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Mutations in *oxyR* Resulting in Peroxide Resistance in *Xanthomonas campestris*

SKORN MONGKOLSUK,[1,2]* WIRONGRONG WHANGSUK,[1,2] MAYUREE FUANGTHONG,[1]† AND SUVIT LOPRASERT[1]

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

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A spontaneous *Xanthomonas campestris* pv. phaseoli H_2O_2-resistant mutant emerged upon selection with 1 mM H_2O_2. In this report, we show that growth of this mutant under noninducing conditions gave high levels of catalase, alkyl hydroperoxide reductase (AhpC and AhpF), and OxyR. The H_2O_2 resistance phenotype was abolished in *oxyR*-minus derivatives of the mutant, suggesting that elevated levels and mutations in *oxyR* were responsible for the phenotype. Nucleotide sequence analysis of the *oxyR* mutant showed three nucleotide changes. These changes resulted in one silent mutation and two amino acid changes, one at a highly conserved location (G197 to D197) and the other at a nonconserved location (L301 to R301) in OxyR. Furthermore, these mutations in *oxyR* affected expression of genes in the *oxyR* regulon. Expression of an *oxyR*-regulated gene, *ahpC*, was used to monitor the redox state of OxyR. In the parental strain, a high level of wild-type OxyR repressed *ahpC* expression. By contrast, expression of *oxyR5* from the *X. campestris* pv. phaseoli H_2O_2-resistant mutant and its derivative *oxyR5*G197D with a single-amino-acid change on expression vectors activated *ahpC* expression in the absence of inducer. The other single-amino-acid mutant derivative of *oxyR5*L301R had effects on *ahpC* expression similar to those of the wild-type *oxyR*. However, when the two single mutations were combined, as in *oxyR5*, these mutations had an additive effect on activation of *ahpC* expression.

Xanthomonas belongs to an important group of bacterial phytopathogens. In response to microbial infection, plants increase production and accumulation of reactive oxygen species (ROS), including H_2O_2, organic peroxide, and superoxide anions, as a component of active plant defense responses (2, 14). Moreover ROS are generated by normal aerobic metabolism (9). Exposure to high levels of ROS leads to inhibition of cell proliferation. Thus, the ability to increase ROS removal could be advantageous to bacteria (7).

OxyR is a peroxide sensor and transcription activator that regulates both catalase and alkyl hydroperoxide reductase (4, 5, 20). OxyR can be converted from the reduced to the oxidized form after exposure to oxidants by formation of a disulfide bond between the highly conserved cysteine residues C199 and C208 (1, 21). This oxidized OxyR then activates transcription of genes in the OxyR regulon (6, 7, 20). In *Xanthomonas*, *oxyR* not only regulates oxidant induction of both catalase and *ahpC* but also mediates the oxidant's inducible H_2O_2 resistance phenotype (17, 18). *Xanthomonas ahpC* and *oxyR* have atypical gene arrangements and transcription organizations. *ahpC* is transcribed as a monocistronic mRNA, while *ahpF-oxyR* and *orfX* are in an operon (15, 17).

We have isolated and partially characterized a spontaneous *Xanthomonas campestris* pv. phaseoli peroxide-resistant mutant, designated *Xp*HR (8). The mutant is highly resistant to killing by peroxide and has over a 50-fold increase in the peroxide-scavenging enzymes catalase and alkyl hydroperoxide reductase subunit C (AhpC) (8). In this paper, we characterize the role of OxyR in the mutant *Xp*HR. The results show not only that the level of OxyR is elevated but also that there are several mutations in the protein. These factors contribute to constitutive activation of genes in the *oxyR* regulon and to the H_2O_2 resistance phenotype.

Increased levels of AhpC, AhpF, and OxyR in *Xp*HR. The levels of AhpC, AhpF, and OxyR in uninduced *Xp*HR and its parental strain were compared by Western analysis (Fig. 1). AhpC, AhpF, and OxyR levels in *Xp*HR were over 20-fold higher than in the parental strain. In *Xanthomonas*, exposure to oxidants leads to a severalfold increase in OxyR levels (17). The OxyR level in *Xp*HR was threefold higher than the OxyR level in an oxidant-induced culture of the parental strain (data not shown). In addition, two forms of OxyR were detected in the mutant. One form (designated N for normal) comigrated with OxyR from the parental strain, while the other form (designated S for slow) had slower migration. In *X. campestris* pv. phaseoli, concentrations of catalase, AhpC, AhpF, and OxyR are increased only in response to oxidant treatments. Elevated levels of these proteins in uninduced cultures of *Xp*HR were highly unusual and suggested deregulation of the peroxide stress response.

Construction of an *Xp*HR *oxyR* mutant. To determine whether the high level of OxyR in the uninduced growth of the mutant was responsible for the H_2O_2 resistance phenotype, a marker-exchanged *oxyR* mutant of *Xp*HR was constructed as previously described (18). *Xp*HR *oxyR* had resistance levels to H_2O_2, organic peroxide, and menadione killing similar to those of *X. campestris* pv. phaseoli *oxyR* (Fig. 2A). We extended these observations by determining the levels of the peroxide-scavenging enzymes catalase and AhpC in these bacteria (Fig. 2B and C). The increases in catalase activities and the amount of AhpC in *Xp*HR were abolished in the *Xp*HR *oxyR* mutant (Fig. 2B and C).

Detection of mutations in *Xp*HR *oxyR5*. PCR of *oxyR* from the *Xp*HR mutant (*oxyR5*) was performed, using primers located at the 5′ end (5′ACGCGCCAGTCGTTCCCCG 3′) and

* Corresponding author. Mailing address: Laboratory of Biotechnology, Chulabhorn Research Institute, Lak Si, Bangkok 10210, Thailand. Phone: (662) 574-0623. Fax: (662) 574-2027. E-mail: skorn@tubtim.cri.or.th.

† Present address: Section of Microbiology, Wing Hall, Cornell University, Ithaca, NY 14853-8101.

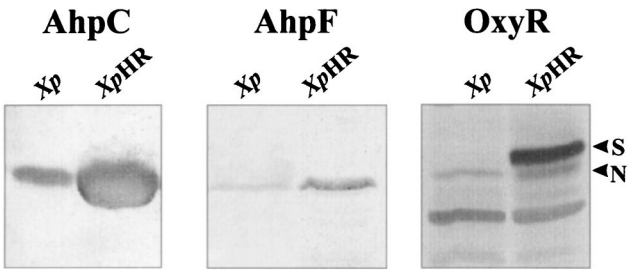

FIG. 1. Western analysis of AhpC, AhpF, and OxyR in *Xp*HR and the parental strain. *X. campestris* pv. phaseoli (*Xp*) and *Xp*HR were grown aerobically to mid-log phase in Silva Buddenhagen (SB) medium at 28°C. Cell lysate preparation, gel electrophoresis, blotting to nitrocellulose membranes, and antibody reactions were performed as previously described (17). Antibody reactions were subsequently detected with an anti-rabbit antibody conjugated to alkaline phosphate. Total protein (50 μg) was loaded into each lane. Western blots were treated with an anti-AhpC (AhpC), an anti-AhpF (AhpF), and an anti-OxyR (OxyR) antibody, respectively.

at the 3′ end (5′ ACCACAGCCAAAGCGATCGCA 3′) of the *oxyR* coding region, with *Pfu* polymerase for 25 cycles. The 960-bp PCR products were cloned into pGEM-T easy (Promega), and their nucleotide sequences were determined with ABI Prism kits on an ABI 310 automated DNA sequencer. *oxyR* from *Xp*HR, designated *oxyR5*, showed three nucleotide changes from the parental gene. The first change, at nucleotide position T213C of the *oxyR* sequence, resulted in a silent mutation. The second and third single-base changes, at positions G590A and T902G, resulted in two amino acid residue changes at the highly conserved position G197 (to D197) and the nonconserved L301 (to R301). No other mutations were detected. To ascertain the effects of these mutations on gene expression, two additional *oxyR5* variants, each with a single-amino-acid difference from the parental gene, were constructed. *oxyR5*G197D, with a single-amino-acid change, was constructed by partial digestion of poxyR5 (*oxyR5* in pBluescript KS) with *Xho*I and *Xba*I. A 150-bp fragment from the internal portion of *oxyR* was removed and replaced by a 150-bp *Xho*I-*Xba*I fragment from poxyR (18). This replaced the mutation at L301R in *oxyR5* with a wild-type sequence. *oxyR5*R301L, with a single-amino-acid change, was constructed by partial digestion of poxyR5 with *Eco*RI and *Xho*I. The 380-bp fragment containing mutated G197D was replaced with a 380-bp *Eco*RI-*Xho*I fragment from a wild-type *oxyR*. All constructs were sequenced to confirm the mutations.

Mutations in *oxyR* affect gene expression. The effects of different *oxyR* mutations on the expression of an *oxyR*-regulated gene, *ahpC*, were determined. In *Xanthomonas*, *ahpC* has a unique pattern of regulation. Its expression can be increased 50-fold in response to oxidants in an *oxyR*-dependent fashion (17, 18). Moreover, expression of the gene is affected by both oxidized and reduced forms of OxyR (18; S. Mougkolsuk, unpublished data). High levels of reduced OxyR lead to repression of *ahpC* (Mougkolsuk, unpublished), while oxidized OxyR activates expression of *ahpC* (18). Thus, expression anal-

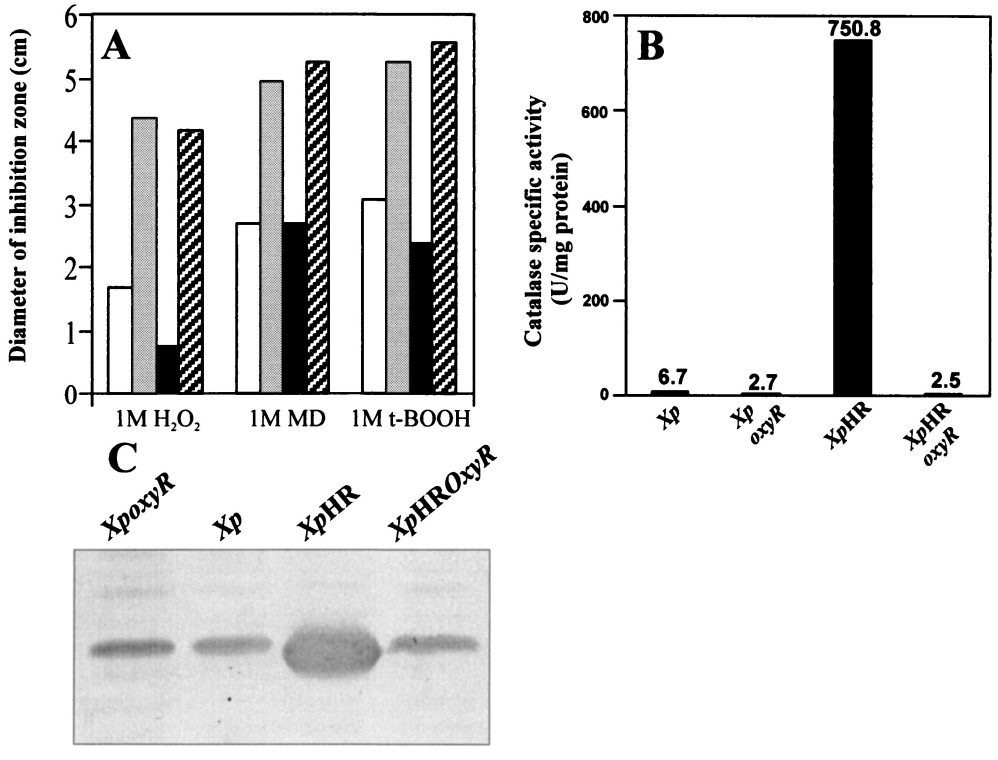

FIG. 2. Resistance to oxidant killing and levels of peroxide-scavenging enzymes in *Xp*HR and its parental strain. (A) Qualitative determination of levels of resistance to killing concentrations of H_2O_2, menadione (MD), and *tert*-butyl hydroperoxide (t-BOOH) in *X. campestris* pv. phaseoli (*Xp*; □), *X. campestris* pv. phaseoli *oxyR* (*Xp oxyR*; ▨), *Xp*HR (■), and *Xp*HR *oxyR* (▨). Essentially, log-phase cells were mixed with Silva Buddenhagen (SB) top agar and poured onto SB plates. Six microliters of the indicated concentrations of oxidants were spotted on paper disks and placed on top of cell lawns. The zone of growth inhibition was measured after 30 h of incubation (16). Experiments were repeated at least three times, and representative data are shown. (B) Catalase levels of various *Xanthomonas* strains. (C) Western analysis of AhpC levels in various *Xanthomonas* strains. Forty micrograms of total protein was loaded into each lane. Western analysis and catalase assays were performed as previously described (17).

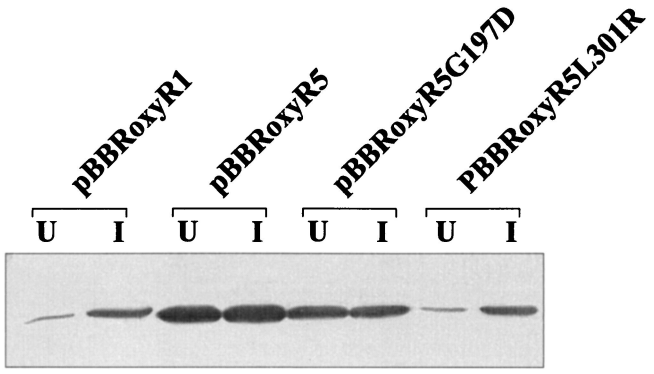

FIG. 3. Effects of various mutations in *oxyR* on levels of AhpC during uninduced and menadione-induced growth. Western analysis of AhpC levels in *X. campestris* pv. phaseoli *oxyR* harboring various *oxyR* genes on an expression vector from a parental strain (pBBRoxyR1), the *Xp*HR mutant (pBBR*oxyR5*), and the gene with one amino acid changed (pBBRoxyR5G197D and pBBR oxyR5L301R). These cells were grown uninduced (U) in Silva Buddenhagen (SB) medium or induced with 100 μM menadione (I) for 30 min. Total protein (20 μg) was loaded into each lane. Lysate preparation, gel electrophoresis, and antibody reactions were performed as described previously (17) and in the legend to Fig. 1.

ysis of the gene would also give an indication of the redox status of the cells and OxyR. In *X. campestris* pv. phaseoli under noninducing growth conditions, *ahpC* is expressed at low levels. By contrast, *ahpC* is expressed at high levels in *Xp*HR without any inducing signals (Fig. 1). We tested whether mutations in *oxyR* were responsible for the altered *ahpC* expression. An *X. campestris* pv. phaseoli *oxyR* mutant was transformed with expression plasmids containing pBBRoxyR5, pBBRoxyR1, pBBRoxyR5G197D, and pBBRoxyR5L301R, and the AhpC levels were monitored (Fig. 3). The *oxyR* mutant harboring pBBRoxyR5 showed a greater-than-50-fold increase in AhpC levels in the uninduced state. On the other hand, cells harboring pBBRoxyR1 showed fivefold repression of AhpC levels. The OxyR mutant harboring pBBRoxyR5L301R repressed AhpC levels in a fashion similar to that of cells harboring pBBRoxyR1, while the mutant harboring pBBRoxy R5G197D produced AhpC at levels 20 times higher than those of a control strain in the absence of inducing signals. Nonetheless, AhpC levels in strains harboring pBBRoxyR5G197D were still about twofold less than the level attained in cells harboring pBBRoxyR5. Next, we examined the effects of an oxidant on mutant OxyR proteins. The levels of AhpC were monitored in *X. campestris* pv. phaseoli *oxyR* cells harboring various *oxyR*-containing plasmids grown under noninducing and inducing conditions (100 μM menadione) (Fig. 3). AhpC levels in cells harboring pBBRoxyR1 or pBBRoxyR5L301R showed strong induction after menadione treatment. By contrast, cells harboring pBBRoxyR5 or pBBRoxyR5G197D expressed *ahpC* at constitutive high levels, and menadione treatment did not result in further increases in the amount of AhpC (Fig. 3).

These results raised the question of the mechanisms responsible for this deregulation. Expression of *oxyR5* from *Xp*HR in *X. campestris* pv. phaseoli *oxyR* led to activation of *ahpC* expression in uninduced cultures, indicating that mutations in *oxyR5* were responsible for unregulated gene expression. *oxyR5* had amino acid changes at two positions, G197D and L301R. G197 is a highly conserved position found in all OxyR proteins (15, 21). The observation that *Xanthomonas* harboring pBBRoxyR5G197D activated *ahpC* expression in the absence of inducing signals confirmed the importance of this mutation in producing altered gene expression. The position of this mutation is in close proximity to the redox-active cysteine C199 (21) and may be responsible for the conversion of OxyR from a reduced to an oxidized form in uninduced cells. In *Escherichia coli*, mutations located close to redox-active C199 (i.e., H198Y, R201C, and C208Y) (13), produce constitutively active proteins similar to G197D in *Xanthomonas*. Oxidation of OxyR occurs at C199 via a sulphenic intermediate and subsequent formation of a disulfide bond with C208 (1, 21). Also, the highly conserved basic residues (H198 and R291) could enhance the activity of C199 (13). Thus, an amino acid change from a neutral G to an acidic D could alter OxyR structure so that either the C199 is more easily accessible to cellular oxidants or the charged residue promotes and stabilizes the formation of sulphenic intermediates. Alternatively, the presence of a carboxylate group at D197 close to the SH group of C199 could result in proton transfer from the SH group to the carboxylate group, resulting in thiolate formation. Thiolate groups are more reactive than SH groups and can subsequently react with carboxyl groups to form relatively stable thiolester bonds. The second mutation, at L301R, introduced a basic residue that had no effect on the transcription activation activity of OxyR. The mutated protein can also be activated by exposure to oxidants (Fig. 3). However, when L301R was combined with the mutation at G197D, as in *oxyR5*, the double mutation enhanced the ability of OxyR to activate transcription of *ahpC* to levels greater than the levels attained by *oxyR5*G197D. The carboxy terminus regions of OxyR and a subclass of LysR transcription activators have been shown to be crucial to protein binding to DNA (12, 19) and in tetramerization or oligomerization of OxyR (12). Mutation at L301R did not seem to affect the ability of mutated *oxyR* to repress *ahpC* expression. Thus, mutation at L301R might possibly affect tetramerization and might enhance the DNA binding of OxyR. Together with G197D, it may enhance binding of OxyR5 and recruiting of RNA polymerase to the promoter. We are attempting to purify the mutated proteins and examine their abilities to bind to the promoter.

G197D mutation was responsible for altered OxyR mobility. We next compared the proteins from several OxyR variants to determine if the mutations in *oxyR* were responsible for the altered protein mobility. The *X. campestris* pv. phaseoli *oxyR*-minus mutant was transformed with a broad-host-range expression vector (pBBR1MCS-4 [11]) containing various constructs of *oxyR*. OxyR Western analysis of lysates prepared from these cells were performed, and the results (Fig. 4) showed that wild-type *oxyR* produced a single OxyR form (N form) that reacted against an anti-OxyR antibody. By contrast, *oxyR5* from *Xp*HR (pBBRoxyR5) produced both S and N forms. This finding was similar to that shown in Fig. 1. Results for *oxyR* variants with single-amino-acid changes (Fig. 4) showed that cells harboring the plasmid containing pBBRoxyR5(G197D) produced S and N forms of OxyR with the S form accounting for greater than 90% of the total OxyR, while cells harboring plasmids containing pBBRoxyR5L301R produced OxyR with mobility similar to that of plasmids containing wild-type *oxyR*. We believe that the S form arises from oxidation of mutant OxyR proteins in the polyacrylamide gel.

All members of the LysR family, including *oxyR*, are autoregulated (19). In *Xanthomonas*, unlike other bacteria, OxyR increased severalfold in concentration as well as changing form in response to oxidants (17). Preliminary data suggest that *oxyR* expression is activated by the oxidized form of the protein (Mongkolsuk, unpublished). This autoregulation could account for the high levels of mutant OxyR detected in *Xp*HR. We are investigating the autoactivation of *Xanthomonas oxyR*.

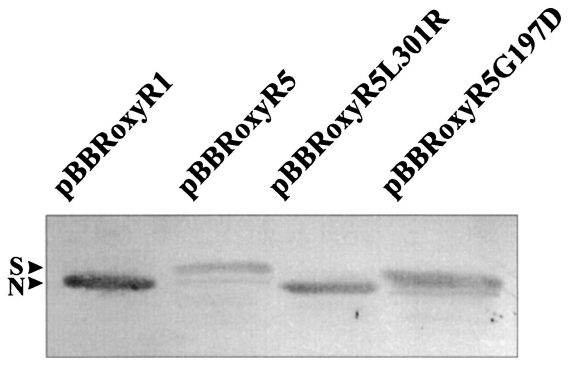

FIG. 4. Effects of mutations in *oxyR* on protein mobility. Cell growth, lysate preparation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western analysis of OxyR were performed as described previously (17) and in Fig. 1. Proteins from lysates (20 μg) were loaded into each lane. N and S indicate two forms of OxyR.

Mutation and deregulation of *oxyR* lead to uncontrolled gene activation in *Xp*HR that is responsible for the H_2O_2-resistant phenotype. In an analogous situation, a *Bacillus subtilis* H_2O_2-resistant mutant (10) has been shown to arise from deregulation of a peroxide repressor, *perR* (3).

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REFERENCES

1. **Aslund, F., M. Zheng, J. Beckwith, and G. Storz.** 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. Proc. Natl. Acad. Sci. USA **96:**6161–6165.
2. **Baker, C. J., and E. W. Orlandi.** 1995. Active oxygen in plant pathogenesis. Annu. Rev. Phytopathol. **33:**299–321.
3. **Bsat, N., A. Herbig, L. Casillas-Martinez, P. Setlow, and J. D. Helmann.** 1998. *Bacillus subtilis* contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol. Microbiol. **29:**189–198.
4. **Christman, M. F., G. Storz, and B. N. Ames.** 1989. OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. USA **86:**3484–3488.
5. **Demple, B.** 1991. Regulation of bacterial oxidative stress genes. Annu. Rev. Genet. **25:**315–337.
6. **Dhandayuthapani, S., M. Mudd, and V. Deretic.** 1997. Interactions of OxyR with the promoter region of the *oxyR* and *ahpC* genes from *Mycobacterium leprae* and *Mycobacterium tuberculosis*. J. Bacteriol. **179:**2401–2409.
7. **Farr, S. B., and T. Kogoma.** 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. **55:**561–585.
8. **Fuangthong, M., and S. Mongkolsuk.** 1997. Isolation and characterization of a multiple peroxide resistant mutant from *Xanthomonas campestris* pv. *phaseoli*. FEMS Microbiol. Lett. **152:**189–194.
9. **Gonzalez-Flecha, B., and B. Demple.** 1997. Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing *Escherichia coli*. J. Bacteriol. **179:**382–388.
10. **Hartford, O. M., and B. C. Dowds.** 1994. Isolation and characterization of a hydrogen peroxide resistant mutant of *Bacillus subtilis*. Microbiology **140:** 297–304.
11. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. I. Roop, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic resistances. Gene **166:**175–176.
12. **Kullik, I., J. Stevens, M. B. Toledano, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for DNA binding and multimerization. J. Bacteriol. **177:**1285–1291.
13. **Kullik, I., M. B. Toledano, L. A. Tartaglia, and G. Storz.** 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. J. Bacteriol. **177:**1275–1284.
14. **Levine, A., R. Tenhaken, R. Dixon, and C. Lamb.** 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:**583–593.
15. **Loprasert, S., S. Atichartpongkun, W. Whangsuk, and S. Mongkolsuk.** 1997. Isolation and analysis of the *Xanthomonas* alkyl hydroperoxide reductase gene and the peroxide sensor regulator genes *ahpC* and *ahpF-oxyR-orfX*. J. Bacteriol. **179:**3944–3949.
16. **Mongkolsuk, S., S. Loprasert, P. Vattanaviboon, C. Chanvanichayachai, S. Chamnongpol, and N. Supsamran.** 1996. Heterologous growth phase- and temperature-dependent expression and H_2O_2 toxicity protection of a superoxide-inducible monofunctional catalase gene from *Xanthomonas oryzae* pv. oryzae. J. Bacteriol. **178:**3578–3584.
17. **Mongkolsuk, S., S. Loprasert, W. Whangsuk, M. Fuangthong, and S. Atichartpongkun.** 1997. Characterization of transcription organization and analysis of unique expression patterns of an alkyl hydroperoxide reductase C gene (*ahpC*) and the peroxide regulator operon *ahpF-oxyR-orfX* from *Xanthomonas campestris* pv. phaseoli. J. Bacteriol. **179:**3950–3955.
18. **Mongkolsuk, S., R. Sukchawalit, S. Loprasert, W. Praituan, and A. Upaichit.** 1998. Construction and physiological analysis of a *Xanthomonas* mutant to examine the role of the *oxyR* gene in oxidant-induced protection against peroxide killing. J. Bacteriol. **180:**3988–3991.
19. **Schell, M. A., P. H. Brown, and S. Raju.** 1990. Use of saturation mutagenesis to localize probable functional domains in the NahR protein, a LysR-type transcription activator. J. Biol. Chem. **265:**3844–3850.
20. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell **78:**897–909.
21. **Zheng, M., F. Aslund, and G. Storz.** 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. Science **279:**1718–1721.