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A Suppressor of the Menadione-Hypersensitive Phenotype of a *Xanthomonas campestris* pv. phaseoli *oxyR* Mutant Reveals a Novel Mechanism of Toxicity and the Protective Role of Alkyl Hydroperoxide Reductase

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We isolated menadione-resistant mutants of *Xanthomonas campestris* pv. phaseoli *oxyR* (*oxyR*$_{Xp}$). The *oxyR*R2$_{Xp}$ mutant was hyperresistant to the superoxide generators menadione and plumbagin and was moderately resistant to H_2O_2 and *tert*-butyl hydroperoxide. Analysis of enzymes involved in oxidative-stress protection in the *oxyR*R2$_{Xp}$ mutant revealed a >10-fold increase in AhpC and AhpF levels, while the levels of superoxide dismutase (SOD), catalase, and the organic hydroperoxide resistance protein (Ohr) were not significantly altered. Inactivation of *ahpC* in the *oxyR*R2$_{Xp}$ mutant resulted in increased sensitivity to menadione killing. Moreover, high levels of expression of cloned *ahpC* and *ahpF* in the *oxyR*$_{Xp}$ mutant complemented the menadione hypersensitivity phenotype. High levels of other oxidant-scavenging enzymes such as catalase and SOD did not protect the cells from menadione toxicity. These data strongly suggest that the toxicity of superoxide generators could be mediated via organic peroxide production and that alkyl hydroperoxide reductase has an important novel function in the protection against the toxicity of these compounds in *X. campestris*.

Aerobic bacteria are always exposed to a variety of reactive oxygen species (ROS) that occur as by-products of normal aerobic metabolism and that arise from external sources. ROS such as superoxide anions, peroxides, and hydroxyl radicals are highly toxic to biological systems. To defend against oxidative stresses, bacteria have evolved inducible responses to protect themselves from oxidative damage. In *Escherichia coli*, two inducible defense regulons, *oxyR* and *soxRS*, have been characterized. OxyR and SoxS are transcription factors capable of activating other antioxidant genes in the regulon in response to sublethal concentrations of H_2O_2 and superoxide-generating compounds, respectively (19). Analyses of these mutants have facilitated the elucidation of resistance mechanisms to oxidants. Biochemical analysis of alkyl hydroperoxide reductase (AhpCF) reveals that it plays crucial roles in oxidative- and nitrosactive-stress protection. The enzyme consists of two subunits, a catalytic unit, AhpC, and a reductase, AhpF. AhpCF is involved in the detoxification of organic hydroperoxide, H_2O_2, and reactive nitrogen species (2, 3, 15, 16). In many bacteria, the expression of *ahpC* and *ahpF* is regulated by OxyR (15, 19).

Xanthomonads are gram-negative, aerobic, and plant-pathogenic bacteria. Exposure of the bacteria to low concentrations of H_2O_2 and the superoxide-generating compound menadione (2-methyl-1,4-naphthoquinone) highly induces expression of genes in the OxyR regulon, with menadione being 10-fold more potent. The *oxyR* gene from *Xanthomonas campestris* pv. phaseoli (*oxyR*$_{Xp}$) has been isolated and characterized (8, 10), and an *oxyR*$_{Xp}$ mutant has been constructed (12). The mutant shows reduced aerobic plating efficiency and is highly sensitive to menadione and peroxide killing.

In this report, we describe the selection of suppressors of the menadione sensitivity phenotype of *oxyR*$_{Xp}$ mutants. Analysis of these mutants suggested a new toxicity mechanism for menadione and a protective role of AhpCF in *Xanthomonas*.

Selection and physiological characterization of menadione-resistant *oxyR*$_{Xp}$ mutants. We assumed that the mechanism responsible for the menadione-hypersensitive phenotype in the *oxyR*$_{Xp}$ mutant is due to an increase in superoxide production and its subsequent dismutation to H_2O_2. To better understand this mechanism, spontaneous mutation of the menadione hypersensitivity suppressor of the *oxyR*$_{Xp}$ mutant was selected by plating stationary-phase cultures on Silva-Buddenhagen (SB) agar plates containing 200 μM menadione. These mutants were designated *oxyR*R$_{Xp}$ mutants. The *oxyR*$_{Xp}$ mutant shows a defect in aerobic plating efficiency that can be negated by the addition of 0.1% sodium pyruvate, a ROS-scavenging compound, to the medium (12). The plating efficiency was defined as the number of CFU obtained with cultures plated on SB medium divided by the number of CFU obtained with cultures plated on SB medium supplemented with pyruvate (0.1% [wt/vol]). This calculation was used as the basis for comparing the plating efficiencies of the *oxyR*R$_{Xp}$ isolates with those of the *oxyR*$_{Xp}$ mutant and the wild-type parental strain. Since all of the *oxyR*R$_{Xp}$ isolates examined showed similar properties, the typical results from one of the *oxyR*R2$_{Xp}$ mutants are shown (Fig. 1A). The aerobic plating efficiency of the *oxyR*R2$_{Xp}$ mutant was about 40-fold higher than that of the *oxyR*$_{Xp}$ mutant

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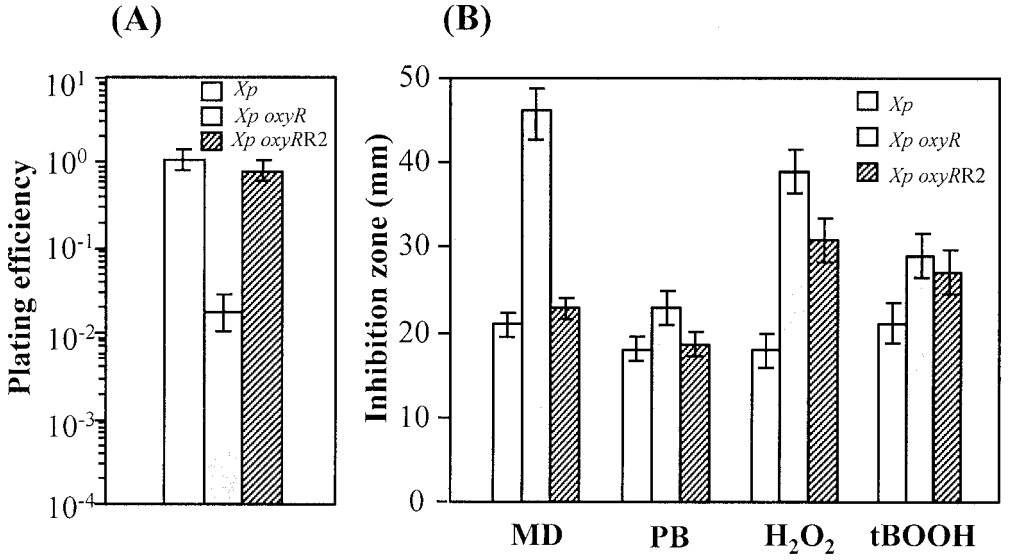

FIG. 1. Characteristics of the $oxyR_{Xp}$, $oxyR2_{Xp}$, and parental wild-type strains. (A) Abilities of the parental wild-type strain and the $oxyR_{Xp}$ and $oxyR2_{Xp}$ mutants to form colonies on SB agar plates. Exponential-phase cells were grown on SB agar and SB agar containing pyruvate. Plating efficiency is defined as the number of CFU on SB agar divided by the number of CFU on SB agar containing 0.1% pyruvate. (B) Determination of the level of resistance to oxidant killing in the $oxyR_{Xp}$, $oxyR2_{Xp}$, and parental strains. The growth inhibition zones developed in response to 1.0 M menadione (MD), 0.5 M plumbagin (PB), 0.5 M H_2O_2, and 0.5 M tBOOH by exponential-phase cultures of the parental wild-type strain and the $oxyR_{Xp}$ and $oxyR2_{Xp}$ mutants were determined as previously described (14).

and was comparable to that of the wild-type strain. In addition, the levels of resistance of the $oxyR2_{Xp}$ and $oxyR_{Xp}$ mutants and the wild-type parental strain to superoxide generators (menadione and plumbagin) and peroxides (H_2O_2 and organic hydroperoxide) were determined with a growth inhibition zone assay. As expected, the menadione resistance level of the $oxyR2_{Xp}$ mutant was increased to almost the same level as that of the wild-type parental strain (Fig. 1B). Since it remains controversial whether menadione toxicity is due to superoxide anion production (18), we measured the resistance levels of the three strains to the superoxide generator plumbagin. The results showed resistance patterns similar to that of menadione, except that the relative difference in plumbagin sensitivities between the $oxyR_{Xp}$ and the $oxyR2_{Xp}$ mutants and the parental wild-type strain was much less than that for menadione sensitivities (Fig. 1B). This suggested that the $oxyR2_{Xp}$ mutant had acquired mechanisms to overcome superoxide anion toxicity. We also determined the resistance levels of the three strains to H_2O_2 and to the organic peroxide *tert*-butyl hydroperoxide (tBOOH). The $oxyR2_{Xp}$ mutant showed a moderate increase in H_2O_2 resistance compared to that of the $oxyR_{Xp}$ strain; however, the level was significantly less than that of the wild type (Fig. 1B). By contrast, both the $oxyR2_{Xp}$ and $oxyR_{Xp}$ strains had similar levels of resistance to *t*BOOH that were lower than that displayed by the wild type (Fig. 1B).

There is a strong possibility that the menadione-resistant phenotype of the $oxyR2_{Xp}$ strain could be due to a mutation leading to a defect in menadione uptake. To test this, we took advantage of the fact that, in *Xanthomonas*, there is an OxyR-independent, menadione-inducible protection against H_2O_2 killing (12). We reasoned that if a mutation resulted in the blocking of menadione uptake into the cell, then the menadione-inducible protective pathway against H_2O_2 toxicity should be abolished. Experimental results clearly showed that a low concentration of menadione (50 μM) induced cross-protection against H_2O_2 (10 mM) killing in both the $oxyR2_{Xp}$ and $oxyR_{Xp}$ strains (12; data not shown), indicating that the menadione-resistant phenotype of the $oxyR2_{Xp}$ mutant was not due to a defect in menadione uptake.

The $oxyR2_{Xp}$ mutant had high levels of the AhpCF subunits AhpC and AhpF. The levels of various enzymes involved in superoxide and peroxide protection were measured in the $oxyR2_{Xp}$ mutant. *Xanthomonas* possesses a manganese (Mn) SOD capable of converting superoxide to H_2O_2 (17). Previous studies have shown that elevated levels of SOD make cells more resistant to superoxide generators (1). Thus, the levels of SOD in the $oxyR2_{Xp}$, $oxyR_{Xp}$, and the parental wild-type strains were determined spectrophotometrically. As shown in Fig. 2A, the SOD activities in all strains were not significantly different. This indicates that the menadione-resistant phenotype in the $oxyR2_{Xp}$ mutant is independent of the SOD level.

Next, the levels of enzymes involved in peroxide metabolism, such as catalase, AhpC, and Ohr, were determined. Previously, we showed a direct correlation between catalase activity and resistance to H_2O_2 killing in *Xanthomonas* (13). Moreover, the hypersensitivity to H_2O_2 of the exponential-phase $oxyR_{Xp}$ mutant is due to a decrease in the catalase level (12). Since we have shown that the catalase activities in exponentially growing cultures of the $oxyR_{Xp}$ and $oxyR2_{Xp}$ strains are similar and significantly lower than that of the wild-type parental strain (Fig. 2A), the data suggest that the increased H_2O_2 resistance level in the $oxyR2_{Xp}$ mutant is not a result of an increase in the level of catalase activity.

There are at least two systems for the detoxification of organic hydroperoxides in *Xanthomonas*. One is the well-known mechanism mediated by AhpCF (15), and another is the re-

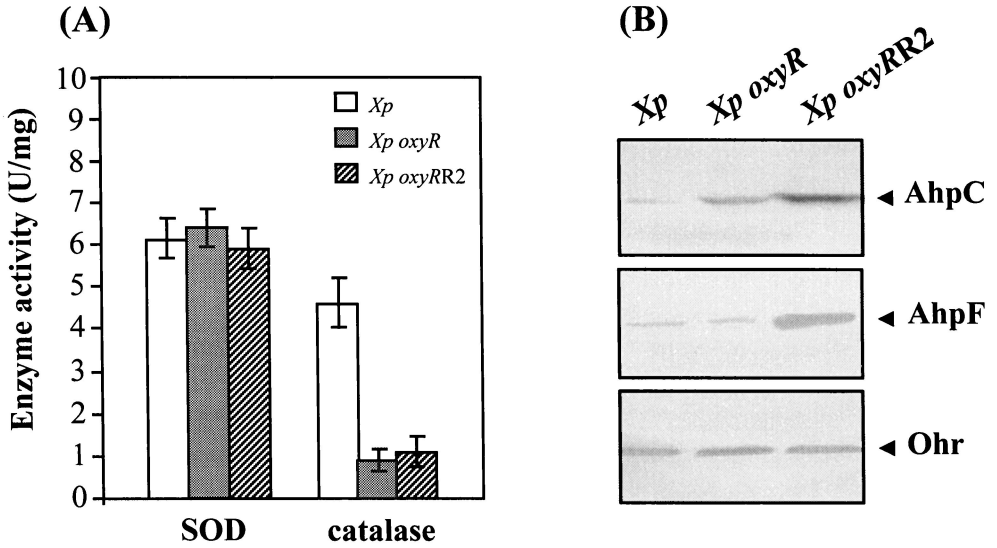

FIG. 2. Levels of antioxidant enzymes in the $oxyR_{Xp}$, $oxyR2_{Xp}$, and the parental wild-type strains. (A) SOD and catalase activities in bacterial lysates prepared from exponential-phase cultures were assayed as previously described (14). (B) Western immunoblot analysis of AphC, AhpF, and Ohr. Crude protein (20 μg) was loaded, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane as previously described (23). Immunodetection was performed as previously described (23).

cently reported Ohr system (11). The levels of Ohr and the AhpCF subunits AhpC and AhpF were measured by Western immunoblotting with polyclonal antibodies raised against *Salmonella* AhpC and AhpF (20) and *Xanthomonas* Ohr. The Western blot analysis showed that the levels of AhpC and AhpF in the $oxyR2_{Xp}$ mutant were 10-fold higher than those of both the $oxyR_{Xp}$ and parental wild-type strains, while the Ohrs level in all strains were not significantly different from each other (Fig. 2B). These data strongly suggest that the elevation of AhpCF levels in the $oxyR2_{Xp}$ mutant was responsible for the suppression of the menadione-hypersensitive phenotype in the $oxyR_{Xp}$ strain. Additional evidence supporting this assumption came from the observation that an $ahpC1_{Xp}$ mutant was hypersensitive to menadione killing (14).

AhpCF plays an important role in the menadione-resistant phenotype. In order to evaluate the role of AhpCF in the menadione-resistant phenotype of the $oxyR2_{Xp}$ mutant, an insertional inactivation of the gene encoding the catalytic subunit AhpC was performed as previously described (14). The resultant $oxyR2_{Xp}$ *ahpC* mutant was confirmed by both Southern and Western blotting (data not shown). Furthermore, the resistance levels of the mutant to menadione, H_2O_2, and organic hydroperoxide were determined with an inhibition zone assay. The results showed that the increased resistance of the $oxyR2_{Xp}$ mutant to menadione was eliminated by the inactivation of *ahpC*, since the $oxyR2_{Xp}$ *ahpC* mutant had a resistance level to menadione that was similar to that of the $oxyR_{Xp}$ strain (data not shown).

It is possible that the increase in the level of AhpCF is a coincidental event and not the cause of the menadione-resistant phenotype. However, the fact that inactivation of *ahpC* in the $oxyR2_{Xp}$ mutant returns the level of menadione resistance to that of the parental wild-type strain strongly suggests that AhpCF plays a critical role in the increased menadione resistance of the $oxyR2_{Xp}$ mutant. Additional experiments were then performed to confirm that the increased level of AhpCF is indeed responsible for the increased menadione resistance of the $oxyR2_{Xp}$ mutant. We reasoned that if no other factors were involved, artificially high levels of AhpCF, attained through the introduction of the *ahpC* and *ahpF* expression plasmid pahpCF (8, 10), should confer increased resistance to menadione in the $oxyR_{Xp}$ mutant. pahpCF was electroporated into the $oxyR_{Xp}$ strain, and the resistance level of the resulting $oxyR_{Xp}$(pahpCF) strain to both menadione and H_2O_2 was determined (Table 1). The presence of the *ahpC* and *ahpF* expression plasmid in the $oxyR_{Xp}$(pahpCF) strain conferred increased menadione resistance to a level similar to that of the parental wild-type strain. In addition, the $oxyR_{Xp}$(pahpCF) strain displayed a sensitivity to H_2O_2 that was slightly higher than that of the parental wild-type strain (Table 1). These results prove that high levels of AhpCF protect *Xanthomonas* against menadione killing. The experiments were then extended to investigate whether the overexpression of other *Xanthomonas* enzymes involved in the detoxification of ROS, such as the monofunctional catalase encoded by *katE* on the plasmid pkat (22) and the MnSOD encoded by *sodA* on the plas-

TABLE 1. Assays of inhibition zones against various oxidants

Strain	Inhibition zone (mm)[a]	
	Menadione	H_2O_2
Wild-type parent	20.7 ± 1.5	22.5 ± 1.5
$oxyR_{Xp}$ (pUFR047) mutant	45.8 ± 2.7	39.5 ± 3.2
$oxyR_{Xp}$ (psod) mutant	43.0 ± 2.5	40.2 ± 2.1
$oxyR_{Xp}$ (pkat) mutant	45.0 ± 2.5	19.0 ± 1.4
$oxyR_{Xp}$ (pahpCF) mutant	23.8 ± 2.4	29.1 ± 2.7

[a] The growth inhibition zones developed in response to menadione (1.0 M) and H_2O_2 (0.5 M) of exponential-phase cultures of the indicated strains were determined as previously described (15). The data represented are the means ± standard deviations of results from three independent experiments.

mid psod (17), could confer increased menadione and H_2O_2 resistance in the $oxyR_{Xp}$ strain. The plasmids were transferred into the $oxyR_{Xp}$ strain, and the resulting strains harboring each plasmid were then checked for their sensitivities to menadione and H_2O_2 with the inhibition zone assay. The results in Table 1 show that high expression of SOD in the $oxyR_{Xp}$(psod) strain and of catalase in the $oxyR_{Xp}$(pkat) strain had no appreciable affect on menadione sensitivity. As expected, the $oxyR_{Xp}$(pkat) strain showed a significant decrease in sensitivity to H_2O_2.

The data described above led to the conclusion that AhpCF has a novel role in protection against menadione in *Xanthomonas*. Exactly how AhpCF protects against the lethal effects of menadione is not known. Menadione is a synthetic derivative of ubiquinone, a membrane-associated compound (4). The most toxic effect of menadione may be due to lipid peroxidation of membrane fatty acids generated via superoxide radicals (21). The organic peroxides that are produced may be substrates of AhpCF. Supporting evidence for this hypothesis comes from the observation that the antioxidant vitamin E (α-tocopherol), a membrane-associated compound, could protect *E. coli* from menadione toxicity (5). Moreover, high expression of cytosolic MnSOD could only partially relieve the menadione hypersensitivity in the $oxyR_{Xp}$ strain by the removal of superoxide anions generated cytosolically (17). The enzyme could not fully dismutate superoxide radicals that occurred in the membrane. In an *E. coli oxyR* mutant, overexpression of catalase (both KatG bifunctional catalase-peroxidase and KatE monofunctional catalase) could suppress hypersensitivity to superoxide generators (6, 7), possibly by the removal of H_2O_2 generated by the dismutation of superoxide anions. In addition, increased levels of AhpCF in a revertant of an *E. coli oxyR* mutant has been observed; however, there was no evidence linking this to increased menadione resistance (6). Our results with *Xanthomonas* show that a high level of monofunctional catalase does not suppress the menadione-hypersensitive phenotype of the $oxyR_{Xp}$ strain. These results suggest that the lethal toxicity of menadione in *Xanthomonas* is likely a result of organic peroxide production and is not due to H_2O_2 production via the dismutation of superoxide anions.

Although the $oxyR2_{Xp}$ mutant produced high levels of AhpCF, the level of tBOOH sensitivity was not fully restored to the wild-type level. This evidence suggests that other *oxyR*-dependent mechanisms are required in order to fully protect *Xanthomonas* from tBOOH toxicity. In addition, the moderate increase in resistance of the $oxyR2_{Xp}$ mutant to H_2O_2 compared to that of the $oxyR_{Xp}$ mutant might be due to the increase in AhpCF, since the purified enzyme can use both organic hydroperoxide and H_2O_2 as substrates (15). Alkyl hydroperoxide reductase has also been shown to protect *E. coli* from H_2O_2 toxicity (16). Thus, elevated levels of AhpCF could increase resistance to H_2O_2 in *Xanthomonas*.

In *Xanthomonas*, the expression of *ahpC* and *ahpF* is under the regulation of OxyR. The increased level of AhpC and AhpF in the $oxyR2_{Xp}$ mutant, in the absence of a functional *oxyR* gene is quite surprising. In *Xanthomonas*, *ahpC* is transcribed as a monocistronic mRNA, while *ahpF* and *oxyR* are transcribed as a polycistronic mRNA. The *ahpC* gene has a strong promoter which is differentially regulated by OxyR. Reduced OxyR represses the promoter, while oxidized OxyR activates it (9). Since the $oxyR2_{Xp}$ mutant resulted from strong selection pressure, rare mutations might have occurred in the *ahpC* and *ahpF* promoters, thereby leading to constitutive high-level expression of these genes without the requirement of OxyR. Alternatively, an increase in *ahpC* and *ahpF* mRNA stability or a decrease in AhpCF degradation could be responsible for the observed high levels of these proteins. The molecular mechanism responsible for the increase in *ahpC* and *ahpF* expression is currently under investigation.

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