to further increase in oxidative stress and synthesis of AhpC (31).

Third, we examined *ahpC* expression in response to various stresses. The effects of temperature and osmotic and pH stresses on the steady-state levels of AhpC were investigated. Thermal stress as either heat (at 37 and 42°C) or cold (at 15 and 10°C) shock for 15 min or osmotic shock at 1.5 M NaCl for 15 min did not cause accumulation or reduction of AhpC (Fig. 3B). In contrast, pH stress did affect the expression of *ahpC*; exposure of *X. campestris* pv. phaseoli to an alkaline pH (8.5) produced a small (twofold) but consistent accumulation of AhpC (Fig. 3B, lane 3). Growth at acid pH (pH 5.0) did not affect the level of AhpC (Fig. 3B). *Xanthomonas* growth in SB medium did cause an increase in medium pH that reached its highest levels during the stationary phase (7). This is coincident with the highest resistance to organic hydroperoxides (40). Thus, alkaline pH induction of *ahpC* may partially contribute to the phenomenon.

Metals and *ahpC* expression. Metal ions play important roles in oxidative stress. They act as important cofactors for oxidants scavenging enzymes and/or regulatory proteins (9, 11, 14). Metal ions also catalyze formation of highly reactive oxygen radicals (17). In *X. campestris* pv. phaseoli, 100 μM $CdCl_2$ was a more potent inducer of *ahpC* than peroxides and superoxide generators (Fig. 3A and 4). We also tested the effect of other metal ions (i.e., cobalt, copper, manganese, nickel, and zinc [Fig. 4] and mercury [data not shown]) at 100 μM. None of these induced accumulation or caused any reduction in the steady-state levels of AhpC. Iron is known to induce oxidative stress. Moreover, iron deprivation induces synthesis of DirA, an AhpC homolog in *Corynebacterium diphtheriae* (39). On the contrary, *X. campestris* pv. phaseoli growth in media with excess iron (Fe^{3+} at 50 or 200 μM [Fig. 4]) or depleted of iron (in the presence of 200 μM dipyridyl [Fig. 3B, lane 5]) did not affect the levels of AhpC. *ahpC* induction by $CdCl_2$ is likely to be mediated by the well-known effects of the heavy metal ions to induce severe oxidative stress. Similarly, induction of an *ahpC* homolog in mice by oxidants, $CdCl_2$, and a thiol reagent has been reported (18).

Northern analysis of *ahpC*. To confirm that accumulation of AhpC induced by various stresses was due to increased transcription of *ahpC* and to assess levels of *ahpC* transcription induction in response to various inducers, a 367-bp sequence containing the coding region of *ahpC* was used to probe total RNA isolated from uninduced and induced cultures. The results are shown in Fig. 5. Uninduced expression levels of the gene were low but detectable. Addition of inducing concentrations of H_2O_2, tBOOH, MD, and $CdCl_2$ caused large increases

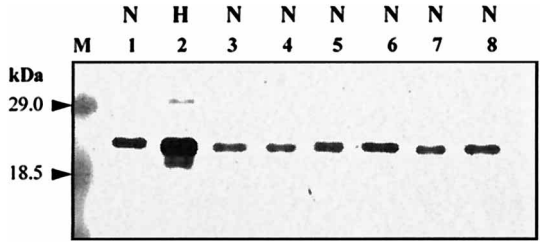

FIG. 4. Effects of metal ions on AhpC levels. Gel electrophoresis and immunological detection of AhpC were done as described in the legend to Fig. 3 and Materials and Methods. Unless otherwise stated, 50 μM metal ions was used as inducers. Lanes: 1, uninduced cells; 2 to 8, cells induced with $CdCl_2$ (lane 2), $CoCl_2$ (lane 3), $CuCl_2$ (lane 4), $FeCl_3$ (lane 5), 200 μM $FeCl_3$ (lane 6), $NiCl_2$ (lane 7), and $MnCl_2$ (lane 8). N, noninduced; H, highly induced (four- to sixfold over the uninduced level).

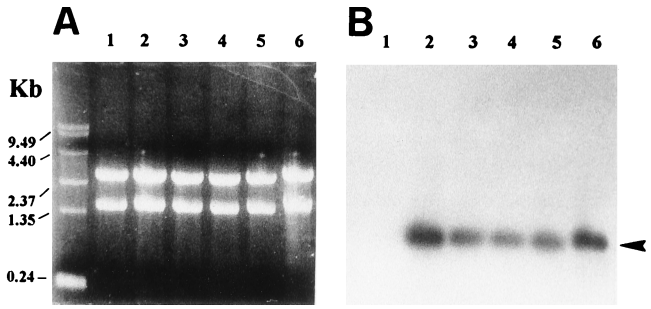

FIG. 5. Northern analysis of the effects of oxidants or $CdCl_2$ on *ahpC* mRNA levels. Total RNA samples were extracted from an uninduced *X. campestris* pv. phaseoli culture grown in SB medium (lane 1) and cultures induced with 50 μM $CdCl_2$ (lane 2), 200 μM H_2O_2 (lane 3), 100 μM MD (lane 4), 100 μM paraquat (lane 5), and 100 μM tBOOH (lane 6). Each lane contained 5 μg of RNA. (A) Ethidium bromide-stained gel showing the rRNA and RNA molecular weight markers (leftmost lane). (B) Hybridization signals of radioactive labelled *Bst*XI-*Bgl*II 367-bp *ahpC* probe with Northern blot of gel in panel A. The arrowheads indicate the position of 600-bp hybridizing transcripts.

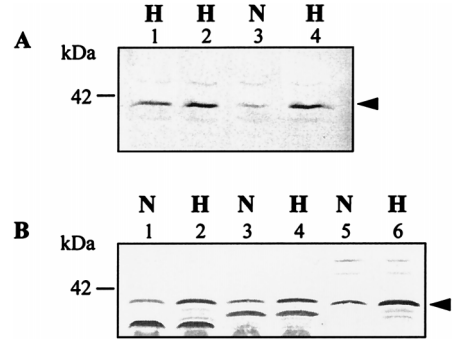

FIG. 7. Analysis of OxyR levels in response to oxidant treatments in *X. campestris* pv. phaseoli (A) and various *Xanthomonas* spp. (B). Sample preparation, gel electrophoresis, blotting, and immunodetection were performed as described in the legend to Fig. 3, except 50 μg of total protein plus anti-*Xanthomonas* OxyR antibody was used. (A) Lanes: 3, uninduced cells; 1, 2, and 4, cells induced with 100 μM MD (lane 1), H_2O_2 (lane 2), and tBOOH (lane 4). (B) Fifty micrograms of protein was prepared from uninduced cells (lanes 1, 3, and 5) and from cells induced with 100 μM tBOOH (lanes 2, 4, and 6). The conditions for electrophoresis, blotting, and antibody detection are as described for panel A. Lanes: 1 and 2, *X. campestris* pv. campestris; 3 and 4, *X. campestris* pv. malvacearum; 5 and 6, *X. oryzae* pv. oryzae. Additional bands detected were from nonspecific interactions. The position of OxyR is indicated by an arrowhead. N, noninduced; H, highly induced (increased four- to sixfold over the uninduced level).

in steady-state levels of *ahpC* mRNA. Densitometer analysis of the *ahpC* hybridization results adjusted for the rRNA bands indicated that $CdCl_2$ induced 20% more *ahpC* mRNA than other inducers. Although all oxidants were equally potent inducers of *ahpC*, the increase in *ahpC* mRNA levels appeared to be more dramatic than the increase in AhpC detected by Western blotting. This could be due to high uninduced levels and stability of AhpC, which reduced the magnitude of the observed induction, although we could not eliminate the possibility that additional regulation at the levels of translation of *ahpC* could exist. Nevertheless, the results confirmed that the increase in AhpC levels in response to inducers resulted from increased *ahpC* expression. These inducers were likely to exert their effects at the transcriptional levels. This is similar to observations in enteric bacteria, in which exposure to oxidants lead to transcription activation of *ahpC* via a peroxide sensor-transcription activator OxyR protein.

Analysis of dimer and monomer forms of AhpC in response to stress. We had observed that in the uninduced sample, all of the AhpC was in the multiple dimeric forms (D1 and D2, Fig. 6). In all cases, exposure of *X. campestris* pv. phaseoli to *ahpC* inducers led to increased formation of the active dimeric form (D1, D2, and D3, Fig. 6) (6). However, the monomer became more apparent with $CdCl_2$ induction (Fig. 6, lane 2), which produced the highest level of AhpC. The conversion of monomeric AhpC to the dimeric form could be important in formation of active enzyme during high rates of enzyme synthesis.

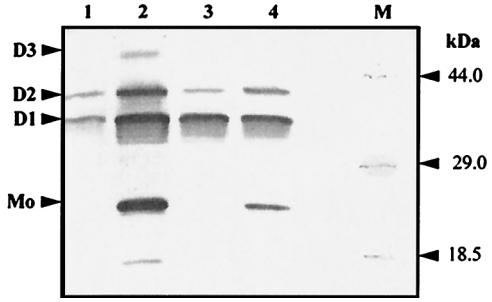

FIG. 6. Effects of oxidants and a heavy metal on AhpC dimerization. The effect of $CdCl_2$ (lane 2), MD (lane 3), and tBOOH (lane 4) on the levels of AhpC dimers (D1, D2, and D3) was investigated. The concentrations of various inducers were the same as in Fig. 3 and 4. The induction and Western blotting procedures were performed as described in Materials and Methods. Thirty-five micrograms of total protein was loaded into each lane, except in lane 1, in which 70 μg of protein from an uninduced sample was used. Mo, monomer.

Oxidant treatment leads to an increased OxyR concentration. In all bacterial systems thus far studied, OxyR responds to oxidative stress by changing from a reduced form to an oxidized form. Depending on the forms of the protein, OxyR can function either as a transcription repressor or as a transcription activator. OxyR is also self-regulated by acting as a repressor for its own gene transcription (20, 21, 37). The unusual arrangement of *oxyR* in *Xanthomonas* prompted us to investigate its response to oxidative stress (24). The level of OxyR was monitored with an anti-*X. campestris* pv. phaseoli OxyR antibody by Western analysis. The results are shown in Fig. 7. A three- to fourfold increase in the amount of OxyR was detected in *X. campestris* pv. phaseoli induced with H_2O_2, tBOOH, and MD. These oxidants produced similar induction levels. The increase in OxyR levels in response to oxidant treatments could be due either to increased protein stability or to increased expression of the gene. Subsequently, Northern analysis was performed with total RNA prepared from uninduced or MD- or tBOOH-induced *X. campestris* pv. phaseoli cultures with the *oxyR* coding region used as a probe. The results in Fig. 8 show that treatments with either tBOOH or MD lead to an increased level of *oxyR* transcripts. H_2O_2 produced induction levels similar to those of tBOOH (data not shown). The data indicated that oxidant treatments induced *oxyR* expression. Also, the oxidants were acting at the levels of transcription and not at the levels of protein stability. Contrary to the results of Western analysis of OxyR levels in response to oxidants, tBOOH induced higher levels of *oxyR* transcripts than MD (Fig. 7). We subsequently investigated the *oxyR* induction kinetic in response to H_2O_2, tBOOH, and MD. The results suggested that H_2O_2 and tBOOH produced a more rapid *oxyR* induction kinetic than MD. However, MD induction of *oxyR* lasts longer than that of peroxides (data not shown). The rapid *oxyR* induction kinetic induced by peroxides suggests that they could directly alter the redox state of the cells and result in immediate activation of *oxyR*, while the slower *oxyR* induction kinetic produced by MD implies that superoxide anions gen-

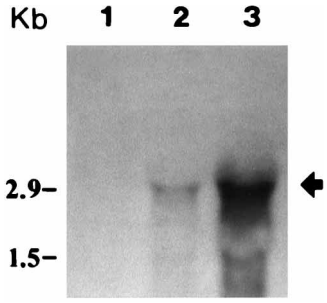

FIG. 8. Effects of oxidants on the levels of *oxyR* transcripts. Total RNA was extracted from uninduced cultures (lane 1) and from cultures induced with 100 μM MD (lane 2) and 100 μM tBOOH (lane 3). Each lane contained 25 μg of total RNA. Gel electrophoresis, blotting, and hybridization were performed as described in Materials and Methods. The filter was probed with the *oxyR* coding region (Fig. 1). The arrow indicated approximately 3.0 kb of polycistronic *ahpF-oxyR-orfX* mRNA. The molecular weights of RNA markers are indicated to the left.

erated by MD may have to be converted to H_2O_2, either enzymatically via superoxide dismutase or nonenzymatically, and H_2O_2 in turn induces *oxyR*. In addition, the rate of MD metabolism is considerably slower than the rate of peroxide metabolism. This could account for a slower but longer-lasting induction of *oxyR* by MD. Nevertheless, we could not rule out that superoxide anions may directly activate *oxyR* transcription via a SoxR-like transcription activator but less efficiently than peroxides. These possibilities are being investigated.

Northern and Western analyses of *oxyR* and *ahpC* expression indicated that under both uninduced and induced conditions, *ahpC* was expressed at much higher levels than the *ahpF-oxyR-orfX* operon (Fig. 3 and 7). This is consistent with the proposed model that AhpF is only required in a catalytic amount to regenerate AhpC (32). Also, OxyR, being a transcriptional regulator, is required in a small amount, and an excessively high concentration of the protein may lead to unregulated gene expression, which could be harmful to the bacteria. We have shown that increased expression of *ahpC* alone conferred only partial protection against alkyl hydroperoxides (24), and cooperative expression of all three genes is essential to the overall response to peroxide stresses.

Questions remain regarding the mechanisms with which peroxides activate *oxyR* expression and the protein that mediates this response. OxyR is a member of a LysR family of transcription activators that autoregulate their expression (33). Preliminary experiments suggested that *X. campestris* pv. phaseoli OxyR also functions as a redox-sensitive transcription activator or repressor (27). It is tempting to suggest that *Xanthomonas* OxyR could act as both a peroxide sensor and a transcription activator of its own gene. Alternatively, other redox-sensitive transcription regulators may activate the *oxyR* operon. The lack of *oxyR* mutants prevents differentiation between these possibilities. We are constructing an *oxyR* mutant with which to investigate the possibility of autoregulation of the *oxyR* operon.

Regulation of *oxyR* is conserved in various *Xanthomonas* strains. We were interested in whether the unique pattern of *X. campestris* pv. phaseoli *oxyR* expression was conserved in other *Xanthomonas* spp. Western analysis with an anti-*X. campestris* pv. phaseoli OxyR antibody was performed with uninduced and MD-induced samples from *X. campestris* pv. campestris, *Xanthomonas oryzae* pv. oryzae, and *X. campestris* pv. malvacearum. The results are shown in Fig. 7B. In three strains tested, MD-induced samples showed an increased amount of OxyR. This indicates that in response to oxidative stress, *Xanthomonas* strains increase the synthesis and accumulation of OxyR, and this unique response is conserved. It would be interesting to see if this novel *oxyR* response to oxidative stress operates in other bacterial systems.

Summary. The transcription organization of *Xanthomonas ahpC* and *ahpF-oxyR-orfX* suggests the possibility of a new regulatory circuit between these genes that is different from those of other bacteria. Analysis of *ahpC* expression patterns showed that the gene was highly induced by peroxides, superoxides, and heavy metals and was moderately induced by alkaline pH. Surprisingly, increased expression of *oxyR* was detected in response to treatments with peroxides and superoxides. These interesting results add another level of complexity to the mechanism by which *oxyR* regulates the oxidative stress response. Various inducers were acting at the levels of transcription which led to increased amounts of *ahpC* and *ahpF-oxyR-orfX* mRNA. In *Xanthomonas*, exposure to oxidants not only changed the redox state of OxyR but also increased its cellular concentration. These observations could be generally important as an alternative strategy by which bacteria respond to oxidative stress. This information may facilitate elucidation of novel regulation of the oxidative stress response in other bacteria. Also, the ability to increase expression of genes involved in stress protection (i.e., catalase [28] and *ahpC*) and regulation (*oxyR*) in response to stress is likely to play a crucial physiological role in oxidant-induced adaptive and cross-protection responses in *Xanthomonas* (29, 40).

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