Exposure of Phytopathogenic *Xanthomonas* spp. to Lethal Concentrations of Multiple Oxidants Affects Bacterial Survival in a Complex Manner

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During plant-microbe interactions and in the environment, *Xanthomonas campestris* pv. *phaseoli* is likely to be exposed to high concentrations of multiple oxidants. Here, we show that simultaneous exposures of the bacteria to multiple oxidants affects cell survival in a complex manner. A superoxide generator (menadione) enhanced the lethal effect of an organic peroxide (*tert*-butyl hydroperoxide) by 1,000-fold; conversely, treatment of cells with menadione plus H₂O₂ resulted in 100-fold protection compared to that for cells treated with the individual oxidants. Treatment of *X. campestris* with a combination of H₂O₂ and *tert*-butyl hydroperoxide elicited no additive or protective effect. High levels of catalase alone are sufficient to protect cells against the lethal effect of menadione plus H₂O₂ and *tert*-butyl hydroperoxide plus H₂O₂. These data suggest that H₂O₂ is the lethal agent responsible for killing the bacteria as a result of these treatments. However, increased expression of individual genes for peroxide (alkyl hydroperoxide reductase, catalase)- and superoxide (superoxide dismutase)-scavenging enzymes or concerted induction of oxidative stress-protective genes by menadione gave no protection against killing by a combination of menadione plus *tert*-butyl hydroperoxide. However, *X. campestris* cells in the stationary phase and a spontaneous H₂O₂-resistant mutant (*X. campestris* pv. *phaseoli* HR) were more resistant to killing by menadione plus *tert*-butyl hydroperoxide. These findings give new insight into oxidant killing of *Xanthomonas* spp. that could be generally applied to other bacteria.

Xanthomonas spp. are soil bacteria and important bacterial plant pathogens. Active plant defense response against microbial invasion involves increased production and accumulation of reactive oxygen species (H₂O₂, organic peroxide, and superoxide) (1). Reactive oxygen species serve several physiological roles, including killing microbes and serving as signals for further activation of the defense response (10). Many chemicals found in the environment are strong oxidants. These can modulate microbial physiological responses, which in turn affect their interaction with the host and their ability to survive in the environment. These changes might alter disease development and progression. To survive in the environment and proliferate in plants, *Xanthomonas* spp. must protect themselves from the harmful effects of oxidants. We have shown that several aspects of the oxidative stress responses of *Xanthomonas* spp. differ from those observed in other bacteria (4, 11, 15, 16); for example, we have isolated and characterized a gene coding for a transcription regulator and a peroxide sensor, *oxyR* (14, 16). OxyR mediates peroxide-induced adaptive responses and regulates expression of genes for peroxide-scavenging enzymes (14, 16). In contrast, superoxide mediation of cross-protection against peroxide killing is governed by an unknown regulator (16). We have also identified a gene, *ohr*, that is responsible for organic peroxide resistance, and it has a novel pattern of regulation in response to oxidative stress (15). These findings suggest that *X. campestris* has complex defense mechanisms

against peroxide toxicity, prompting us to investigate further the protective mechanisms against oxidant killing.

All previous studies of oxidant killing of microbes were performed with one oxidant at a time. This does not truly reflect the conditions that bacteria encounter in nature; nonetheless, the effects of simultaneous exposure of bacteria to killing concentrations of multiple oxidants have not previously been investigated. Here, we report the results of experiments with *X. campestris* pv. *phaseoli* undertaken to investigate the interactions of various oxidants in terms of bacterial survival and the insight gained into the protective mechanisms which some bacteria employ to protect themselves from these harmful chemicals.

MATERIALS AND METHODS

Bacterial strains, growth, and electroporation conditions. *X. campestris* pv. *phaseoli* was grown aerobically in Silva-Buddenhagen (SB) medium (0.5% sucrose, 0.5% yeast extract, 0.5% peptone, 0.1% glutamic acid [pH 7.0]) at 28°C. Overnight cultures were subcultured into fresh SB medium to give an *A*₆₀₀ of 0.1. Bacterial growth was monitored spectrophotometrically at *A*₆₀₀. Both log-phase (*A*₆₀₀ of 0.5 after 4 h) and stationary-phase (*A*₆₀₀ of 5.5 after 24 h) cells were used in the experiments (4, 20). *X. campestris* strains were transformed with plasmids by electroporation performed as previously described (13).

Quantitative determination of resistance to oxidants. Quantitative determinations of resistance of *X. campestris* to oxidants were performed by exposing cells to lethal concentrations of menadione (MD; 40 mM), *tert*-butyl hydroperoxide (tBOOH; 15 mM), and H₂O₂ (20 mM). The effects of combinations of oxidants on *X. campestris* survival were determined by treating cells with 40 mM MD plus 15 mM tBOOH, 40 mM MD plus 20 mM H₂O₂, and 20 mM H₂O₂ plus 15 mM tBOOH. At indicated times, cells were removed from the culture vessel, washed twice with fresh SB medium, and then plated onto SB agar. In the case of oxidant killing under anaerobic conditions, cells from log-phase cultures of *X. campestris* grown aerobically in SB medium were pelleted and resuspended in oxygen-depleted SB medium. The suspensions were placed in an anaerobic jar with an anaerobic gas-generating kit for 30 min before addition of the oxidants. After addition of oxidant(s), cultures were returned to the anaerobic jar. Aliquots of cells were removed after a further 30-min incubation, rapidly diluted with oxygen-depleted SB medium, and pelleted. Cell pellets were resuspended in SB medium, washed once, and plated on SB agar. Colonies were counted after

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FIG. 1. Killing of *X. campestris* pv. phaseoli by multiple oxidants. *X. campestris* culture growth and oxidant treatment were performed as described in Materials and Methods. (A) *X. campestris* cultures were exposed to 40 mM MD (●), 15 mM tBOOH (▲), 40 mM MD plus 15 mM tBOOH (□), and 40 mM MD plus 15 mM tBOOH under anaerobic conditions (▽). (B) *X. campestris* cultures were exposed to 40 mM MD (●), 20 mM H₂O₂ (■), 40 mM MD plus 20 mM H₂O₂ (△), and 40 mM MD plus 20 mM H₂O₂ under anaerobic conditions (▽). (C) *X. campestris* cultures were exposed to 15 mM tBOOH (▲), 20 mM H₂O₂ (■), and 15 mM tBOOH plus 20 mM H₂O₂ (○). The data shown are means of four independently performed experiments. Error bars indicate the standard error of the mean.

48 h of incubation at 28°C. The lethal concentrations of individual oxidants have been established previously (17, 20). All experiments were performed independently four times. The data shown represent analysis of the four experiments.

Statistical analysis. The significance of differences among oxidant treatments was statistically determined by using the Student's *t* test when comparing two conditions and one-way analysis of variance and a post hoc pairwise comparison with the least significant difference (LSD) test when more than two conditions were compared. Statistical analysis was performed only with results obtained after 30 min of treatment, and a significant difference is taken as $P < 0.05$.

RESULTS AND DISCUSSION

Simultaneous exposure to lethal concentrations of multiple oxidants. The effects of exposure to combinations of oxidants on *X. campestris* survival were investigated. Bacteria were treated with lethal concentrations of a superoxide generator (MD), an organic peroxide (tBOOH), H₂O₂, and combinations of these oxidants (MD plus tBOOH, MD plus H₂O₂, and tBOOH plus H₂O₂). The results are shown in Fig. 1. *X. campestris* was resistant to MD killing, but was highly and moderately sensitive to H₂O₂ and tBOOH, respectively. Treatment of *X. campestris* with MD plus tBOOH for 30 min enhanced the killing by more than 1,000-fold compared to treatment with the individual oxidants (Fig. 1A). Experiments were then repeated under anaerobic conditions to reduce the rate of superoxide production. In this case, MD plus tBOOH did not enhance killing compared with the individual oxidants (Fig. 1A). These results support a direct role for superoxide anions in intensifying the lethal effects of tBOOH; however, the effects of MD plus tBOOH are not specific to these chemicals. We have determined the response to other superoxide generators, such as paraquat, in combination with tBOOH or cumene hydroperoxide. Regardless of the combination of superoxide generator and organic peroxide, these combination treatments always enhanced the lethal effect compared to treatment with the individual agents (data not shown). There are several possible explanations for the observations. Organic peroxide is metabolized to the corresponding alcohol by alkyl hydroperoxide reductase, an NADH- or NADPH-requiring enzyme (3). MD is an intracellular redox cycling agent, an activity that generates high levels of toxic superoxide anions, which in turn promotes oxidation of iron and inactivation of

superoxide-sensitive enzymes, such as aconitase (7) and many enzymes involved in amino acid biosynthesis (2). These effects could alter the intracellular ratios of small antioxidant molecules, oxidized glutathione/reduced glutathione, NAD/NADH, and NADP/NADPH, making the cells more susceptible to organic peroxide killing. In addition, exposure to superoxide anions has been shown to result in increased production of organic peroxide and organic radicals (8), which could act synergistically with MD plus tBOOH to kill the cells. In contrast, treatment of *X. campestris* for 30 min with a combination of MD plus H₂O₂ gave a 100-fold increase in protection compared to killing by H₂O₂ alone (Fig. 1B). This increased protection, resulting from MD-plus-H₂O₂ treatment, was eliminated when the experiment was repeated under anaerobic conditions (Fig. 1B). The results support the idea that superoxide anions are responsible for induced resistance following MD-plus-H₂O₂ exposure. We have shown that MD pretreatment induces neither resistance to MD killing nor superoxide dismutase, an enzyme responsible for dismutation of superoxide anions (8). *Xanthomonas* spp. are naturally very resistant to superoxide anions, while they are susceptible to H₂O₂ (4, 11), suggesting that intracellular superoxide anions are converted to H₂O₂, by either enzymatic or nonenzymatic reactions, and that H₂O₂ is responsible for *Xanthomonas* killing. We have shown that exposure of *X. campestris* pv. phaseoli to low concentrations of MD induces high-level resistance to H₂O₂ by increasing levels of catalase in an OxyR-dependent fashion (16, 17). Moreover, *X. campestris* pv. phaseoli also has an additional OxyR-independent, MD-inducible resistance to H₂O₂ killing (16). Thus, it is likely that MD-induced resistance to H₂O₂ killing was responsible for the observed increased resistance to MD-plus-H₂O₂ killing. This idea is supported by the data presented in Fig. 2A showing that high levels of catalase conferred protection against MD-plus-H₂O₂ killing.

Treatment of the bacteria with a combination of tBOOH plus H₂O₂ neither enhanced nor protected them from the lethal effects of these agents. Although high concentrations of H₂O₂ are known to cause formation of organic peroxide (8), no additive lethal effects arising from tBOOH-plus-H₂O₂

FIG. 2. Effects of high levels of catalase on oxidant killing. *X. campestris* pv. phaseoli cells harboring pUFR047 (□), pUFRkat (△) (13), and pUFRoxyR (●) (14) were grown as described in Materials and Methods and treated with the indicated concentrations of oxidants: 40 mM MD plus 20 mM H₂O₂ (A), 15 mM tBOOH plus 20 mM H₂O₂ (B), or 40 mM MD plus 15 mM tBOOH (C). The data shown are means of four independently performed experiments. Error bars indicate the standard error of the mean.

treatment were observed. The survival after treatment with a combination of these peroxides was similar to the survival observed following H₂O₂ treatment alone (Fig. 1C). This suggests that H₂O₂ was responsible for killing the bacteria. The results in Fig. 2B show that high levels of catalase conferred protection against the tBOOH-plus-H₂O₂ treatment, which is consistent with this idea.

The effects of high levels of oxidant detoxification enzymes on oxidant killing of *X. campestris* pv. phaseoli. The different responses caused by MD to H₂O₂ and tBOOH killing prompted us to investigate protective mechanisms against these oxidant treatments. High-level expression of genes for oxidative stress-protective enzymes has been shown to protect *Xanthomonas* spp. from oxidant killing (13, 14). Thus, the role of catalase in protecting cells from MD-plus-H₂O₂ and tBOOH-plus-H₂O₂ treatments was investigated. *X. campestris* pv. phaseoli harboring the plasmid pUFRkat (13) or the vector alone (pUFR047) had catalase-specific activities of 148 and 6.8 U/mg of protein, respectively. These cells were treated with MD plus H₂O₂ and tBOOH plus H₂O₂. The results (Fig. 2A and B) show that *X. campestris* harboring pUFRkat were more than 100-fold more resistant to MD-plus-H₂O₂ and tBOOH-plus-H₂O₂ killing than the bacteria harboring the vector alone. The ability of catalase alone to efficiently protect *X. campestris* from these treatments supports the idea that H₂O₂ was responsible for killing the bacteria. Additional support for this conclusion came from the observation that *X. campestris* pv. phaseoli harboring the recombinant plasmid (pUFRoxyR) (14) had high levels of catalase (190 U/mg of protein) and was more resistant to MD-plus-H₂O₂ and tBOOH-plus-H₂O₂ treatments (Fig. 2A and B) than the strain carrying only the cloning vector pUFR047. However, *X. campestris* strains harboring pUFRkat or pUFRoxyR were no more resistant to MD-plus-tBOOH treatment ($P > 0.05$ at 30 min of treatment) (Fig. 2C) than the host strain with or without the cloning vector.

These findings raised the question of how *X. campestris* cells protect themselves from MD-plus-tBOOH killing. We determined the effects of high-level expression of genes involved in scavenging superoxide anions (superoxide dismutase, *sod* [19]) and organic peroxides (alkyl hydroperoxide reductase, *ahpCF* [14]) and the organic hydroperoxide resistance gene (*ohr* [15])

on MD-plus-tBOOH killing. *X. campestris* pv. phaseoli cells harboring pUFR047 (5), pUFRsod (16), pUFRahpCF (14), or pUFRohr (15) were treated with MD plus tBOOH, and the percentage of survival was determined after 30 min. The degrees of survival of all strains following MD-plus-tBOOH treatment were essentially the same, indicating that high-level expression of individual genes for oxidant-scavenging enzymes is not sufficient to confer protection against MD plus tBOOH (data not shown). In *X. campestris*, MD induces resistance to peroxide killing by coordination of both *oxyR*-dependent and *oxyR*-independent activation of peroxide stress defense genes (16). Accordingly, we tested whether there are concerted increases in peroxide and superoxide detoxification enzymes and other protective proteins upon exposure to a low concentration of MD (200 μ M) and whether these responses can protect the bacteria from MD plus tBOOH. The results indicate that uninduced and MD-induced cultures had similar levels of resistance to the treatment (data not shown). Hence, the mechanism of MD plus tBOOH killing of *X. campestris* appears not to be a simple additive lethal effect of individual oxidants.

Cells in the stationary phase of growth and an H₂O₂-resistant mutant were resistant to multiple oxidants. *X. campestris* cells in the stationary phase are highly resistant to peroxide and superoxide killing (20). In general, the degree of resistance to oxidant killing shown by stationary-phase cells does not correlate with the levels of oxidant detoxification enzymes, suggesting that other protective mechanisms are involved (4, 20). Hence, the effects of MD plus tBOOH on cells from different stages of growth were investigated. Stationary-phase cells were found to be 50-fold more resistant to MD-plus-tBOOH killing than log-phase cells after 30 min of treatment ($P < 0.05$) (Fig. 3A). The data suggest that resistance to multiple-oxidant killing requires growth-phase-dependent products and/or structural changes. Increased expression of a DNA binding protein (Dps) that protects DNA from oxidants and alterations in membrane structure and composition that reduce oxidant permeability have been shown to be involved in stationary-phase multiple-stress resistance (9, 12). This conclusion is supported by previous findings that, during the early stages of growth, bacteria are most susceptible to oxidative stress killing (20). Stationary-phase resistance and starvation-induced resistance

FIG. 3. MD-plus-tBOOH killing of cells. (A) Log-phase cells (□) or stationary-phase cells (○) of *X. campestris* pv. *phaseoli* were treated with 40 mM MD plus 15 mM tBOOH, as described in Materials and Methods. (B) *X. campestris* pv. *phaseoli* (□) and a spontaneous H₂O₂-resistant mutant, *X. campestris* pv. *phaseoli* HR (●), were grown to log phase and treated with 40 mM MD plus 15 mM tBOOH, as described in Materials and Methods. The data shown are means of four independently performed experiments. Error bars indicate the standard error of the mean.

to multiple stresses are important survival mechanisms and have been observed in many bacteria (9).

A spontaneous multiple-peroxide-resistant mutant (*X. campestris* pv. *phaseoli* HR) has been isolated and characterized (6). When in log-phase growth, the mutant is more resistant than the parent strain to H₂O₂ and tBOOH killing, but not to MD killing (6). Therefore, experiments were undertaken to determine the effect of MD-plus-tBOOH treatment on survival of the mutant. Log-phase cells of the mutant and the parental strain were treated with MD plus tBOOH. The results in Fig. 3B show that the mutant is 1,000-fold more resistant to the treatment than the parental strain ($P < 0.05$). We have shown that *X. campestris* pv. *phaseoli* HR has mutations in *oxyR*. In *Xanthomonas*, upon exposure to oxidants, OxyR not only changes from the reduced to the oxidized form but also increases in concentration (15). These mutations in the *oxyR* gene change OxyR structure so that the protein appears to be in an oxidized form in uninduced cells. This might be expected to activate expression of *oxyR* and genes in the OxyR regulon in the absence of an inducing signal. This results in over 100-fold-higher levels of products of OxyR-regulated genes such as the *ahpC* and catalase genes (18). Thus, the mutant has a significantly increased capacity for detoxification of organic peroxides; therefore, it is likely that in the HR mutant, tBOOH is detoxified before its concentration can reach toxic levels, and hence MD cannot exert a synergistic killing effect.

In the case of most microbial pathogens, in vitro sensitivity to oxidant killing shows no direct correlation with ability to survive in the host. One of the reasons for this discrepancy is that, during interactions with the host, bacteria are exposed to multiple oxidants. A major concern raised with respect to oxidant killing of microbes in vivo is whether concentrations of oxidants generated by the host would be sufficient to kill the bacteria, since the results of in vitro studies indicate that bacteria are resistant to high concentrations of oxidants. Our observations that MD potentiates tBOOH killing of *X. campestris* suggest that individual concentrations of oxidants need not be very high to kill bacteria, given that some oxidants act synergistically. Depending on the combination of oxidants, simultaneous exposure may act antagonistically, as in the case of

MD plus H₂O₂, or synergistically, as in the case of MD plus tBOOH. These preliminary findings could be generally useful in helping us to understand the oxidative killing of other microbes. Oxidative killing of bacteria and the roles of the various genes involved in protecting bacteria from this process need to be reevaluated and to accommodate the interactions of different oxidants. More important, common well-characterized bacterial stress responses such as adaptive or cross-protection and induction of oxidant-scavenging enzymes did not protect log-phase cells from killing by combinations of oxidants. This suggests a target for a novel treatment strategy to control proliferation during the early stages of bacterial growth.

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