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Construction and Physiological Analysis of a *Xanthomonas* Mutant To Examine the Role of the *oxyR* Gene in Oxidant-Induced Protection against Peroxide Killing

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We constructed and characterized a *Xanthomonas campestris* pv. phaseoli *oxyR* mutant. The mutant was hypersensitive to H_2O_2 and menadione killing and had reduced aerobic plating efficiency. The oxidants' induction of the catalase and *ahpC* genes was also abolished in the mutant. Analysis of the adaptive responses showed that hydrogen peroxide-induced protection against hydrogen peroxide was lost, while menadione-induced protection against hydrogen peroxide was retained in the *oxyR* mutant. These results show that *X. campestris* pv. phaseoli *oxyR* is essential to peroxide adaptation and revealed the existence of a novel superoxide-inducible peroxide protection system that is independent of OxyR.

Inducible stress responses are important components of bacterial survival under stressful conditions. Exposure to a low level of one stress can induce a protective response against subsequent exposure to lethal levels of the same (adaptive response) or unrelated (cross-protective response) stresses (3, 5, 7, 23, 32). OxyR, a global regulator for peroxide stress response, is a bifunctional protein that acts as a peroxide sensor and a transcription activator in response to oxidative stress (2, 31, 33). It regulates many genes involved in the scavenging of peroxides (i.e., catalase and alkyl hydroperoxide reductase [ahpR] [5, 30]) and the prevention and repair of oxidative damage for macromolecules (i.e., glutathione reductase and *dps*) (5, 17, 19, 29).

The inducible adaptive and cross-protective responses against peroxide killing could play important roles in plant-microbe interactions. Active plant defense response against microbes involves increased production of H_2O_2, organic peroxides, and superoxides (14). These reactive oxygen species can inhibit growth and kill invading microbes. During initial interactions, bacteria are exposed to low-concentration mixtures of superoxide anions and peroxides (14). These could induce protection against subsequent exposure to higher concentrations of reactive oxygen species that prolong bacterial survival in the plant and may affect disease progression. Moreover, normal aerobic metabolism also generates significant quantities of reactive oxygen species (8, 9), which have to be rapidly detoxified.

We have isolated and characterized an *oxyR* from *Xanthomonas campestris* pv. phaseoli (15, 22). The gene has unique organization and transcription regulation (1, 16, 23). This fact, coupled with observations that many aspects of *Xanthomonas* oxidative stress response differ from those of other bacteria (1, 16), leads us to investigate OxyR function in *X. campestris* pv. phaseoli.

Construction of the *oxyR* mutants. Inactivation of the *oxyR* gene was achieved by insertion of a *Kpn*I-digested gentamicin resistance gene from pUCGM (27) into a *Kpn*I site located in the coding region of *oxyR* on plasmid pUC18 (15). The new recombinant plasmid, designated poxyR::Gm, was electroporated into *X. campestris* pv. phaseoli as previously described (21). Transformants were selected on SB (0.5% yeast extract, 0.5% peptone, 0.5% sucrose, 0.1% glutamic acid; pH 7.0) plates containing 15 μg of gentamicin per ml. Gm^r colonies were subsequently scored for an Ap^s phenotype. Many colonies had Ap^s Gm^r phenotypes, indicating an exchange of the mutated *oxyR* for its functional counterpart. These colonies were selected for further characterization by both Southern and Western analyses, which confirmed that the mutated *oxyR* had replaced the functional gene in these cells with an Ap^s Gm^r phenotype (data not shown).

Physiological characterization of the mutant. We noticed that the *oxyR* mutants formed smaller colonies than did the parental strain on SB plates. Mutations in genes involved in oxidative stress response often lead to defects in aerobic plating efficiency (18, 34). All of the *X. campestris* pv. phaseoli *oxyR* mutant strains tested showed a 10^4 decrease in aerobic plating efficiency on SB plates compared to that for the parental strain. This effect could be reversed by the addition of 10 mM sodium pyruvate (18, 24, 34) to SB plates (Fig. 1), suggesting that accumulation of peroxides in the *oxyR* mutants probably caused the defect. To test the hypothesis, plasmids containing *Xanthomonas* genes involved in oxidative stress protection were transformed into the mutant and their plating efficiency was determined. The results are shown in Fig. 1. A high level of superoxide dismutase (pUFR-SOD [28]) or microaerobic growth conditions had no effect on the plating efficiency of the mutant. An increased level of enzymes directly involved in peroxide metabolism (e.g., monofunctional catalase [pkat] [21] and AhpR subunits C and F [pUFR-ahpCF]) restored the plating efficiency of the mutant so that it was close to that of the parental strain. An increased level of catalase was less efficient than AhpR at complementing the defect, probably due to the inability of catalase to metabolize organic peroxide. Unexpectedly, increased levels of AhpF (pUFR-ahpF) alone restored the level of plating efficiency similar to the level attained by overexpression of catalase, while high levels of AhpC (pahpC [15]) alone were not as effective (Fig. 1). Purified

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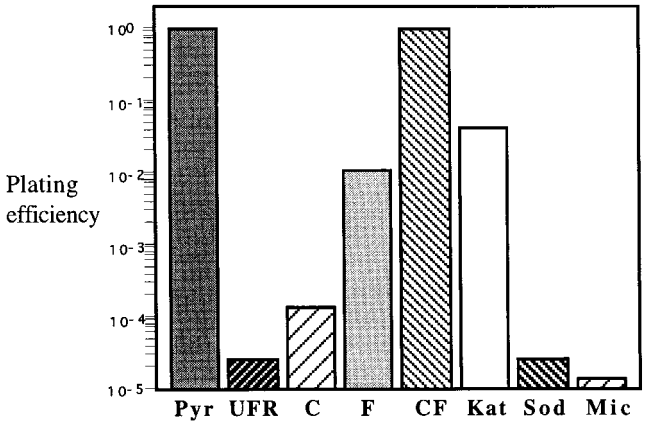

FIG. 1. Plating efficiency of an *oxyR* mutant harboring various expression plasmids containing genes involved in oxidative stress response or conditions that affected oxidative stress. In all experiments, a mid-log-phase *X. campestris* pv. phaseoli *oxyR* mutant grown in SB was serially diluted and plated on SB plates with or without 10 mM pyruvate. Plating efficiency is defined as the number of cells on SB plates divided by the number of cells on SB plates with pyruvate. Pyr, *X. campestris* pv. phaseoli *oxyR* mutant on 10 mM pyruvate SB plates; Mic, the mutant was plated on SB plates and incubated in an anaerobic jar under microaerobic conditions (Oxoid gas generating kit); UFR, *X. campestris* pv. phaseoli *oxyR* mutant harboring only pUFR047 (4) expression vector; C, pahpC (15); F, pahpF (ahpF subunit of *X. campestris* pv. phaseoli [15] in pUFR047); CF, pahpCF (*ahpC* and *ahpF* [15] in pUFR047); Kat, pkat (21); Sod, psod (*Xanthomonas sod* [28] coding region in pUFR047).

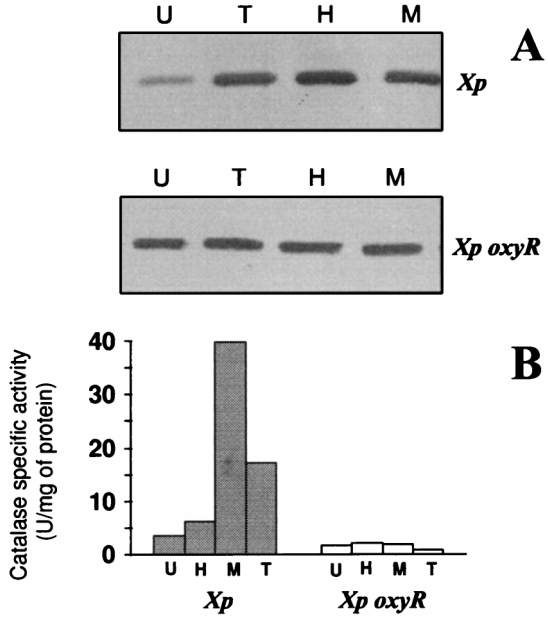

FIG. 2. Levels of AhpC and catalase activities in response to various oxidants in *X. campestris* pv. phaseoli (*Xp*) and an *X. campestris* pv. phaseoli *oxyR* mutant (*Xp oxyR*). Mid-log-phase *X. campestris* pv. phaseoli or an *X. campestris* pv. phaseoli *oxyR* mutant grown in SB was induced with 100 μM H_2O_2 (H) or tBOOH (T) or 20 μM MD (M) for 30 min. Various concentrations of oxidants were chosen to give maximum induction and minimal effects on *X. campestris* pv. phaseoli growth. Uninduced (U) and induced samples were collected by centrifugation, and lysates were prepared as previously described (21). AhpC levels (A) were determined by Western immunoblotting with an anti-*E. coli* AhpC (22, 30). Forty micrograms of total protein was loaded into each lane, and immunodetection was performed according to the method of Mongkolsuk et al. (22). At the right of each panel is indicated whether lysates were from *X. campestris* pv. phaseoli or an *X. campestris* pv. phaseoli *oxyR* mutant. Catalase levels were determined spectrophotometrically (21). (B) Closed and open bars represent catalase activities of *X. campestris* pv. phaseoli and the *X. campestris* pv. phaseoli *oxyR* mutant, respectively. Letters above the lanes (A) or below the bars (B) indicate that lysates were prepared from uninduced or oxidant-induced cultures, respectively. Experiments were performed three times, and typical results are shown.

AhpC and AhpF can use both H_2O_2 and organic peroxide as substrates (25, 26). On the other hand, we have observed in *X. campestris* pv. phaseoli that increased expression of either *ahpC* (15) or *ahpC-ahpF* in vivo does not increase resistance to H_2O_2 killing. We interpreted these data as evidence that *oxyR* mutants accumulate both H_2O_2 and organic peroxides, consistent with the observation in *Escherichia coli* that *oxyR* mutants have higher levels of peroxides than a wild-type strain (9). This fact and increased susceptibility to oxidative damage during the early stages of colony formation when bacterial density is low (17) could have been responsible for the lower aerobic plating efficiency seen for the mutants.

Next we qualitatively determined the sensitivity of the log-phase *oxyR* mutant to killing concentrations of various oxidants by a killing zone method (15). Essentially, 6 μl of indicated concentrations of oxidants applied to 6-mm-diameter paper discs was subsequently placed on lawns of cells. Experiments were performed in triplicate. To ensure reproducibility, only log-phase cells were used. The killing zones for H_2O_2 (500 mM), menadione (MD) (500 mM), tert butyl hydroperoxide (tBOOH) (500 mM), and cumene hydroperoxide (CuOOH) (500 mM), respectively, were 13, 17, 11, and 16 mm for a wild-type *X. campestris* pv. phaseoli and 34, 42, 13, and 18 mm for an *oxyR* mutant. The *oxyR* mutant showed increased sensitivity to all oxidants tested, with MD and H_2O_2 causing the most severe effects. The high sensitivity of the *oxyR* mutant to H_2O_2 was expected, but the hypersensitivity to MD implied that its killing mechanism could partly be mediated via superoxide anion metabolism to H_2O_2 (11, 12). By contrast to an *E. coli oxyR* mutant, the *X. campestris* pv. phaseoli *oxyR* mutant had only a minor increase in sensitivity to organic peroxide killing. This could be due to presence of an additional novel organic peroxide-protective system (*ohr*) in *X. campestris* pv. phaseoli that may functionally compensate for regulatory defects of AhpC (20).

Regulation of oxidant induction of catalase and AhpC by *oxyR*. We have observed in *Xanthomonas* that the peroxide-scavenging enzymes, catalase and AhpC, are highly induced by low concentrations of peroxides and superoxide generators (1, 22). However, the regulator of these responses could not be identified. Experiments were performed to determine catalase and AhpC levels in response to low concentrations of oxidants in *X. campestris* pv. phaseoli and *X. campestris* pv. phaseoli *oxyR*. The results are shown in Fig. 2. In *X. campestris* pv. phaseoli, H_2O_2, tBOOH, and MD induced both catalase and AhpC to high levels, consistent with previous observations (1, 16, 21). However, induction of both enzymes by all oxidants tested did not occur in the *oxyR* mutant. This finding is consistent with a notion that OxyR is acting as a peroxide sensor and a transcription activator of genes for peroxide-scavenging enzymes. These functions are conserved for *oxyR* in all bacteria thus far studied (28, 31, 33, 34). An increase in the basal level of AhpC in the *oxyR* mutant was observed. This could be due to OxyR in its reduced form functioning as a repressor of *ahpC*; thus, in the absence of OxyR, this leads to an increase in *ahpC* expression (20). The induction of these peroxide-scavenging enzymes by a superoxide generator (MD) was likely to occur via the breakdown of superoxide anion to H_2O_2 that, in turn, activated OxyR, not via a superoxide sensor transcription activator protein such as SoxRS (11, 12).

Basal levels of catalase and AhpC in the mutant appeared to

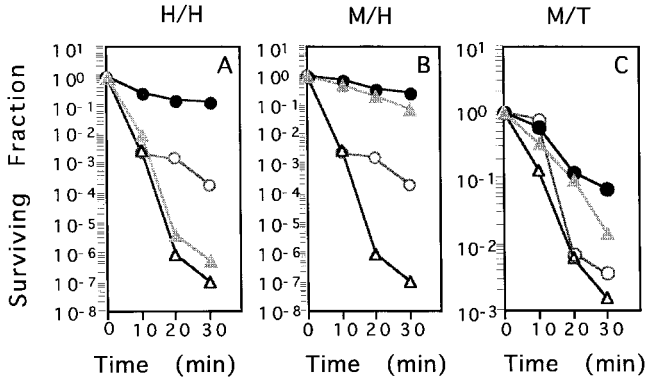

FIG. 3. Adaptive and cross-protective responses against peroxide killing in *X. campestris* pv. phaseoli and an *X. campestris* pv. phaseoli *oxyR* mutant. Log-phase uninduced *X. campestris* pv. phaseoli (○) and an *X. campestris* pv. phaseoli *oxyR* mutant (△) and oxidant-induced (30-min treatment with either 100 μM H_2O_2 [A] or 50 μM MD [B and C]) *X. campestris* pv. phaseoli (●) and *X. campestris* pv. phaseoli *oxyR* mutant (▲) grown in SB were treated with killing concentrations of either 30 mM H_2O_2 (A and B) or 100 mM tBOOH (C) as previously described (15). At the indicated times, aliquots of cells were removed and washed twice before viable cells were counted (23). Experiments were repeated three times, and representative results are shown.

be sufficient for normal aerobic growth. The lack of an induction mechanism for peroxide-scavenging enzymes and the increased oxidant sensitivity of *oxyR* mutants support the interpretation that up-regulation of these scavenging enzymes is important to bacterial survival under stressful conditions. Consistent with this notion, *oxyR* suppressor mutants with high levels of AhpC-AhpF and catalases have been isolated (10).

***oxyR* roles in adaptive and cross-protective responses.** In *Xanthomonas*, peroxide and superoxide anions induce protective responses to peroxide killing (23). These responses are mediated by OxyR in *E. coli* (32), and the *oxyR* mutant was used to investigate whether the situation in *Xanthomonas* was similar. The results of the experiment are shown in Fig. 3. H_2O_2 induced protection against H_2O_2 killing in wild-type *X. campestris* pv. phaseoli. This response was abolished in the *oxyR* mutant (Fig. 3A). In contrast to previous observations with other bacteria (6, 10, 18), MD could induce protection against H_2O_2 and tBOOH killing in both the parental strain and the *oxyR* mutant (Fig. 3B and C). The data indicate that OxyR is essential to peroxide adaptation and also to the existence of a novel superoxide-inducible peroxide-protective system independent of OxyR. This novel peroxide-protective system does not depend on up-regulation of the well-known peroxide-scavenging enzymes catalase and AhpR, since their induction by superoxide anions was abolished in the *oxyR* mutant (Fig. 2).

It is noteworthy that resistance levels to peroxide killing in the MD-induced *oxyR* strain were similar to those attained by the similarly induced parental strain, even though the uninduced *oxyR* mutant was more sensitive than the parental strain to peroxide killing. Thus, the novel superoxide-inducible peroxide-protective system is likely to play a crucial role in protection against peroxide killing in *X. campestris* pv. phaseoli. We believe this system differs from the starvation-induced or the general stress-protective systems (13). In *Xanthomonas*, MD does not induce protection against itself or against a nonoxidative stress such as heat killing (23). We are investigating the mechanism of this novel superoxide anion-induced peroxide-protective system.

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