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

**SUVIT LOPRASERT,1 SOPAPAN ATICHARTPONGKUN,1 WIORGRONG WHANGSUK,1 AND SKORN MONGKOLSUK1,2*  

Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok 10210, 1 and Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, 2 Thailand

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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 H₂O₂ 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 H₂O₂, 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 H₂O₂ 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.

**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 (*ahpCFA* [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 *Eco* site to an *Hin* site from pAhp1 (Fig. 1) was subcloned into pKS vector, resulting in pKS-ahpC. Similarly, pUFR-ahpC was constructed by ligation of the 1-kbp *Eco* fragment into pUFR047, a broad-host-range IncW expression vector (6) digested with SmaI.

**Nucleotide sequencing.** pAhp1-1 was sequenced in both directions from a *Cul* site to a vector *EcoRI* site. Similarly, a 0.2-kbp DNA fragment between an *EcoRI* site and the second *Xho* site of pOX1 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 (10⁹) 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 (GEVCP
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 (56%); 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, 21) and Bacillus subtilis (11, 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-
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). However, little homology was detected in the region close to the carboxy terminus 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 H2O2-sensitive phenotype of GSO8 (see "Isolation of oxyR").

Analysis of OrfX.

The fourth ORF identified encoded a protein of 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 39 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), UM2 (katG katE), and K-12 (wild type). The results of peroxide sensitivity tests with oxidants by the disc diffusion method indicated that pKS-ahpC could complement all three peroxide-sensitive mutants (Fig. 3). These results suggested that ahpC could play a role in the protection of E. coli against peroxide stress.

FIG. 3. Multiple alignment of bacterial AhpF amino acid sequences. Comparison of AhpF amino acid sequences from S. typhimurium (SAL;G15368), 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 (V) are shown. Residues in the conserved NAD(P)H binding site are overlined.

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.
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) 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). X. campestris pv. phaseoli oxyR was isolated on the basis of the gene’s ability to functionally complement hypersensitivity to the H2O2 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 H2O2 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 2.8 h) (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 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 H2O2, menadione, N-ethylmaleimide, and CdCl2, 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 H2O2 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 H2O2 as a substrate (19, 20). Nonetheless, expression of cloned ahpC in X. campestris pv. phaseoli did not enhance protection against H2O2 toxicity. On the contrary, we observed a small (30%) decrease in catalase activity in X. campestris pv. phaseoli harboring pUFR-ahpC (data not shown).

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

<table>
<thead>
<tr>
<th>X. campestris pv. phaseoli plasmid</th>
<th>Growth inhibition zone value (cm)</th>
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<tbody>
<tr>
<td></td>
<td>tBOOH (0.5 M)</td>
</tr>
<tr>
<td>pUFR047</td>
<td>3.0</td>
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<tr>
<td>pUFRAhpc</td>
<td>2.2</td>
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*The experiments were performed as described in Materials and Methods and were repeated at least three times. The results shown represent average values.*
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 (5). In X. campestris pv. phaseoli, ahpC, ahpF, oxyR, and oxyF 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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**REFERENCES**


**FIG. 7.** Conservation of ahpC and ahpF organization in various Xanthomonas species. The primer set corresponded to 3′ahpC (5′ ATCGGCGCAAGGTTCACAA 3′) and 5′ahpF (5′TGATGCGTTGATTTGAATC 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. oryzae; 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′ ATGGGCCGAAAGGTTCACAA 3′) and 5′oxyR (5′ GGCCTGACAA 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. oryzae; 4, X. vesicatoria; 5, X. oryzae pv. oryzae; and 6, X. translucens; 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.


