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Isolation and Analysis of the *Xanthomonas* Alkyl Hydroperoxide Reductase Gene and the Peroxide Sensor Regulator Genes *ahpC* and *ahpF-oxyR-orfX*

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From *Xanthomonas campestris* pv. phaseoli, we have isolated by two independent methods genes involved in peroxide detoxification (*ahpC* and *ahpF*), a gene involved in peroxide sensing and transcription regulation (*oxyR*), and a gene of unknown function (*orfX*). Amino acid sequence analysis of AhpC, AhpF, and OxyR showed high identity with bacterial homologs. OrfX was a small cysteine-rich protein with no significant homology to known proteins. The genes *ahpC*, *ahpF*, *oxyR*, and *orfX* were arranged in a head-to-tail fashion. This unique arrangement was conserved in all of the *Xanthomonas* strains tested. The functionalities of both the *ahpC* and *oxyR* genes were demonstrated. In *X. campestris* pv. phaseoli, increased expression of *ahpC* alone conferred partial protection against growth retardation and killing by organic hydroperoxides but not by H_2O_2 or superoxide generators. These genes are likely to have important physiological roles in protection against peroxide toxicity in *Xanthomonas*.

The genus *Xanthomonas* belongs to an important family of plant bacterial pathogens. During bacterial interactions with plants, bacteria are exposed to plant-generated H_2O_2, organic peroxides, and superoxides, which are important components of the plant defense response (14, 26). Bacterial pathogens must overcome these reactive oxygen species to colonize the host. Thus, bacterial genes responsible for oxidative stress regulation and detoxification enzymes are likely to play major roles in disease development and progression.

Microbial defense against oxidative stress involves both primary detoxification of the stress and secondary repair processes. Expression of these enzymes is coordinated by several regulatory proteins, i.e., OxyR and SoxRS (7, 10, 23). In *Xanthomonas*, we have shown that high-level expression of catalase provides protection against H_2O_2 toxicity but not against alkyl hydroperoxides. The best-characterized bacterial defense factor against organic hydroperoxides is alkyl hydroperoxide reductase (AhpR) (3, 10, 24). The enzyme has two subunits, AhpC (a 22-kDa protein) and AhpF (a 54-kDa protein [20, 24]). AhpC belongs to the highly conserved family of AhpC/TSA proteins involved in reduction of highly toxic organic hydroperoxides to corresponding alcohols (4). AhpF shares homology to other thioredoxin reductase enzymes, and its main function is to regenerate AhpC (19). In enteric bacteria and *Mycobacterium* spp., *ahpC* is regulated by OxyR (5, 7, 8). OxyR is a global regulator of the peroxide stress regulon (7, 23, 25). It functions both as a peroxide sensor and as a transcription regulator of genes involved in peroxide stress protection (25).

Homologs of *ahpC*, *ahpF*, and *oxyR* have been identified in several bacteria. In most bacteria, the *ahpC* and *ahpF* genes are arranged in close proximity, and in some cases they have been shown to be coregulated (1, 3, 19, 24). While *oxyR* is usually not located nearby, an exception to this typical organization is in *Mycobacterium* strains in which *oxyR* is located 5′ of *ahpC* and transcribed in the opposite direction to it. No *ahpF* homolog has been found in close proximity to these genes (8, 29). Here, we reported the isolation of the *ahpC*, *ahpF*, and *oxyR* homologs and their genome and transcription organization in various *Xanthomonas* strains.

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MATERIALS AND METHODS

Bacterial strains, growth, and transformation. The following *Escherichia coli* strains and their relevant genotypes were used: K-12 (wild type), GSO8 (*oxyR* [12, 13]), TA4315 (*ahpCF*Δ [24]), and UM2 (*katE katG* [P. Loewen]). All *E. coli* and *Xanthomonas* strains were grown aerobically at 37 and 28°C on Luria-Bertani and Silva-Buddenhagen media, respectively. Ampicillin was used at 100 μg/ml for both *E. coli* and *Xanthomonas* strains. Routinely, *E. coli* was transformed by a chemical method, while *Xanthomonas* was electroporated under previously described conditions (17).

Construction of pKS-ahpC and pUFR-ahpC. The 1-kbp sequence from an *Nco*I to an *Hin*cII site from pAhp4-1 (Fig. 1) was subcloned into pKS vector, resulting in pKS-aphC. Similarly, pUFR-ahpC was constructed by ligation of the 1-kbp *Nco*I-*Hin*cII fragment into pUFR047, a broad-host-range *Inc*W expression vector (6) digested with *Sma*I.

Nucleotide sequencing. pAhp4-1 was sequenced in both directions from a *Cal*I site to a vector *Eco*RI site. Similarly, a 2.0-kbp DNA fragment between an *Eco*RI site and the second *Xho*I site of pOXX was sequenced. Both plasmids were sequenced by the primer walking technique with an ABI Prism kit on an ABI 373 automated DNA sequencer.

Disc diffusion killing zone method. Log-phase cells (10^8) were mixed with top agar (0.5% SB agar) and poured on top of SB plates. Various chemicals at appropriate concentrations were placed on 6-mm-diameter paper discs made from Whatman filter paper and put on top of a lawn of cells. The diameter of the cleared zone was measured after 24 h of incubation. For *E. coli*, SB medium was replaced with Luria-Bertani medium.

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in GenBank and has been assigned accession no. U94336.

RESULTS AND DISCUSSION

Cloning of *ahpC* by reverse genetics. Comparison of amino acid sequences of the AhpC family of proteins revealed highly conserved regions (4), which were suitable for application of reverse genetics and PCR gene isolation techniques. The corresponding nucleotides of the conserved amino acid motifs at positions 42 to 50 (DFTFVCPTE) and 163 to 170 (GEVCPA

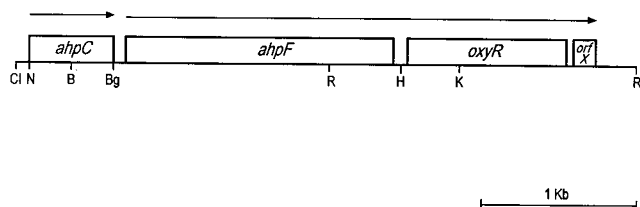

FIG. 1. Organization of *ahpC*, *ahpF*, *oxyR*, and *orfX* in *X. campestris* pv. phaseoli. The arrows indicate the direction and length of the transcripts. B, *Bst*XI, Bg, *Bgl*II; Cl, *Cla*I; *H, Hin*dIII; K, *Kpn*I; N, *Nco*I; R, *Eco*RI.

KW) of *E. coli* AhpC were used to synthesize degenerate oligonucleotide primers for PCRs (4). To reduce primer degeneracy and complexity, it was taken into account that *Xanthomonas* frequently used G or C in the last position of the codons. The following primers were used to amplify *Xanthomonas campestris* pv. phaseoli genomic DNA: 5′ahpC, 5′ GAC TTC ACX TTC GTX TGC CCX ACX GA 3′; and 3′ahpC, 5′ CCA CTC XGC XGG ACA XAC CTC XCC 3′ (where X is for G and C). The resulting 390-bp PCR product corresponding in size to that expected of *ahpC* was cloned into pGEM-T vector (Promega), and the sequence was determined. The predicted translation products of this partially sequenced clone of *ahpC* showed a high degree of homology to AhpC sequences from several bacteria. Thus, they were used as probes to screen an *X. campestris* pv. phaseoli genomic library constructed in a ZipLox vector (Bethesda Research Laboratories [24]). Several positive clones were isolated, and plaques were purified. Many isolates shared internal fragments that cross-hybridized with the *ahpC* probe. One such clone, pAhp 4-1, was completely sequenced in both directions from a *Cla*I site to the vector *Eco*RI site. Analysis of the sequence revealed three open reading frames (ORFs). The predicted amino acid sequences of these ORFs were used to search GenBank, and the results showed that the first ORF had high homology with the AhpC family of proteins. The complete second and the third truncated ORFs showed homology to AhpF, a subunit of AhpR (1, 19, 24), and OxyR, a peroxide stress sensor and transcription regulator, respectively (8, 11, 25).

Isolation of *oxyR*. A plasmid, pOXX, was isolated from an *X. campestris* pv. phaseoli plasmid expression library by complementation of an H_2O_2-hypersensitive phenotype of an *E. coli oxyR* mutant, GSO8 (12, 13). GSO8 harboring pOXX was more resistant to H_2O_2, with a killing zone diameter of 2.2 cm compared with 2.8 cm for GSO8 harboring pKS vector only. Deletion analysis of pOXX indicated that the *oxyR* complementation activity was located on a 2.0-kb DNA fragment between an *Eco*RI site and the second *Xho*I site. The fragment was completely sequenced. Sequence analysis indicated a partial first ORF with homology to AhpF, a complete second ORF homologous to OxyR, and an unknown protein, ORFX (Fig. 1). Although pAhp 4-1 and pOXX were independently isolated, they had overlapping regions.

Gene organization in *X. campestris* pv. phaseoli. In *Xanthomonas*, *ahpC*, *ahpF*, and *oxyR* showed an unusual organization. These genes were arranged in head-to-tail fashion in the following order: *ahpC*, *ahpF*, *oxyR*, and *orfX* (Fig. 1). Each of the ORFs had a strong ribosome binding site preceding the translation initiation codons. The first and the second ORFs were separated by 213 bp, the second and the third by 91 bp, and the third and the fourth by 73 bp.

Analysis of *ahpC* expression in several microbes shows unusual patterns, suggesting the possibility that more than one copy of the gene could exist (1, 27). We performed Southern hybridization of *X. campestris* pv. phaseoli genomic DNA digested individually with five restriction enzymes and probed with the coding regions of *ahpC*, *ahpF*, and *oxyR*. The hybridization results suggested that only one copy of these genes was present in *X. campestris* pv. phaseoli (data not shown).

In the accompanying paper, we have analyzed the transcription organization of these genes (18). The results indicate that *ahpC* is organized as a monocistronic gene, whereas the *ahpF-oxyR-orfX* genes are arranged in an operon (18).

Primary structural analysis of AhpC and AhpF. The predicted first ORF (AhpC) encoded a 20.4-kDa protein that had size similar to that of the other bacterial AhpC. *Xanthomonas* AhpC showed highest identity to AhpC from *E. coli* (57%) and *Staphylococcus aureus* (50%); the percentage of identity to other bacterial AhpC homologs dropped dramatically to around 30% compared with those of homologs from *Mycobacterium tuberculosis*, *Sulfolobus* sp., and *Corynebacterium diphtheriae* (Fig. 2). This suggests a possible subgroup of AhpC. There is higher sequence identity within members of each group than between the groups. The low identity between the two groups could reflect differences in enzyme mechanisms or substrate specificity. Lack of biochemical characterization of AhpR in many of these bacteria prevents a more definitive analysis.

In general, the family of AhpC proteins can be subdivided into two groups on the basis of whether they contain one or two cysteine residues (4). *Xanthomonas* AhpC belonged to the family of antioxidant proteins containing two cysteines (3).

Amino acid sequence comparisons of the second ORF showed that *Xanthomonas* AhpF shared 67 and 61% identity to *Salmonella typhimurium* (20, 24) and *Bacillus subtilis* (1, 3) AhpF (Fig. 3). The high degree of homology between these proteins suggested that they might have similar enzyme mechanisms. Cysteine residues involved in disulfide bridges, an ac-

```
     1
ECO  MSLINT----   ---KIKPFKN   QAF---KNGE   ----FIEITE   KDTEGRWSVF
XAN  ......----   ---QVQ...A   N.YH---..N   ----...V..   ASLK.K...L
STA  G...K-----   ---E.L..TA   ...DDP.KDQ   ----.K.V.Q   E.LK.S...V
DIR  ..ILTVGEKF   PEFNLTAL.G   GDLHDVNASQ   PEDY.ETVSL   DKY..K.K.V
MYC  .P.LTIGDQF   PAYQLTALIG   GDLSKVDAKQ   PGDY.TT..S   DEHP.K.R.V
SUL  .K.Y---QKF   PETQVITT.G   ----------   PLDFYRDVF.   ---K.K.LFL

    37            ∇
     FFYPADFTFV   CPTELGDVAD   HYEELQKLGV   DVYAVSTDTH   FTHKAWHSSS
     I.M..A...N   ....VE.A..   N.AAF..A.A   E..I.T....   .S..V..ET.
     C......S..   .....E.LQN   Q.........   N.FS......   .V.....DH.
     ....K.....   ....IAAFGK   LD..F.DRDT   QILGG.I.NE   .S.FN.RATH
     ..W.K.....   ....IAAFSK   LND.FEDRDA   QILG..I.SE   .A.FQ.RAQH
     .AH.....P.   .T..FVGFSK   V...FKR.N.   ELVGM.V.SI   YS.IE.LKDI

    87
     ETI--AKIKY   AMIGDPTGAL   TRNFDNMRED   EGLADRATFV   VDPQGIIQAI
     PAV--G.AQF   PL.....HK.   ..A.GVHI.E   ....L.G..I   IN.E.V.KTL
     DAI--S..T.   T.....SQTI   .....VLD.A   T...Q.G..I   I..D.VV..S
     PEL--KTVPF   PLFS.IKHD.   IKALGVE-NE   ..V......I   I..D....FV
     NDL--KTLPF   P.LS.IKRE.   SQAAGVL-NA   D.V...V..I   ...NNE..FV
     QERYGIQVPF   PL.A..DKR.   A.LL.IID.A   S.VTI..V.L   .N.E...RFM

   135                                     ∇
     EVTAEGIGRD   ASDLLRKIKA   AQYVASHPGE   VC-PAKWKEG   EATLAPSLDL
     .IHDNS.A..   VTET...LT.   ..F..NN..Q   ..-.......   AK........
     .IN.D.....   ..T.AH....   ....RKN...   ..-....E..   AK..Q.G...
     S..PDAV.CN   VDEV..VLD.   L.-----SE.   ..-ACN.QKN   DP.KNIDKFA
     SA..GSV..N   VDEV..VLDA   L.-----SD.   L.-ACN.RK.   DP..DAGEL.
     AYYPIEY..K   IEE...IT..   .--LVNYKAK   .SL.VD.EP.   QEVIV.APST

   184
     VGKI
     ....
     ....
     ELEKGLN
     KASA
     IDEAQIRMKL   PNAKTWYLTF   KKYDELPQDQ   RVV
```

FIG. 2. Comparison of bacterial AhpC amino acid sequences. The sequences were aligned by the Clustal W program (28). ECO, *E. coli* D13187); XAN, *X. campestris* pv. phaseoli (U94336); STA, *S. aureus* (2); DIR, *C. diphtheriae* (27); MYC, *M. tuberculosis* (8); and SUL, *Sulfolobus* sp. (U36479). Gaps were introduced to maximize the fit. Numbers on top are according to the sequence of *E. coli* AhpC. Arrowheads indicate highly conserved cysteine residues, dashes represent gaps, and dots represent amino acid residues identical to those in the *E. coli* sequence.

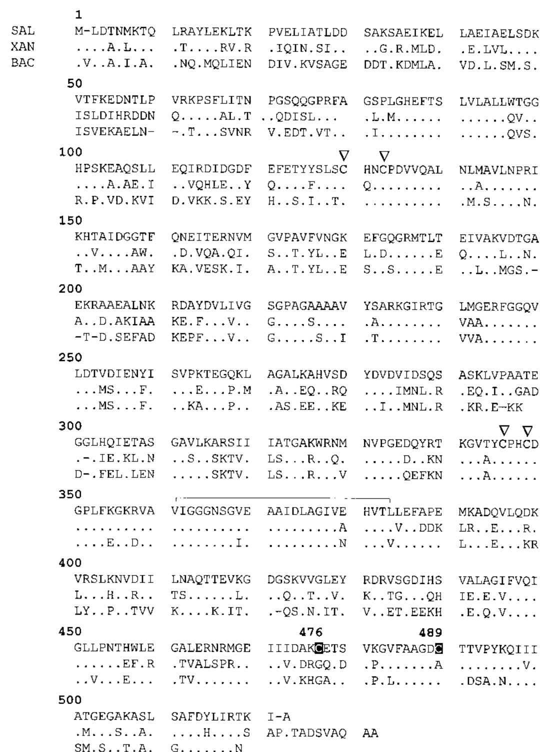

FIG. 3. Multiple alignment of bacterial AhpF amino acid sequences. Comparison of AhpF amino acid sequences from *S. typhimurium* (SAL;G153863), *X. campestris* pv. phaseoli (XAN;U94336), and *B. subtilis* (BAC,D78193) aligned by the Clustal W program (28). Gaps were introduced to maximize the fit. The numbers on top were according to *S. typhimurium* AhpF numbering. The cysteine residues involved in a disulfide bridge and in an active site (▽) are shown. Residues in the conserved NAD(P)H binding site are overlined.

tive site (C129 to C132 and C345 to C348 in *Salmonella*), and the NAD(P)H binding domain were all conserved in *Xanthomonas* AhpF (Fig. 2) (15). Two cysteine residues at C476 and C489 were substituted for with G and A residues, respectively, in *Xanthomonas* AhpF, indicating that these residues were not essential for enzyme activity.

Amino acid sequence analysis of OxyR. Comparison of *Xanthomonas* OxyR with OxyR from *E. coli* (12, 13), *Erwinia carotovora*, *Haemophilus influenzae* (11), and *Mycobacterium* (4) showed overall 47, 47, 45, and 42% identity, respectively (Fig. 4). Extensive structure-function analysis has been done for *E. coli* OxyR, and detailed examination of OxyR amino acid sequences revealed many important features, such as the helix-turn-helix motif, the redox-sensitive C199 residue, and residues involved in DNA binding and multimerization (12, 13). These residues were highly conserved among all four OxyR homologs. Amino acid residues involved in OxyR peroxide-inducible activation of transcription were also highly conserved, except at residues H114 and G253 (8, 9), which were changed to R and E residues, respectively, in *X. campestris* pv. phaseoli OxyR. Interestingly, the H114 residue was not conserved among the five homologs, while the G253 residues were identical in *E. coli* and *H. influenzae* (Fig. 4). These two nonconserved residues may reflect minor differences in the ability of OxyR homologs to inducibly activate transcription.

OxyR belongs to a well-characterized LysR family of transcription activators (12, 13, 25). For at least two members of the LysR family (i.e., OxyR and NahR), the region around the carboxy terminus of each protein has been shown to be crucial for their function (12, 13, 21). However, little homology was detected in the region close to the carboxy termini of *X. campestris* pv. phaseoli OxyR and other OxyR homologs. On the other hand, there was some conservation in this region for *E. coli* and *H. influenzae* OxyR sequences. Despite differences in the carboxy-terminal regions, other amino acid residues important to the *E. coli* OxyR repression mechanism were all highly conserved in OxyR. The disparity in the *X. campestris* pv. phaseoli OxyR carboxy-terminal regions could be due to differences in the mechanisms by which these proteins negatively regulate their own expression. We are investigating these possibilities. Nonetheless, *X. campestris* pv. phaseoli *oxyR* can functionally substitute for *E. coli oxyR* in activation of the catalase gene that results in complementation of the H_2O_2-sensitive phenotype of GSO8 (see "Isolation of *oxyR*").

Analysis of OrfX. The fourth ORF identified had a coding potential for 78 amino acid residues, an 8-kDa protein. The putative protein, designated OrfX, was an alanine (19 alanine residues)- and cysteine (7 cysteine residues)-rich protein (Fig. 5). A search of GenBank did not reveal any homolog to the OrfX amino acid sequence. OrfX had a pI of 8.9, indicating that at physiological pH it would have a positive charge. This suggested that it could interact with negatively charged cellular components (proteins or DNA). *orfX* was located 3′ of *oxyR* and was transcribed in an operon with *ahpF-oxyR* (18).

Functional integrity of the cloned *ahpC* and *oxyR*. The functionality of the cloned *ahpC* was tested by complementation analysis with various peroxide-sensitive *E. coli* mutants. pKS-ahpC was used to transform *E. coli* strains TA4315 (*ahpCF*Δ), UM2 (*katG katE*), and K-12 (wild type). The results of peroxide sensitivity tests with oxidants by the disc diffusion method

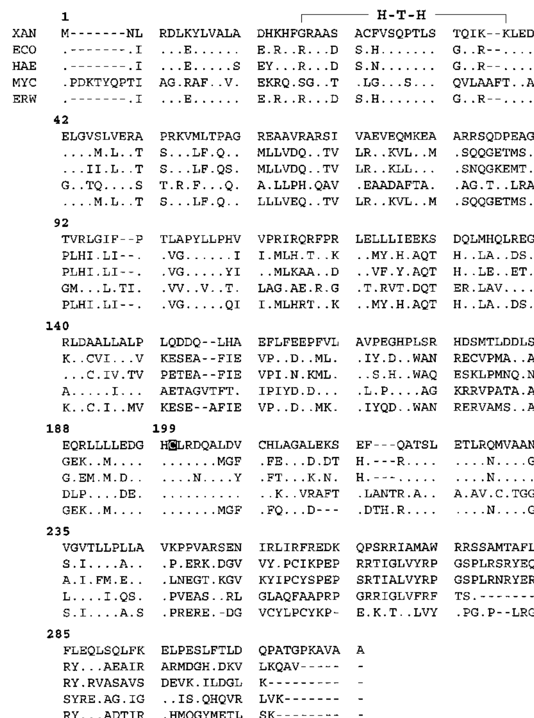

FIG. 4. Amino acid sequence comparison of OxyR from *E. coli* (ECO; X16531), *X. campestris* pv. phaseoli (XAN;U94336), *E. carotovora* (ERW; U74302), *M. avium* (MYC; U18263), and *H. influenzae* (HAE;U32847). Amino acid sequence alignment was carried out with the Clustal W program (28). H-T-H, helix-turn-helix motif.

```
    --------------- Oxy R --------------- ►
  1 CCGGCAACGGGACCGAAGGCGGTTGCGGCTTAGGGAATCGGGAATCGGGAATCGTAAAAG   60
     P  A  T  G  P  K  A  V  A  A  *

 61 CGTATCGCGGTTGCGATCGCTTTGGCTGTGGTTGGAGGTTTGGAAATGGGCGGCGCGCGA  120
                                                M  G  G  A  R

121 GCGTCGCTTTGTGTTTGTGCGCAGCCCTCTTGCCCAAGGTTGGCGGCGCATCAATCCGCC  180
     A  S  L  C  V  C  A  Q  P  S  C  P  R  L  A  A  H  Q  S  A

181 GCTGCCTGCTCGACGCGGCTGGCGGCGGCGCTGCCCACGCGACCATGCAATGTACGCG  240
     A  A  C  S  T  R  L  A  A  A  R  C  P  R  D  H  A  M  Y  A

241 CAGCGTCTTGTTCGCTTGCTTGACACCGATAGTGCGGCGTTTGCGGCATGCGCTGGGAGC  300
     Q  R  L  V  R  L  L  D  T  D  S  A  A  F  A  A  C  A  G  S

301 AGGCTCGTGTGCAGCGGCCGTCATGCCATCCCCTCCAATTGACCCAACCGCAGCGCTTGC  360
     R  L  V  C  S  G  R  H  A  I  P  S  N  *

361 TCGCCGCCCCAGGCCCGCAGCGACTGCCTGGCGGCCCTGACGGTGTCCTGGCTTGGCACC  420
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FIG. 5. Nucleotide and deduced amino acid sequences of *orfX*. A putative ribosome binding site of *orfX* is in boldface. The amino terminus of the OxyR sequence is also shown.

showed that TA4315, UM2, and K-12 cells harboring pKS-ahpC were more resistant to tBOOH than mutants harboring only the vector plasmid. (Typical growth inhibition zone values from four independently performed experiments were 2.3, 1.7, and 1.6 cm for the *ahpC* transformants and 3.3, 2.5, and 2.3 cm for the mutants.) The results suggested that cloned *ahpC* was functional and that increased expression of the *ahpC* subunit alone was sufficient to confer resistance to ROOH (i.e., increased resistance to tBOOH killing in TA4315, a mutant lacking both AhpC and AhpF, harboring pKS-ahpC). This is consistent with the proposed model that the AhpC subunit alone can directly reduce ROOH to corresponding alcohols and that AhpF is only required for regeneration of AhpC (19, 20, 24).

X. campestris pv. phaseoli *oxyR* was isolated on the basis of the gene's ability to functionally complement hypersensitivity to the H_2O_2 phenotype of an *E. coli oxyR* mutant. Deletion analysis was performed to localize the complementation activity of pOXX. Removal of the non-*oxyR* coding sequence from pOXX and subsequent placement of the *oxyR* coding region into an expression vector showed that the new recombinant plasmid retained the ability to confer H_2O_2 resistance to an *oxyR* mutant (data not shown). This confirmed that we had isolated a functional *oxyR* gene.

***ahpC* expression and organic peroxide resistance in *X. campestris* pv. phaseoli.** To investigate the effects of increased expression of a cloned *ahpC* gene on the physiological response of *X. campestris* pv. phaseoli to oxidative stress, *ahpC* was cloned into pUFR047, and the resulting plasmid, pUFR-ahpC, was used to transform *X. campestris* pv. phaseoli. *X. campestris* pv. phaseoli harboring pUFR-ahpC produced about twofold more AhpC than *X. campestris* pv. phaseoli harboring pUFR047 vector (data not shown). The effects of low concentrations of oxidants on growth and high concentrations of oxidants on survival were examined. *X. campestris* pv. phaseoli harboring pUFR-ahpC showed better growth in the presence of growth-inhibitory concentrations of tBOOH (doubling time [Td] of 3.7 h compared with a Td of >8 h in *X. campestris* pv. phaseoli harboring only the pUFR047 vector) (Fig. 6). However, increased *ahpC* expression alone did not fully protect *X. campestris* pv. phaseoli from the growth inhibition effects of tBOOH. This was evident from the lower growth of *X. campestris* pv. phaseoli harboring pUFR-ahpC in the presence of tBOOH (Td, 3.7 h) than in its absence (Td, 2.8 h) (Fig. 6). Similar effects on the growth rate were observed when tBOOH was replaced with CuOOH (data not shown). By the disc diffusion killing zone method, *X. campestris* pv. phaseoli cells harboring pUFR-ahpC were exposed to killing concentrations

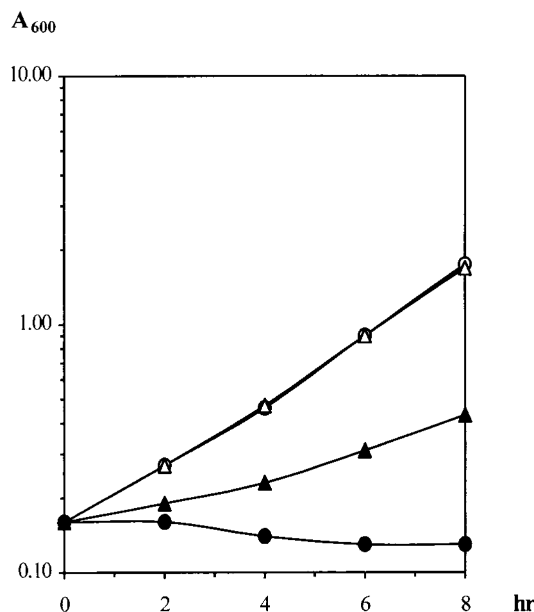

FIG. 6. Growth curves of *X. campestris* pv. phaseoli harboring pUFR047 (○) or pUFR-ahpc (△) in the absence (○, △) or presence (●, ▲) of a growth-inhibitory concentration (0.4 mM) of tBOOH.

of various agents, and the results are shown in Table 1. Increased *ahpC* expression alone was sufficient to confer protection against killing concentrations of tBOOH and CuOOH. No protection was evident for H_2O_2, menadione, *N*-ethylmaleimide, and $CdCl_2$, all potent inducers of *ahpC* (18).

In *Mycobacterium*, increased expression of *ahpC* is thought to be a compensatory mutation to a mutation in *katG* which makes cells vulnerable to H_2O_2 toxicity (8, 9, 22, 29). Conversely, in *B. subtilis*, *ahpC* mutants show increased expression of a *kat* gene. These observations suggest a close interregulated relationship between *kat* and *ahpC*. Additionally, purified AhpR enzyme can use H_2O_2 as a substrate (19, 20). Nonetheless expression of cloned *ahpC* in *X. campestris* pv. phaseoli did not enhance protection against H_2O_2 toxicity. On the contrary, we observed a small (30%) decrease in catalase activity in *X. campestris* pv. phaseoli cells harboring pUFR-ahp (data not shown). Additionally, AhpR might play a less important role than catalase in the protection against H_2O_2 toxicity. This is consistent with our observations that increased catalase levels alone are sufficient to protect *Xanthomonas* from H_2O_2 killing (17).

The partial protection against ROOH in *X. campestris* pv. phaseoli by the cloned *ahpC* gene (Fig. 7) can be accounted for

TABLE 1. Effect of increased expression of *ahpC* alone on sensitivity of *X. campestris* pv. phaseoli to various oxidants and chemicals[a]

X. campestris pv. phaseoli plasmid	Growth inhibition zone value (cm)					
	tBOOH (0.5 M)	CuOOH (0.1 M)	Menadione (0.5 M)	*N*-Ethylmaleimide (0.1 M)	$CdCl_2$ (0.2 M)	H_2O_2 (0.2 M)
pUFR047	3.0	1.7	2.4	2.8	2.1	1.5
pUFR-ahpC	2.2	1.3	2.3	2.9	2.0	1.6

[a] The experiments were performed as described in Materials and Methods and were repeated at least three times. The results shown represent average values.

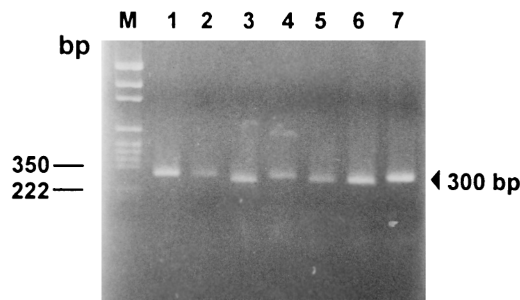

FIG. 7. Conservation of *ahpC* and *ahpF* organization in various *Xanthomonas* species. The primer set corresponded to 3′*ahpC* (5′ ACCTGGTCGGCAA GATCTAA 3′) and 5′*ahpF* (5′TCGATGCGTTGATTTGAATC 3′). The following PCR conditions were used: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min. PCR products were obtained from genomic DNA with the following (by lane): 1, *X. campestris* pv. phaseoli; 2, *X. campestris* pv. glycine; 3, *X. vesicatoria*; 4, *X. campestris* pv. campestris; 5, *X. translucens*; 6, *X. oryzae* pv. oryzae; and 7, *X. oryzae* pv. orizicola; M, pGEM (Promega) molecular weight markers. The arrowhead to the right indicates the position of the expected 300-bp PCR products.

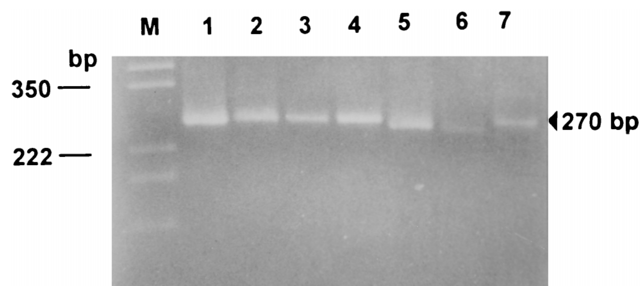

FIG. 8. Organization of *ahpF* and *oxyR* in various *Xanthomonas* species. PCR conditions were as described in the legend to Fig. 4. The primer set corresponded to 3′*ahpF* (5′ ATGGGCGAAGGTTCCAA 3′) and 5′*oxyR* (5′ GGCTGACAA AGCAGGC 3′). PCR products were obtained from genomic DNA with the following (by lane): 1, *X. campestris* pv. phaseoli; 2, *X. campestris* pv. glycine; 3, *X. oryzae* pv. orizicola; 4, *X. vesicatoria*; 5, *X. oryzae* pv. oryzae; 6, *X. translucens*; and 7, *X. campestris* pv. campestris. M, pGEM (Promega) molecular weight markers. The arrow to the right indicates the position of the expected 270-bp PCR products.

by the fact that AhpC can undergo one round of ROOH reduction and that AhpF is required in catalytic amounts to regenerate AhpC for additional rounds of ROOH reduction. Thus, under a condition of increased *ahpC* expression alone, the level of AhpF could be a limiting factor in regenerating AhpC (19, 20). This indicates that coordinate expression of *ahpC* and *ahpF* is crucial to overall levels of resistance to ROOH. In some bacteria, *ahpC* and *ahpF* are coregulated in an operon (1, 3, 24). In *X. campestris* pv. phaseoli, the atypical organization of *ahpC* as a monocistronic gene and *ahpF* in an operon together with *oxyR* (a known regulator of other bacterial *ahpC*) raises important questions regarding the regulation of these genes. We have shown that peroxide stress induced expression of both *ahpC* and *ahpF-oxyR* (18). This suggests that coordinate regulation of these three genes is required for full protection against ROOH. A possible mechanism is that OxyR, in addition to acting as a transcription regulator of *ahpC*, could self-regulate the *ahpF-oxyR* operon. This has interesting implications regarding the regulation of these genes, which we are investigating. Additionally, we have identified in *Xanthomonas* a second novel ROOH protection system not related to AhpR (16). Protection against ROOH toxicity is likely to be a result of combined contributions from both systems. Thus, overexpression of AhpC alone may not have dramatic effects on levels of resistance to ROOH.

This highly conserved structural and regulatory mechanism of the *ahpC* gene from bacteria to mammals (4) suggests important roles the enzyme plays in oxidative stress protection. We have attempted unsuccessfully to make a marker exchange *ahpC* mutant and are currently investigating whether the gene is essential to *X. campestris* pv. phaseoli.

Organization of *ahpC*, *ahpF*, and *oxyR* in *Xanthomonas* species. The organization of genes is usually conserved among strains of a single species of bacteria and sometimes among species of a single genus. However, variations in the organization of *ahpC*, *ahpF*, and *oxyR* homologs have been found even among strains of a single species of bacterium (8). In *X. campestris* pv. phaseoli, *ahpC*, *ahpF*, *oxyR*, and *orfX* were arranged in a head-to-tail fashion (Fig. 1). To determine whether this organization was conserved in other *Xanthomonas* species, PCR of genomic DNA was carried out with two sets of primers. Each primer set was designed to correspond to the 3′ end of one gene and the 5′ end of an adjacent gene. Two sets of primers were made to localize *ahpC-ahpF* (3′*ahpC* and 5′*ahpF*) or *ahpF-oxyR* (3′*ahpF* and 5′*oxyR*). The results of the PCRs are shown in Fig. 7 and 8. With the first set of primers (3′*ahpC* and 5′*ahpF*), PCRs with genomic DNA from various *Xanthomonas* species gave the expected 300-bp fragments (Fig. 7). PCRs with the second set of primers (3′*ahpF* and 5′*oxyR*) in the same way yielded the expected PCR products of 270 bp from *X. campestris* pv. phaseoli and similar-size fragments for other *Xanthomonas* species (Fig. 8). In both sets of PCRs, minor differences in length were observed among the different *Xanthomonas* strains. These results were not entirely unexpected. Minor variations in length in the nonconserved intergenic regions between conserved gene sequences have been noted. The results support the notion that *ahpC*, *ahpF*, and *oxyR* are arranged in a head-to-tail fashion and are also separated by similar distances for all *Xanthomonas* species examined.

In *Xanthomonas*, *ahpC* was located close to *ahpF*, and this arrangement is similar to that in other bacteria (1, 3, 19). On the other hand, the location of *oxyR* behind *ahpF* and in an operon has not been observed in other bacteria. The conservation in this novel gene arrangement suggests that it may play an important role in the regulation of these peroxide stress protection genes and in the overall physiological response to peroxide stress in *Xanthomonas*. It remains to be seen whether other bacteria have an arrangement of genes and a pattern of peroxide stress response similar to those of *Xanthomonas.*

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