รายงานฉบับสมบูรณ์

โครงการ "การศึกษาพันธุศาสตร์ และสรีรวิทยาของยีนส์ที่มีความสำคัญในการทำลาย Organic Hydroperoxides ในเชื้อแบคทีเรีย *Xanthomonas*"

Genetics and Physiological Characterization of genes involve in detoxification of organic hydroperoxides in phytopathogen, *Xanthomonas*.

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และ

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

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SUMMARY.

The original goal of the project was to investigate the toxic effect of organic hydroperoxide and how *Xanthomonas*, soil bacteria and bacterial phytopathogen protect themselves from its toxicity. However, in order to under this important question, it is necessary to investigate the general oxidative stress response and how different processes are co-ordinated. In this project, we are the first group to successfully identified a new family of organic peroxide resistance gene (*ohr*). Subsequently, this gene family has been shown by us and many other investigators to be widely distributed in both gram negative and gram positive bacteria suggesting that it is generally important in protecting bacteria against organic peroxide toxicity. In addition, we have characterized in details the widely distributed alkyl hydroperoxide reductase gene that coded for the organic peroxide metabolising enzyme. The gene structure, physiological functions and regulation levels were investigated. The research has revealed novel aspects of its regulation and complex physiological function which are differed from other bacteria. Moreover, the global regulator and peroxide sensor, OxyR from *Xanthomonas* has been investigated. The regulator has crucial role in mediating general peroxide and oxidative stress responses and mutation in the gene resulted in pleotropic changes in bacterial stress physiology. As previously, mention that understanding of peroxide stress alone is not sufficient, the overall process of oxidative stress response must also be investigated. We have performed basic bacterial physiological studies that revealed complex processes of *Xanthomonas* oxidative stress response.

Key Words: *ahpC*, *ohr*, organic peroxide toxicity, *OxyR*.

บทคัดย่อ

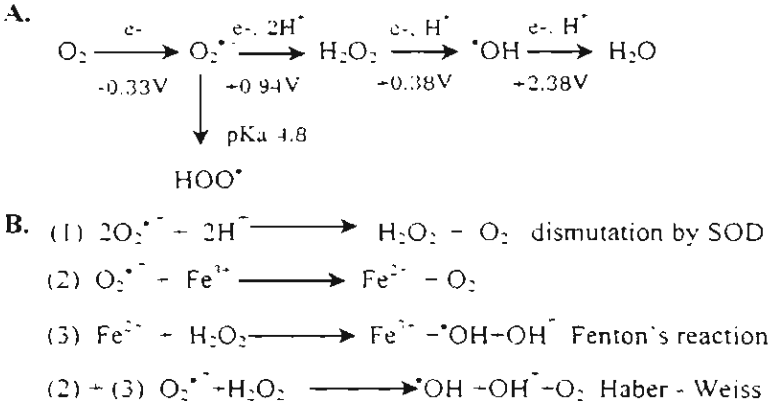
วัตถุประสงค์ของโครงการวิจัยครั้งนี้คือ การศึกษา toxic effect ของสาร organic hydroperoxide และกลไกที่แบคทีเรีย *Xanthomonas* (ซึ่งเป็นแบคทีเรียที่อยู่ในดินที่สามารถก่อโรคในพืชเศรษฐกิจ) ใช้ในการป้องกันและกำจัดพิษของสารดังกล่าว เพื่อให้บรรลุวัตถุประสงค์ คณะวิจัยจำเป็นต้อง เข้าใจถึงกลไกโดยทั่วไปที่แบคทีเรียใช้ตอบสนองต่อสภาวะ oxidative stress ผลของงานวิจัยจากโครงการวิจัยนี้ทำให้คณะผู้วิจัยสามารถพบและรายงานยีนชนิดใหม่ที่เกี่ยวข้องกับการต้านทานต่อสาร organic hydroperoxide ที่ให้ชื่อว่า *ohr* (organic hydroperoxide resistance) ซึ่งต่อมาได้มีรายงานวิจัย จากคณะผู้วิจัยและนักวิจัยกลุ่มอื่นว่า พบยีนนี้ ปรากฏอยู่ในแบคทีเรียทั้งแกรมลบและแกรมบวก นอกจากนี้คณะผู้วิจัยยังได้ทำการแยกยีน alkyl hydroperoxide reductase (*ahpCF*) ที่ควบคุมการสร้างเอนไซม์ที่แบคทีเรียใช้ในการทำลายพิษของ organic hydroperoxide ทำให้คณะผู้วิจัยสามารถศึกษาถึงโครงสร้างของยีน การควบคุมการแสดงออกของยีน และความสำคัญของยีนนี้ต่อแบคทีเรีย *Xanthomonas* โดยพบว่ามีหลายลักษณะที่แตกต่างจากในแบคทีเรียชนิดอื่นที่เคยมีรายงานมา คณะผู้วิจัยยังสามารถแยกยีนที่ควบคุม (regulate) การแสดงของยีน *ahpCF* คือ ยีน *oxyR* ซึ่งเป็นทั้ง global peroxide sensor และ regulator ของยีนที่เกี่ยวข้องกับการทำลายพิษจากสาร peroxide ต่างๆ เพื่อเข้าใจถึงบทบาทของยีนนี้ต่อการตอบสนองต่อสาร peroxide ในแบคทีเรีย *Xanthomonas* โดยสรุป คณะผู้วิจัยได้ทำการศึกษาและแสดงให้เห็นถึงกระบวนการอันซับซ้อนที่แบคทีเรีย *Xanthomonas* ใช้ในการตอบสนองต่อสภาวะ oxidative stress

คำหลัก : *ahpC*, *ohr*, organic peroxide toxicity, *OxyR*.

CONTENT

INTRODUCTION.

Xanthomonas are gram negative aerobic soil bacteria and important bacterial phytopathogen. During normal growth, large quantity of reactive oxygen species (ROS) including superoxide anions, hydrogen peroxide and organic peroxide (Fig.1) are produced as by products of aerobic metabolism. During plant microbe interaction, one of active plant defense responses to microbial invasion is increased production of reactive oxygen species (ROS) (Baker and Orlandi, 1995; Levine *et al.*, 1994). There are evidence correlated increased production of ROS with the ability of plant to successfully defense against pathogen attack (Baker and Orlandi, 1995; Levine *et al.*, 1994). These ROS could react with macromolecules in the cells leading to genetics and cellular damages. ROS need to be rapidly removed before their concentrations build up to toxic levels resulted in inhibition of cells growth. These observations indicate that production



of ROS is an important part of plant defense response.

Fig.1 Formation of reactive oxygen species (ROS).

In A: The four electron reduction of molecular oxygen to water.

B: Reactions involve in the generation of ROS.

General microbial oxidative stress protective genes.

The microbial oxidative stress response is a well-orchestrated reactions involving synthesis of many proteins and small molecules. The function of these responses can be broadly classified into three categories namely to detoxify the toxic molecules, to repair damage macromolecules and to regulate various processes. Enzymes involved in detoxification of toxic molecules such as superoxide dismutase (SOD) that breaks down superoxide anions to H_2O_2. H_2O_2 is then break down by both monofunctional and bifunctional catalase (Demple, 1991; Farr and Kogoma, 1991). The second category of enzymes involved in oxidative stress response are various repair enzymes such as exonuclease III and Fappy glycosylase that repairs oxidative damaged DNA and methionine sulfoxide reductase that repairs oxidative damaged proteins (Demple, 1991; Farr and Kogoma, 1991)(Table 1).

Table1

Antioxidant genes, activities and their regulators in *Escherichia coli.*

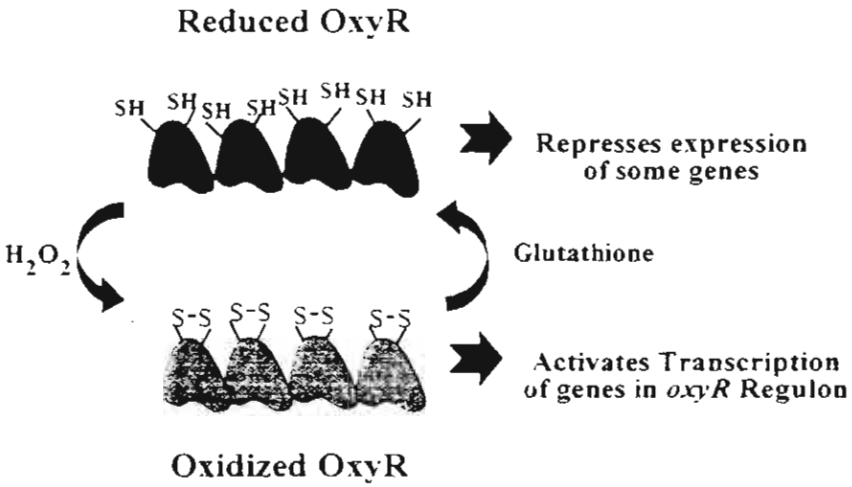
Gene	Activity	Regulators
sodA	Manganese superoxide dismutase	$SoxRS^+$,ArcAB, FNR,Fur,IHF
fumC	Fumarase C	$SoxRS^+$,ArcAB,σ^S
acnA ,σ^S	Aconitase A	$SoxRS^+$,ArcAB,FNR, Fur
zwf	Glucose-6-phosphate dehydrogenase	$SoxRS^+$
fur	Ferric uptake repressor	$SoxRS^+$, OxyR
micF	RNA regulator of *ompF*	$SoxRS^+$, OmpR, LRP
acrAB	Multidrug efflux pump	$SoxRS^+$
tolC	Outer membrane protein	$SoxRS^+$
fpr	Ferredoxin reductase	$SoxRS^+$
fldA	Flavodoxin	$SoxRS^+$
nfo	Endonuclease IV	$SoxRS^+$
sodB	Iron superoxide dismutase	
sodC	Copper-zinc super oxide dismutase	σ^S, FNR
katG	Hydroperoxidase I	OxyR, σ^S

ahpCF	Alkyl hydroperoxide reductase	OxyR
gorA	Glutathione reductase	OxyR, σ^S
grxA	Glutafedoxin 1	OxyR
dps	Non-specific DNA binding protein	OxyR, σ^S,IHF
oxyS	Regulatory RNA	OxyR
katE	Hydroperoxidase II	σ^S
xthA	Exonuclease III	σ^S
polA	DNA polymerase I	
recA	RecA	RecA, LexA
msrA	Methionine sulfoxide reductase	
hslO	Molecular chaperone	
mutM(fpg)	8-hydroxyguanine endonuclease	FNR[70]
hmp	Flavohemoglobin	MetR

The third category of proteins involved in oxidative stress response are regulatory proteins. These proteins such as RpoS, SoxRS and OxyR (Demple, 1991, Rosner and Storz 1997; Storz and Imlay, 1999) regulate and coordinate the global responses to oxidative stress. Some of the proteins are also involved in signal transduction (Table 1)(Storz and Imlay, 1999). *oxyR* and *soxRS* regulons are best characterized regulators involved in oxidative stress response in *E.coli* (Demple, 1991, Rosner and Storz 1997; Storz and Imlay, 1999) SoxR is activated by superoxide possibly via the Fe-S centre of the protein. Activated SoxR then enhances transcription of *soxS* (Demple, 1997) High levels of SoxS activates genes involved in superoxide protection in *soxRS* regulon (Nunoshiba *et al.* 1993)(Table 1). Many genes in *oxyR* regulon are activated by H_2O_2 and involved in protection against peroxide toxicity (Table 1)(Storz and Imlay, 1999). OxyR belongs to a LysR family of transcription regulator. OxyR is one of the first regulators shown to have both oxidative stress sensing and transcription regulation activities (Storz et al, 1990; Storz and Imlay, 1999). Recently, the mechanism of OxyR activation and deactivation have been worked out in details (Fig.2)(Zheng *et al*, 1998). Essentially intracellular H_2O_2 reacts with highly conserved C199 and C208 residues to form a disulphide bond (Fig.2)(Storz and Imlay, 1999). The

disulphide bond formation is thought to occur via sulfenic acid intermediate of C199. Formation of the bond changes OxyR from reduced to oxidized form (Zheng *et al*, 1998). Oxidized OxyR activates transcription of genes in the *oxyR* regulon. Once the peroxide stress has been neutralized, oxidized OxyR must be converted back to reduced form. This process involves glutaredoxin and reduced glutathione. Reduced OxyR act as repressor of *oxyR* itself and some genes in the regulon (Zheng *et al*, 1998).

Fig.2 Oxidation and Reduction of OxyR



DISCUSSION.

Oxidative stress response in *Xanthomonas*.

Many aspects of *Xanthomonas* oxidative stress response are differed from other bacteria. In *Xanthomonas*, we have shown that stationary phase cells are more resistance to H_2O_2, organic peroxides and a superoxide generator killing than exponential phase cells (Chamnongpol *et al.*, 1995a). The stationary phase resistance to peroxides and superoxide killing do not depend upon *de novo* protein synthesis (Vattanaviboon *et al.*, 1995). This observation is in agreement with observations in other bacteria.

Oxidants induced adaptive and cross protection responses.

Exposure of bacteria to a sub-lethal dose of one agent can induce protection against subsequent challenged with lethal doses of the same agent (adaptive) or non related agents (cross protection). These responses are important stress survival strategies for bacteria. Exposure of *Xanthomonas* to a low concentration of H_2O_2 confers resistance to subsequent challenges with a killing concentration of H_2O_2 . Surprisingly, no adaptive response to organic peroxides (tert butyl or cumene hydroperoxides) or a superoxide generator (menadione) have been observed (Vattanaviboon *et al.* 1995). While, exposure to a low concentration of a superoxide generator (menadione) confers cross protection against lethal concentrations of H_2O_2 and organic hydroperoxides (tert butyl and cumene hydroperoxides)(Mongkolsuk *et al.* 1997). Both adaptive and cross protection responses require de novo protein synthesis and aerobic growth. These responses to oxidants could be important for *Xanthomonas* during interactions with plant. Plant generated ROS might induce catalase which in turn protects *Xanthomonas* from the growth inhibiting effects of H_2O_2 produced by plant defense responses. *Xanthomonas* cells in the early log phase cells are more sensitive to oxidative killing than cells at other stages of growth. Thus, induced protection against oxidative stress allows the bacteria to overcome oxidative stress and grow in plant. During plant-microbe interactions and in the environment, *Xanthomonas*

campestris pv. phaseoli are likely to be exposed to high concentrations of multiple oxidants. Here, we showed that simultaneous exposures of the bacteria to multiple oxidants affected cells survival in a complex manner. A superoxide generator (menadione) enhanced the killing effect of an organic peroxide (*tert*-butyl hydroperoxide) by 1000-fold; conversely, treating cells with menadione plus H_2O_2 resulted in 100-fold protection compared to cells treated with individual oxidants. *Xanthomonas* treated with a combination of H_2O_2 and *tert*-butyl hydroperoxide showed no additive or protective effects. High levels of catalase alone were sufficient to protect cells against killing effects of menadione plus H_2O_2 and *tert*-butyl hydroperoxide plus H_2O_2. These data suggested that H_2O_2 was responsible for killing of bacteria in 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 pretreatment had no protective effect against a combination of menadione plus *tert*-butyl hydroperoxide killing. Nonetheless, *Xanthomonas* cells in stationary phase and a spontaneous H_2O_2 resistant mutant (*Xanthomonas campestris* pv. phaseoli HR) showed increased resistance to menadione plus *tert*-butyl hydroperoxide killing. The findings give new insight into oxidants killing of *Xanthomonas* that could be generally applied to other bacteria.

Isolation and characterization of genes involved in peroxide protection.

Catalase is the major H_2O_2 scavenging enzyme in bacteria. We have identified at least two forms of monofunctional catalase designated KatA and KatE and one form of bifunctional catalase peroxidase enzymes. KatA is the major form of catalase in *Xanthomonas campestris* pv. *phaseoli* (Vattanavibbon and Mongkolsuk, 2000). We are working on isolation of the gene. KatE is a growth phase regulated catalase. KatE belongs to a family of "long" bacterial catalase that shared high degree of amino acid sequence homology with mammalian catalases. Mutations in *katE* show no alterations in catalase levels and sensitivity to H_2O_2 killing. This is due to

compensatory increase in other forms of catalase (Vattanavibbon and Mongkolsuk, 2000). In *X.oryzae* pv. *oryzae*, a homologue of *katE* designated *katY* is inducible with menadione and H_2O_2 (Mongkolsuk, et al. 1996). The ability of *Xanthomonas* to protect themselves from killing concentrations of a man made (n ethylmaleimide, NEM) and an endogenously produced (methyl glyoxal, MG) electrophiles were investigated. Pretreatment of *Xanthomonas* with a low concentration of NEM induced protection against killing concentrations of NEM and MG. While, MG pretreatment only weakly induced protection against NEM killing but not against itself. NEM induced protection against electrophile killing required new protein synthesis and was abolished by addition of a protein synthesis inhibitor. By contrast, MG induced protection against NEM killing was independent of new protein synthesis. *Xanthomonas* harbouring an expression vector containing a catalase gene was over 100 fold more resistance to MG and NEM killing. High expression levels of genes for other peroxide protective enzymes such as alkyl hydroperoxide reductase (*ahpC* and *ahpF*) and *ohr* had no protective effects against electrophile killing. Thus, catalase appears to have novel protective roles against electrophile toxicity. Moreover, this suggests that in *Xanthomonas* NEM and MG toxicity might involve accumulation and/or increased production of H_2O_2. The idea was further supported by the observation that addition of 10 mM sodium pyruvate, a compound that could chemically react with peroxide protected *Xanthomonas* from MG and NEM killing. The protective role of catalase and the role of H_2O_2 in electrophile toxicity are novel and could be generally important in different bacteria (Vattanaviboon et al., 2001).

Alkyl hydroperoxide reductase (AhpR) is the best characterized bacterial defense enzyme against organic hydroperoxides. The enzyme has two subunits, a 22 kDa catalytic subunit AhpC and a 57 kDA flavoprotein-reductase subunit, AhpF (Poole, 1996). Both components are required for NADH or NADPH dependent reduction of organic peroxides to corresponding alcohols (Poole, 1996). AhpC belongs to a family of evolutionary highly conserved enzymes which have

been found from bacteria to man (Chae *et al.* 1994). AhpF shares homology with thioredoxin reductases (Poole, 1996). Purified AhpR can use wide range of substrate from H_2O_2 and simple organic peroxides to more complex lipid and nucleic acid hydroperoxides (Poole, 1996). This suggests that the enzymes probably have important physiological roles in protection against peroxide toxicity (Storz and Imlay, 1999). In many bacteria, mutations in the gene lead to defects in the bacterial ability to protect themselves from organic peroxide toxicity. We have isolated and characterized an *ahpC* homologue, from *Xanthomonas campestris* pv. *phaseoli*. The predicted amino acid sequence shared over 50% identity to AhpC from *E.coli*. Western analysis shows that oxidants (peroxides and superoxides), a thiol reagent (NEM) and a heavy metal, $CdCl_2$ induce large increased in the steady state level of AhpC (Fig.5)(Loprasert *et al.*, 1997; Mongkolsuk *et al.* 1997). Northern analysis confirms that oxidant induced AhpC accumulation is due to increase transcription of the gene. In *Xanthomonas*, increased expression of *ahpC* alone confers protection against growth retardation and killing effects of organic peroxides (Mongkolsuk *et al.* 1997). However, high levels of the enzyme did not cross protect the bacteria from H_2O_2 or superoxide generators toxicity.

In *Xanthomonas*, *ahpC*, *ahpF*, and a gene involved in peroxide sensing transcription regulation (*oxyR*) are located in close proximity. *ahpC*, *ahpF* and *oxyR* are arranged in head to tail fashion (Fig.3) (Loprasert *et al.*, 1997).

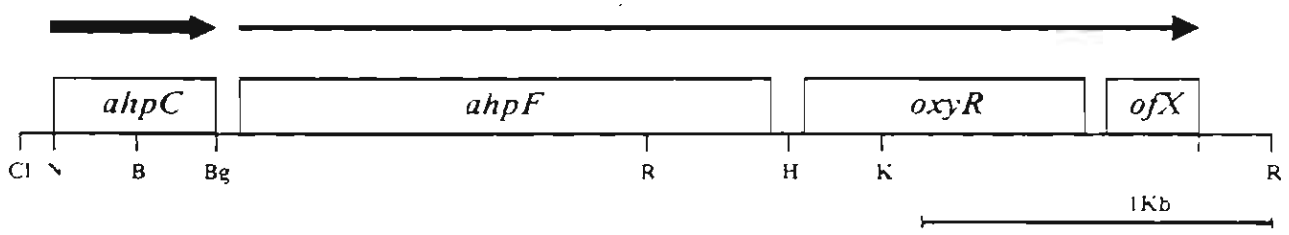


Fig.3 Structural and transcription organization of *ahpC*, *ahpF-oxyR-orfX*.

The arrows represent transcription organization of the genes. B, *Bcl*I; Bg, *Bgl*II; Cl, *Cla*I; H, *Hind*III, K, *Kpn*I; N, *Not*I.

This arrangement is conserved in all *Xanthomonas* strains tested. Transcription organization of these genes are shown in Fig. 6. *ahpC* is transcribed as a monocistronic 0.6 kb mRNA, while *ahpF, oxyR* are transcribed as a polycistronic 3.0 kb mRNA (Loprasert *et al.*, 1997). At present, we do not have *ahpC* or *ahpF* mutants. However, expression analysis in *Xanthomonas* suggests that both genes are inducible by oxidants (Fig.5)(Mongkolsuk *et al.* 1997).

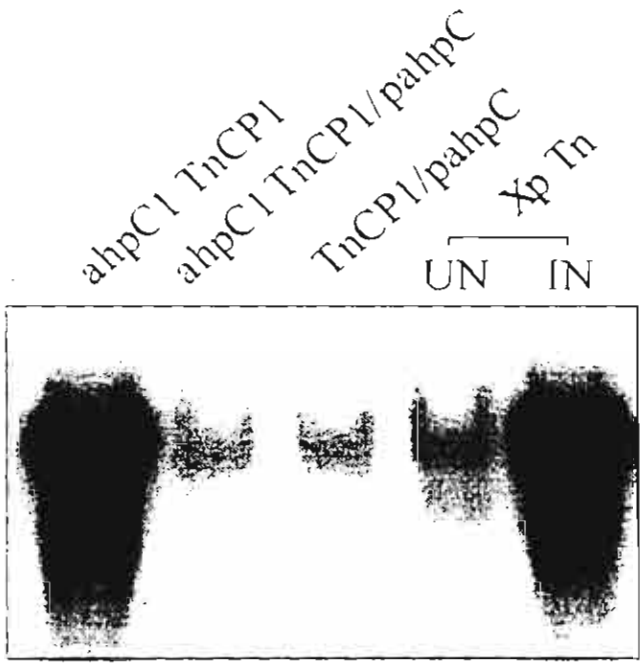


Fig.4 Northern analysis of *ahpC* promoter fused to cat in both ahpC1 and the parental strain.

All *Xanthomonas* strains used contained TnCP1. RNA was extracted by hot phenol method and probed with radioactively labelled *cat*.

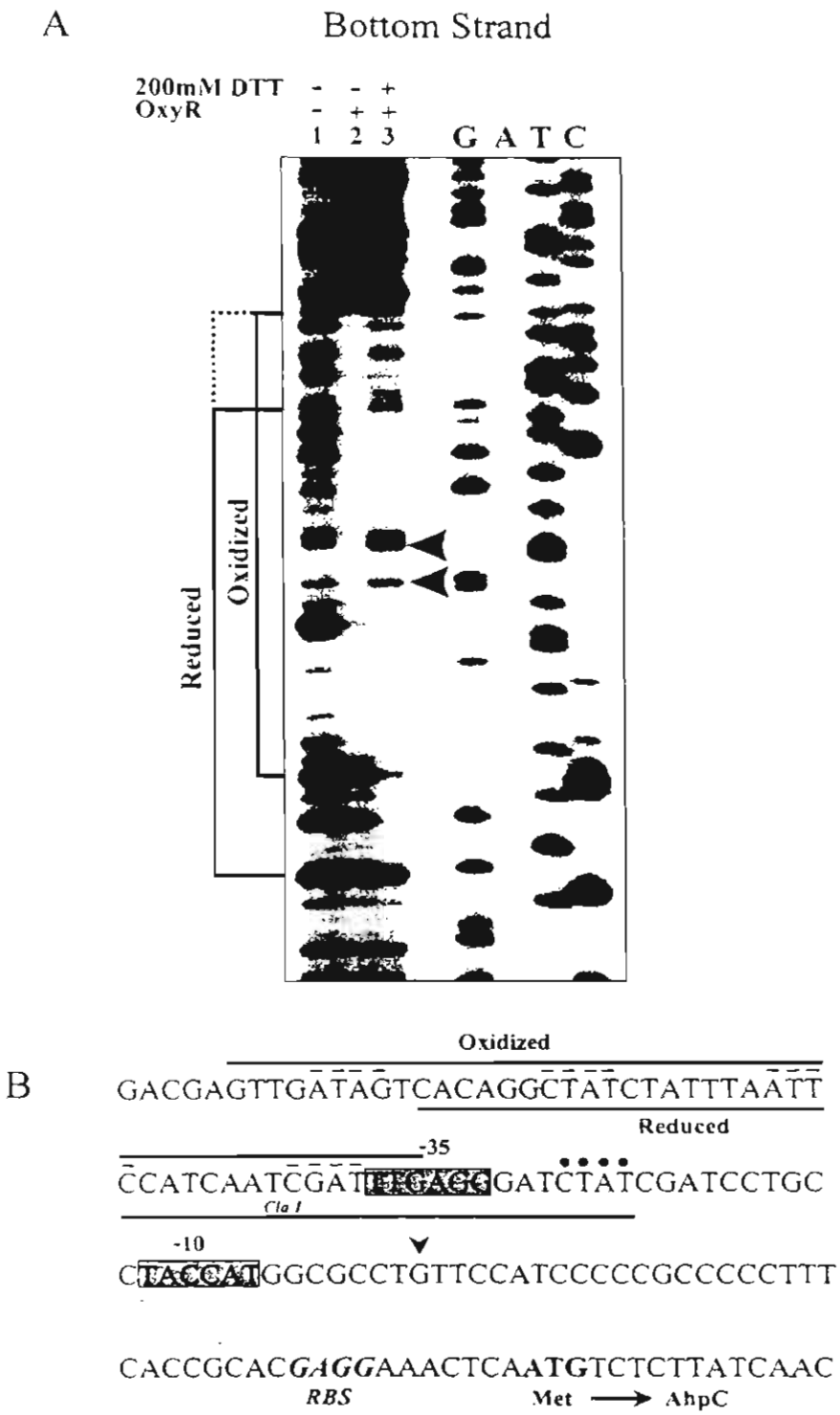


Fig.5 Differential binding of reduced and oxidized OxyR to the *ahpC* promoter.

In A. DNAseI footprint of the *ahpC* promoter fragment performed as described in Loprasert et al., 2000 and in B the *ahpC* promoter and OxyR binding sites showed different binding site for reduced and oxidized OxyR.

We have recently characterized the *ahpC* promoter and OxyR binding sites. Alkyl hydroperoxide reductase subunit C (*ahpC*) had unique patterns of regulation by various forms of OxyR. Reduced OxyR repressed expression of the gene while oxidized OxyR activated its expression. This dual regulation of *ahpC* is unique and unlike all other OxyR regulated genes. The *ahpC* transcription start site was determined. Analysis of the region upstream of the site revealed promoter sequences that had high homology to the *Xanthomonas* consensus promoter sequence. Data from gel shift experiments indicated that both reduced and oxidized OxyR could bind to the *ahpC* regulatory region. Moreover, the reduced and the oxidized forms of OxyR gave different DNAseI footprint patterns indicating that they bound to different sites (Fig.5)(Loprasert et al., 2000). The oxidized OxyR binding site overlapped the -35 region of the *ahpC* promoter by a few bases. This position is consistent with the role of the protein in activating transcription of the gene. Binding of reduced OxyR to the *ahpC* promoter showed an extended DNAseI footprint and DNAseI hypersensitive sites suggesting that binding of the protein caused a shift in the binding site and bending of the target DNA. In addition, binding of reduced OxyR completely blocked the -35 region of the *ahpC* promoter and prevented binding of RNA polymerase leading to repression of the gene (Fig.5). Monitoring of the *ahpC* promoter activity in vivo confirmed the location of the oxidized OxyR binding site required for activation of the promoter. A mutant that separated OxyR regulation from basal *ahpC* promoter activity was constructed. The mutant was unable to respond to oxidants by increasing *ahpC* expression. Physiologically, it had a slower aerobic growth rate and was more sensitive to organic peroxide killing. This indicated that oxidant induction of *ahpC* had important physiological roles in normal growth and during oxidative stress.

Alkyl hydroperoxide reductase subunit C (AhpC) is the catalytic subunit responsible for alkyl peroxide metabolism. An *ahpC* mutant in *Xanthomonas* was constructed. The mutant had

increased sensitivity to organic peroxide killing but was unexpectedly hyper-resistant to H_2O_2 killing. Analysis of peroxide detoxification enzymes in this mutant revealed differential alteration in catalase activities in that its bifunctional catalase/peroxidase and major monofunctional catalase (Kat1) increased several fold while levels of its third growth phase regulated catalase (KatE) did not change. The increase in catalase activities was a compensatory response to lack of AhpC and the phenotype was complemented by expression of a functional *ahpC*. Regulation of the catalase compensatory was complex. Compensatory increase in Kat1 was mediated by OxyR since it was abolished in an *oxyR* mutant. By contrast, compensatory increase in the bifunctional catalase/peroxidase was mediated by an unknown regulator, independent of OxyR. Moreover, the mutation in *ahpC* appeared to convert OxyR from a reduced to oxidized form that activated genes in the OxyR regulon in uninduced cells Fig.4. This complex regulation of peroxide stress response in *Xanthomonas* differed from that in other bacteria (Mongkolsuk et al., 2000).

We have identified a new gene responsible for organic hydroperoxide resistance (*ohr*) from *Xanthomonas campestris* pv.*phaseoli*. The gene is isolated by complementation of an *E.coli* alkylhydroperoxide reductase (*ahpC* and *ahpF*) mutant that has an organic hydroperoxide hypersensitive phenotype. *ohr* encodes a 14.5 kDa protein. The predicted Ohr amino acid sequence has highest homology (63%) to an unknown protein from *Acinetobacter calcoaceticus* and moderate homology (46%) with two unknown proteins (YkIA, and YkzA) from *B.subtilis*. There is lower homology to OsmC (an osmotic inducible protein) from *E.coli* (31%, 18) and an ORF of unknown function from *Mycoplasma genitalium* (31%, 13). All four proteins have similar size and two highly conserved redox sensitive cysteine residues suggesting they could be important in structure and functions of Ohr (Mongkolsuk et al . 1998a). A knockout mutant of *ohr* has been constructed in *Xanthomonas* to evaluate *ohr* physiological roles. The mutant growth

is sensitive to a low concentration of tBOOH and also more sensitive (over 100 fold) to tBOOH killing than the parental strain. The mutant shows no difference in sensitivity to either H_2O_2 or MD killing. These defects in the mutant can be complemented by expression of a functional *ohr* on an expression plasmid (Mongkolsuk et al . 1998a). *ohr* has an unique expression pattern. The gene is highly induced by organic hydroperoxide, weakly by H_2O_2 and not at all by a superoxide generator. Furthermore, the peroxide inducible expression is not under OxyR regulation. The data suggest that Ohr is a new family of organic hydroperoxides protective protein.

Characterization of a *Xanthomonas oxyR*.

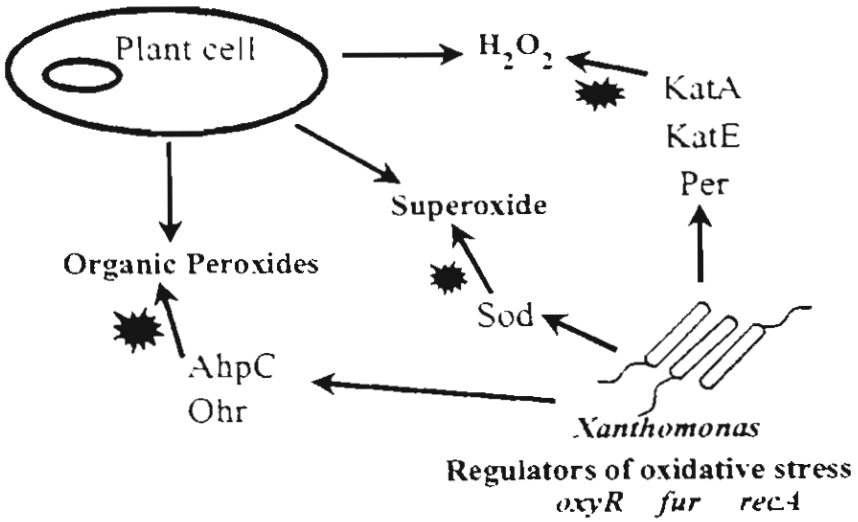
Analysis of OxyR amino acid sequence shows highly conserved C residues at C199 and C208 (Loprasert *et al*. 1997)(Fig.2). These redox active C residues are important for conversion of OxyR from reduced to oxidized form. We have constructed a maker exchanged *oxyR* mutant by replacing the functional copy of the gene with a mutated copy. The mutant show interesting phenotypes which have not been observed in other *oxyR* mutants. First the mutant has defective aerobic plating efficiency. This defect could be complemented by expression of *katE*, *ahpF* genes on an expression vector or addition of pyruvate (a compound that could chemically inactivate toxic peroxide) to growth medium. These observations suggest that the *oxyR* mutant accumulated toxic peroxides leading to growth inhibition and cell death (Mongkolsuk *et al*. 1998b). In other bacteria, OxyR is the regulator of the oxidants induced expression of genes for peroxide scavenging enzyme such as *ahpC*, *kat*. Similar observations have been made in *Xanthomonas*, (Mongkolsuk *et al*. 1998b). Oxidants induction of catalase and *ahpC* were abolished in an *oxyR* mutant. Moreover, the mutant loses the ability to mount H_2O_2 induced adaptive response against H_2O_2 killing but surprisingly it retains the menadione induced cross protection against H_2O_2 killing. The data confirm important role of OxyR in the oxidative stress response.

The regulation of *Xanthomonas oxyR* is unique. In all other bacteria, upon exposure to

oxidants OxyR changes from reduced to oxidized forms. The concentration of the protein remains constant. By contrast, in *Xanthomonas* exposure to oxidants not only changes OxyR from reduced to oxidized forms but also induced synthesis and accumulation of the protein (Fig.2). High levels of oxidized OxyR then activates genes in the regulon. This raise an important question on regulation of *Xanthomonas oxyR*. We are current examined the mechanism responsible for oxidants induced expression of *oxyR*.

Spontaneous mutant of *X.campestris* pv. *phaseoli* H_2O_2 resistant mutant emerge upon selection with 1 mM H_2O_2. we show that growth of this mutant under non-inducing 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* was responsible for the phenotype. Nucleotide sequence analysis of the mutant *oxyR* 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 non conserved location (L301 to R301) in the OxyR. Furthermore, these mutations in *oxyR* effected expression of genes in the *oxyR* regulon. Expression of an *oxyR* regulated gene, *ahpC* was used to monitor redox state of OxyR. In the parental strain, high level of wild type OxyR repressed *ahpC* expression. By contrast, expression of *oxyR5* from *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 similar effects on *ahpC* expression as 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 show complex response to oxidative stress. Different scavenging enzymes and regulator of their genes are summarized in Fig.6.



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Vattanaviboon P., R.Sriprang, S. and Mongkolsuk, S (2001). Catalase has a novel protective role against electrophile killing of *Xanthomonas*. Microbiology. 147: 491–498.

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OUTPUT.

The output in term of publication in international journals and number of students trained have far exceeded the original expectation. Total number of 14 papers have been published in international journals with impact factor ranging from 1.673 to 6.36. In addition, one Ph.D and five M.Sc. students have been trained. The output is summarized in Fig. 1 and 2. The list of graduate student supported is in Fig.3.

Fig1

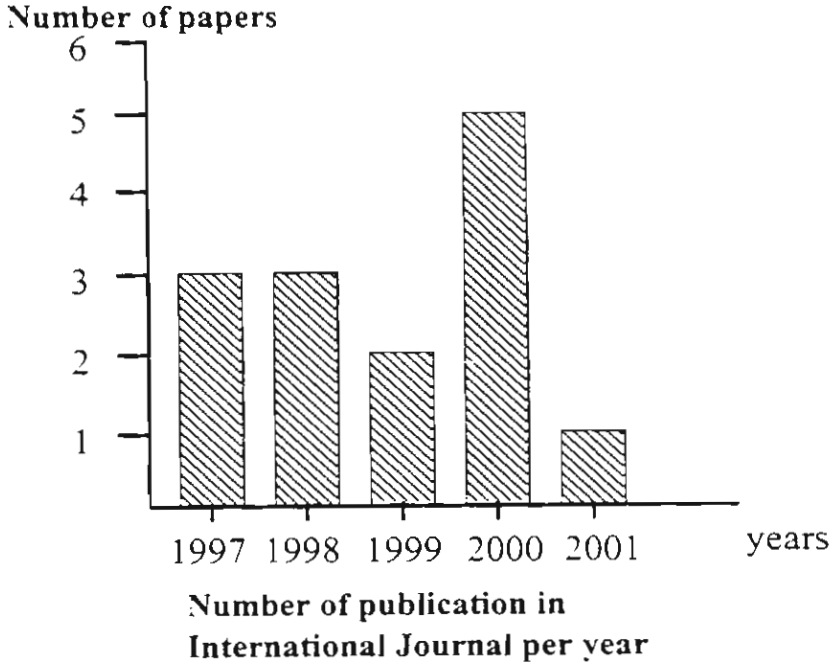

Fig2

Fig3

Number of Publication and journal impact factor

Year	Journal	Impact factor
1997	1. Journal of Bacteriology	3.721
	2. Journal of Bacteriology	3.721
	3. FEMS Microbiology Letter	1.673
1998	1. Journal of Bacteriology	3.721
	2. Journal of Bacteriology	3.721
	3. FEMS Microbiology Letter	1.673
1999	1. FEMS Microbiology Letter	1.673
	2. FEMS Microbiology Letter	1.673
2000	1. Gene	2.258
	2. Journal of Bacteriology	3.721
	3. Applied and Environmental Microbiology	3.541
	4. Molecular Microbiology	6.361
	5. Journal of Bacteriology	3.721
2001	1. Microbiology	2.307

นักศึกษาระดับบัณฑิตศึกษาที่งานวิจัยในโครงการที่ได้รับทุน เป็นส่วนหนึ่งของงานในวิทยานิพนธ์

1. ระดับปริญญาเอก

   ร.อ.ไพบูลย์ วัฒนวิบูลย์ (ปี 2539-2542)

2. ระดับปริญญาโท

   นางสาวรัชดาภรณ์ ศรีปรางค์ (ปี 2539-2542)

   นายรติบุตร สัลละพันธ์ (ปี 2538-2542)

   นางสาวฐานุตรา วรลักษณ์สิทธิ์ (ปี 2540-2543)

   นางสาววิรงรอง หวังสุข (ปี 2541-2543)

The completed list of publications is also shown:

1. Loprasert, S., S.Atichartpongkul, 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.

2. **Mongkolsuk, S.**, S.Loprasert, W.Whangsuk, M.Fuangthong and S.Atichartpongkul. 1997. Characterization of transcription organization and analysis of unique expression patterns of an alkyl hydroperoxide reductase C gene (*ahpC*) and peroxide regulator operon *ahpF-oxyR-orfX* from *Xanthomonas campestris* pv. *phaseoli*. J.Bacteriol. **179:** 3950-3955.

3. 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.

4. **Mongkolsuk, S.**, W. Praitum, S.Loprasert, M.Fuangthong and S. Chamnongpol. 1998. Identification and characterization of a new organic hydroperoxide resistance (*ohr*) gene with a novel pattern of oxidative stress regulation from *Xanthomonas campestris* pv. *phaseoli*. J.Bacteriol. **180:** 2636-2643.

5. **Mongkolsuk, S.**, R. Sukchawalit, S.Loprasert, W. Praitum and A. Upaichit. 1998. Construction and physiological analysis of a *Xanthomonas* mutant to examine the role of the *oxyR* gene in oxidants induced protection response to peroxide killing. J.Bacteriol. **180:** 3988-3991.

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9. Vattanaviboon, P. and **S. Mongkolsuk**. 2000. Expression analysis and characterization of the mutant of a growth phase and starvation regulate monofunctional catalase gene from *Xanthomonas campestris* pv.*phaseoli*. Gene. 241: 259-265.

10. **Mongkolsuk, S.**, W. Whangsuk, M. Fuangthong, S. Loprasert. 2000. Mutations in *oxyR* resulting in peroxide resistance in *Xanthomonas campestris*. J.Bacteriol. 182: 3846-3849.

11. Sriprang, R., P. Vattanaviboon and **S. Mongkolsuk**. 2000. Exporsure of phytopathogenic *Xanthomonas* spp. to lethal concentrations of multiple oxidants affects bacterial survival in a complex manner. Appl. Environ. Microbiol. 66: 4017-4021.

12. Loprasert, S., M. Fuangthong, W. Whangsuk, S. Atichartpongkul and **S. Mongkolsuk**. 2000. Molecular and physiological analysis of an OxyR regulated *ahpC* promoter in *Xanthomonas campestris* pv. *phaseoli*. Mol. Microbiol. 37: 1504-1514.

13. **Mongkolsuk S.**, W. Whangsuk, P. Vattanaviboon, S. Loprasert, M. Fuangthong. 2000. A *Xanthomonas* alkyl hydroperoxide reductase subunit C (*ahpC*) mutant showed an altered peroxide stress response and complex regulation of the compensatopy response of peroxide detoxification enzymes. J.Bacteriol. 182: 6845-6849.

14. Vattanaviboon, P., R. Sriprang and **S. Mongkolsuk**. 2001. Catalase has a novel protective role against electrophile killing of *Xanthomonas*. Microbiology. 147: 491-498.

Appendix

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

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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 H_2O_2 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 H_2O_2, 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 H_2O_2 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.

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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 (*ahpCF*Δ [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 *Nco*I to an *Hin*cII site from pAhp4-1 (Fig. 1) was subcloned into pKS vector, resulting in pKS-ahpC. Similarly, pUFR-ahpC was constructed by ligation of the 1-kbp *Nco*I-*Hin*cII fragment into pUFR047, a broad-host-range *Inc*W expression vector (6) digested with *Sma*I.

Nucleotide sequencing. pAhp4-1 was sequenced in both directions from a *Cal*I site to a vector *Eco*RI site. Similarly, a 2.0-kbp DNA fragment between an *Eco*RI site and the second *Xho*I site of pOXX 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 (10^8) 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 (GEVCPA

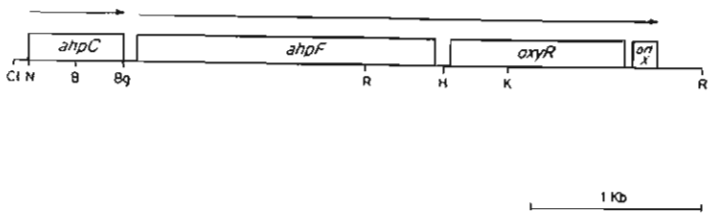

FIG. 1. Organization of *ahpC*, *ahpF*, *oxyR*, and *orfX* in *X. campestris* pv. phaseoli. The arrows indicate the direction and length of the transcripts. B, *Bst*XI; Bg, *Bgl*II; Cl, *Cla*I; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; R, *Eco*RI.

KW) of *E. coli* AhpC were used to synthesize degenerate oligonucleotide primers for PCRs (4). To reduce primer degeneracy and complexity, it was taken into account that *Xanthomonas* frequently used G or C in the last position of the codons. The following primers were used to amplify *Xanthomonas campestris* pv. phaseoli genomic DNA: 5'ahpC, 5' GAC TTC ACX TTC GTX TGC CCX ACX GA 3'; and 3'ahpC, 5' CCA CTC XGC XGG ACA XAC CTC XCC 3' (where X is for G and C). The resulting 390-bp PCR product corresponding in size to that expected of *ahpC* was cloned into pGEM-T vector (Promega), and the sequence was determined. The predicted translation products of this partially sequenced clone of *ahpC* showed a high degree of homology to AhpC sequences from several bacteria. Thus, they were used as probes to screen an *X. campestris* pv. phaseoli genomic library constructed in a ZipLox vector (Bethesda Research Laboratories [24]). Several positive clones were isolated, and plaques were purified. Many isolates shared internal fragments that cross-hybridized with the *ahpC* probe. One such clone, pAhp 4-1, was completely sequenced in both directions from a *Cla*I site to the vector *Eco*RI site. Analysis of the sequence revealed three open reading frames (ORFs). The predicted amino acid sequences of these ORFs were used to search GenBank, and the results showed that the first ORF had high homology with the AhpC family of proteins. The complete second and the third truncated ORFs showed homology to AhpF, a subunit of AhpR (1, 19, 24), and OxyR, a peroxide stress sensor and transcription regulator, respectively (8, 11, 25).

Isolation of *oxyR*. A plasmid, pOXX, was isolated from an *X. campestris* pv. phaseoli plasmid expression library by complementation of an H_2O_2-hypersensitive phenotype of an *E. coli oxyR* mutant, GSO8 (12, 13). GSO8 harboring pOXX was more resistant to H_2O_2, with a killing zone diameter of 2.2 cm compared with 2.8 cm for GSO8 harboring pKS vector only. Deletion analysis of pOXX indicated that the *oxyR* complementation activity was located on a 2.0-kb DNA fragment between an *Eco*RI site and the second *Xho*I site. The fragment was completely sequenced. Sequence analysis indicated a partial first ORF with homology to AhpF, a complete second ORF homologous to OxyR, and an unknown protein, ORFX (Fig. 1). Although pAhp 4-1 and pOXX were independently isolated, they had overlapping regions.

Gene organization in *X. campestris* pv. phaseoli. In *Xanthomonas*, *ahpC*, *ahpF*, and *oxyR* showed an unusual organization. These genes were arranged in head-to-tail fashion in the following order: *ahpC*, *ahpF*, *oxyR*, and *orfX* (Fig. 1). Each of the ORFs had a strong ribosome binding site preceding the translation initiation codons. The first and the second ORFs were separated by 213 bp, the second and the third by 91 bp, and the third and the fourth by 73 bp.

Analysis of *ahpC* expression in several microbes shows unusual patterns, suggesting the possibility that more than one copy of the gene could exist (1, 27). We performed Southern 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* (50%); 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, 24) and *Bacillus subtilis* (1, 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-

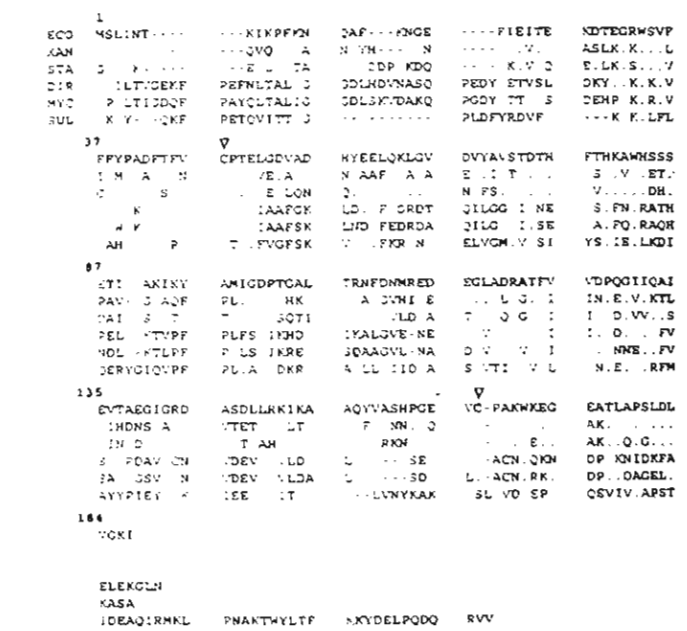

FIG. 2. Comparison of bacterial AhpC amino acid sequences. The sequences were aligned by the Clustal W program (28). ECO, *E. coli* D13187); XAN, *X. campestris* pv. phaseoli (U94336); STA, *S. aureus* (2); DIP, *C. diphtheriae* (27); MYC, *M. tuberculosis* (8); and SUL, *Sulfolobus* sp. (U36479). Gaps were introduced to maximize the fit. Numbers on top are according to the sequence of *E. coli* AhpC. Arrowheads indicate highly conserved cysteine residues, dashes represent gaps, and dots represent amino acid residues identical to those in the *E. coli* sequence.

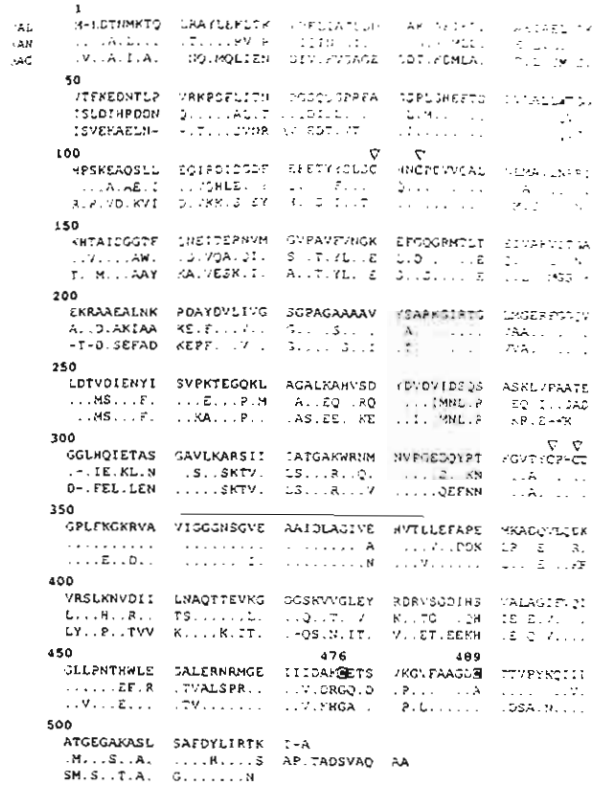

FIG. 3. Multiple alignment of bacterial AhpF amino acid sequences. Comparison of AhpF amino acid sequences from *S. typhimurium* (SAL:G153863), *X. campestris* pv. phaseoli (XAN:U94336), and *B. subtilis* (BAC:D78193) aligned by the Clustal W program (28). Gaps were introduced to maximize the fit. The numbers on top were according to *S. typhimurium* AhpF numbering. The cysteine residues involved in a disulfide bridge and in an active site (▽) are shown. Residues in the conserved NAD(P)H binding site are overlined.

ive site (C129 to C132 and C345 to C348 in *Salmonella*), and the NAD(P)H binding domain were all conserved in *Xanthomonas* AhpF (Fig. 2) (15). Two cysteine residues at C476 and C489 were substituted for with G and A residues, respectively, in *Xanthomonas* AhpF, indicating that these residues were not essential for enzyme activity.

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, 25). For at least two members of the LysR family (i.e., OxyR and NahR), the region around the carboxy terminus of each protein has been shown to be crucial for their function (12, 13, 21). However, little homology was detected in the region close to the carboxy termini 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 H_2O_2-sensitive phenotype of GSO8 (see "Isolation of *oxyR*").

Analysis of OrfX. The fourth ORF identified had a coding potential for 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 3′ 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*Δ), UM2 (*katG katE*), and K-12 (wild type). The results of peroxide sensitivity tests with oxidants by the disc diffusion method

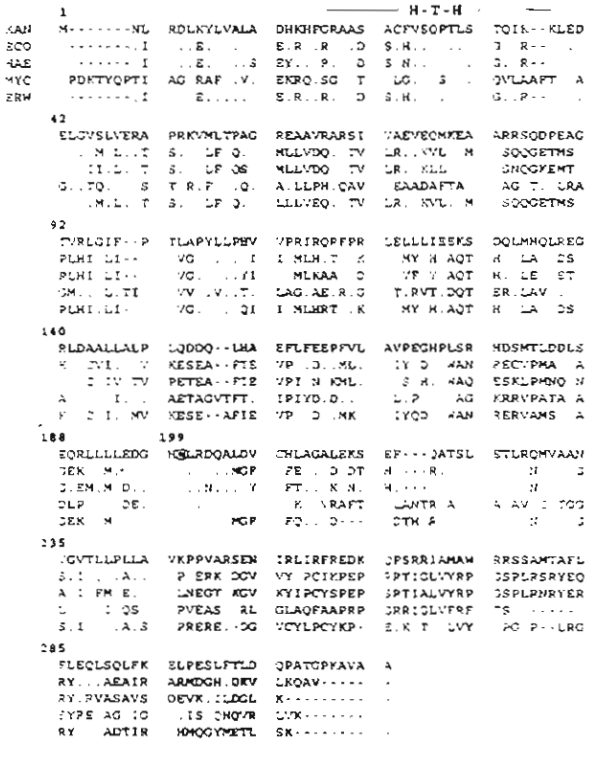

FIG. 4. Amino acid sequence comparison of OxyR from *E. coli* (ECO; X16531), *X. campestris* pv. phaseoli (XAN:U94336), *E. carotovora* (ERW; U74302), *M. avium* (MYC; U18263), and *H. influenzae* (HAE:U32847). Amino acid sequence alignment was carried out with the Clustal W program (28). H-T-H, helix-turn-helix motif.

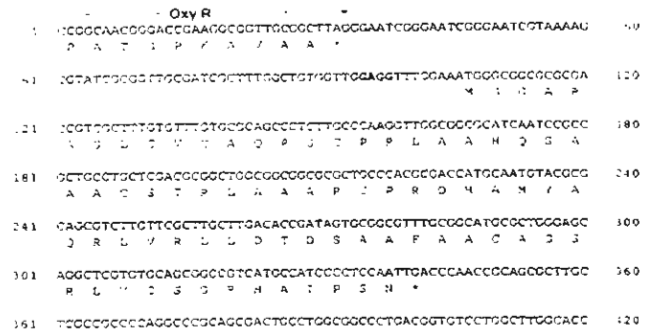

FIG. 5. Nucleotide and deduced amino acid sequences of *orfX*. A putative ribosome binding site of *orfX* is in boldface. The amino terminus of the OxyR sequence is also shown.

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 killing 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, 24).

X. campestris pv. phaseoli *oxyR* was isolated on the basis of the gene's ability to functionally complement hypersensitivity to the H_2O_2 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 H_2O_2 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 >8 h in *X. campestris* pv. phaseoli harboring only the pUFR047 vector) (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

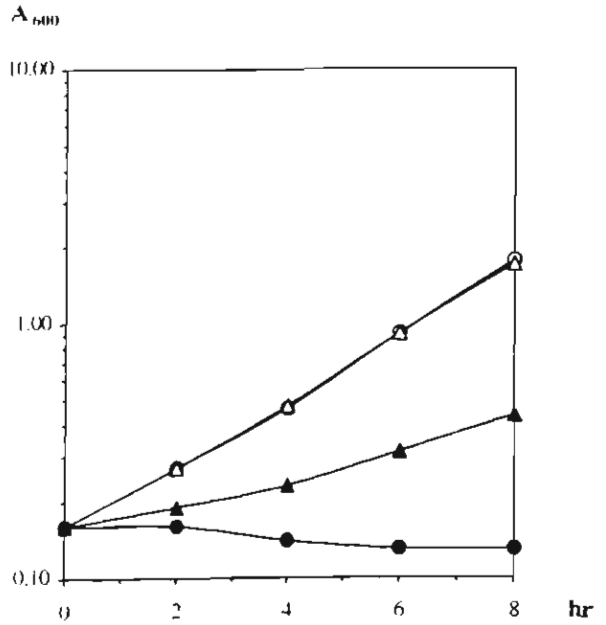

FIG. 6. Growth curves of *X. campestris* pv. phaseoli harboring pUFR047 (○) or pUFR-ahpc (△) in the absence (○, △) or presence (●, ▲) of a growth-inhibitory concentration (0.4 mM) of tBOOH.

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 H_2O_2, menadione, *N*-ethylmaleimide, and $CdCl_2$, 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 H_2O_2 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 H_2O_2 as a substrate (19, 20). Nonetheless expression of cloned *ahpC* in *X. campestris* pv. phaseoli did not enhance protection against H_2O_2 toxicity. On the contrary, we observed a small (30%) decrease in catalase activity in *X. campestris* pv. phaseoli cells harboring pUFR-ahp (data not shown). Additionally, AhpR might play a less important role than catalase in the protection against H_2O_2 toxicity. This is consistent with our observations that increased catalase levels alone are sufficient to protect *Xanthomonas* from H_2O_2 killing (17).

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[a]

X. campestris pv. phaseoli plasmid	Growth inhibition zone value (cm)					
	tBOOH (0.5 M)	CuOOH (0.1 M)	Menadione (0.5 M)	*N*-Ethylmaleimide (0.1 M)	$CdCl_2$ (0.2 M)	H_2O_2 (0.2 M)
pUFR047	3.0	1.7	2.4	2.8	2.1	1.5
pUFR-ahpC	2.2	1.3	2.3	2.9	2.0	1.6

[a] The experiments were performed as described in Materials and Methods and were repeated at least three times. The results shown represent average values.

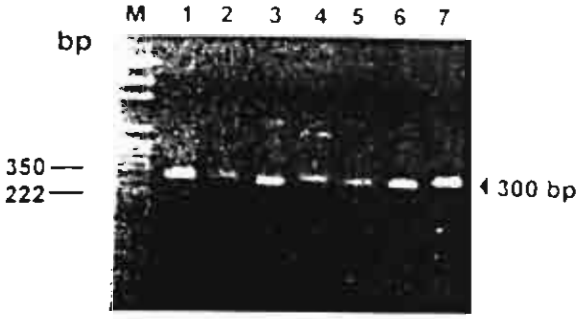

FIG. 7. Conservation of *ahpC* and *ahpF* organization in various *Xanthomonas* species. The primer set corresponded to *3'ahpC* (5' ACCTGGTCGGCAA GATCTAA 3') and *5'ahpF* (5' TCGATGCGTTGATTTGAATC 3'). The following PCR conditions were used: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, 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. oryzicola. M, pGEM (Promega) molecular weight markers. The arrowhead to the right indicates the position of the expected 300-bp PCR products.

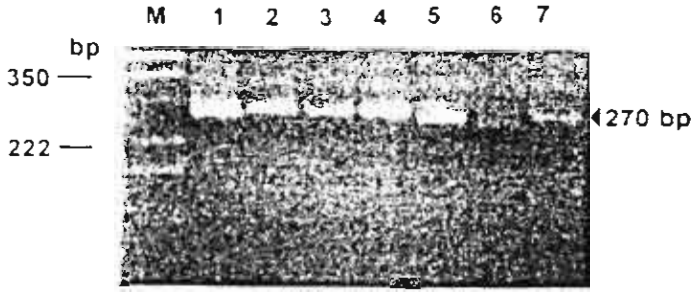

FIG. 8. Organization of *ahpF* and *oxyR* in various *Xanthomonas* species. PCR conditions were as described in the legend to Fig. 4. The primer set corresponded to *3'ahpF* (5' ATGGGCGAAGGTTCCAA 3') and *5'oxyR* (5' GGCTGACAA AGCAGGC 3'). PCR products were obtained from genomic DNA with the following (by lane): 1, *X. campestris* pv. phaseoli; 2, *X. campestris* pv. glycine; 3, *X. oryzae* pv. oryzicola; 4, *X. vesicatoria*; 5, *X. oryzae* pv. oryzae; 6, *X. translucens*; and 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.

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 (8). In *X. campestris* pv. phaseoli, *ahpC*, *ahpF*, *oxyR*, and *orfX* 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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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

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We have analyzed the transcription organization of *ahpC*, *ahpF*, *oxyR*, and *orfX* from *Xanthomonas campestris* pv. phaseoli. *ahpC* was transcribed as a monocistronic 0.6-kb mRNA, while *ahpF-oxyR-orfX* were transcribed as a polycistronic approximately 3.0-kb-long mRNA. The novel transcription organization of these genes has not observed in other bacteria. Western analysis showed that oxidants (peroxides and superoxide anions), a thiol reagent (*N*-ethylmaleimide), and $CdCl_2$ caused large increases in the steady-state level of AhpC. Growth at alkaline pH also moderately induced AhpC accumulation. Thermal and osmotic stresses did not alter the levels of AhpC. Northern blotting results confirmed that oxidant- and $CdCl_2$-induced AhpC accumulation was due to increased levels of *ahpC* transcripts. Analysis of *oxyR* expression revealed a unique pattern. Unlike other bacterial systems, peroxides and a superoxide generator induced accumulation of OxyR. Northern blotting results confirmed that these oxidants induced expression of *oxyR* operon. This novel regulatory pattern could be generally important. The transcription organization and patterns of chemicals and stress induction of *ahpC* and *oxyR* differed from those of other bacteria and are likely to be important for *X. campestris* pv. phaseoli survival during exposure to oxidants.

During plant-microbe interactions, the initial plant defense response involves increased production of reactive oxygen species, including H_2O_2, organic peroxides, and superoxides. They function as bactericidal agents and as secondary signal molecules to further activate plant defense responses (22, 38). To survive and proliferate, bacterial pathogens must overcome reactive oxygen species.

Microbial defense against oxidative stress required well-orchestrated enzyme reactions involving both detoxification of the stress and repair processes. Catalases have important protective roles against H_2O_2 toxicity (11, 14). So far, the best-characterized bacterial scavenging enzyme against organic hydroperoxides is alkyl hydroperoxide reductase (AhpR), which consists of two components, a 22-kDa protein, AhpC, and a 57-kDa flavoprotein, AhpF (19, 36). Both components are required for NADH- or NADPH-dependent reduction of organic peroxides to corresponding alcohols (31, 32). The genes coding for these enzymes have been isolated from several microbes, but extensive analyses of their regulation have been done in few cases (1–3, 5, 13, 32, 40). In these cases, the inducing conditions of *ahpC* vary a great deal for different bacterial species. Increased expression of *ahpC* has been shown to confer resistance to organic hydroperoxides and in some cases can compensate for the lack of catalase enzyme (34, 41). Thus, conditions which affect *ahpC* expression are likely to play important physiological roles in the peroxide stress response.

Oxidative stress response is regulated by several redox-sensitive transcription regulators, such as OxyR (for the peroxide regulon [6, 10, 11, 35, 37]) and SoxRS (for the superoxide regulon [11, 17]). In general, alterations in the cellular redox state lead to changes in the redox status of these proteins that result in activation of genes under their regulation (20, 21, 35, 37). This permits a concerted response to the stress. The regulation of the regulatory genes themselves is important; minor alterations in their patterns of expression would have profound effects on the bacteria. *oxyR* expression is autoregulated, in addition to having a posttranscriptional regulation step (20, 21). *oxyR* homologs have been identified in several bacteria. Details of the structural function and analyses of expression have mostly been done for enteric bacteria (12, 36). Interestingly, many *Mycobacterium tuberculosis* strains are natural *oxyR* mutants with altered expression in peroxide stress protection genes and a drug resistance phenotype (12, 34, 41). OxyR regulates a number of genes involved in peroxide protection and detoxification, i.e., those coding for catalase, AhpR, and Dps (11, 14). OxyR can act either as a transcription repressor or transcription activator, depending on the target promoters and the redox state of the protein (20, 21).

Many aspects of oxidative stress response in *Xanthomonas* are different from those of other bacteria (7, 8, 25). To investigate the regulation of the peroxide stress response and genes involved in protection against alkyl hydroperoxides, we have isolated *ahpC*, *ahpF*, and *oxyR* from *Xanthomonas campestris* pv. phaseoli (24). We have shown that expression of *ahpC* alone provides partial protection against organic hydroperoxides (ROOH) but not H_2O_2 toxicity (24). The aim of this study was to ascertain the transcription organization and expression patterns of *ahpC*, *ahpF*, *oxyR*, and *orfX* in *Xanthomonas*.

MATERIALS AND METHODS

Bacterial strains and induction of *X. campestris* pv. phaseoli cultures. All *Xanthomonas* strains were grown aerobically at 28°C on SB medium (30). To ensure the reproducibility of the experiments, it was important to use bacteria at

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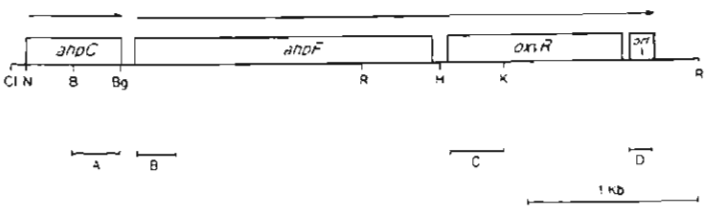

FIG. 1. Organization of *ahpC* and *ahpF-oxyR-orfX* in *X. campestris* pv. phaseoli. The arrows indicate the direction and length of transcripts. Regions marked A, B, C, and D were used as gene-specific probes in Northern and Southern blotting experiments. B, *Bst*XI; Bg, *Bgl*II; Cl, *Cla*I; H, *Hind*III; K, *Kpn*I; N, *Nco*I; R, *Eco*RI.

similar stages of growth. Overnight (15-h) late-log-phase cultures were subcultured into fresh SB medium at equal density and grown for 1 h. Various inducers were then added (7, 8). Unless otherwise stated, the induced cultures were grown for half an hour before cells were harvested and used in lysate preparation for Western blot analysis. For Northern analysis, total RNA was extracted from cultures after 10 min of incubation with inducers. Bacterial growth was monitored spectrophotometrically at A_{600}.

Construction of an OxyR expression plasmid and antibody production. OxyR for antibody production was purified from an overexpression plasmid. Essentially, oligonucleotide primers corresponding to the N terminus (5′ TAG GAT CCG AAT CTG CGT GAC 3′) and C terminus (5′ ACC ACA GCC AAA GCG TAC GCA A 3′) of OxyR were used in a PCR with pOXX (24) as a DNA template. The 950-bp PCR product was digested with *Bam*HI and cloned into pBluescript KS digested with *Bam*HI and *Eco*RV (26). The resultant plasmid, pKS-OXX, was digested with *Bam*HI and *Hind*III, and 0.9-kb fragments containing *oxyR* were cloned into a His-tagged gene fusion vector, pQE 31 (Qiagen, Inc.). The resultant plasmid, pQE-OXX, overproduced His-tagged OxyR fusion protein at high levels. The fusion protein was purified from a 1-liter culture with Ni-nitrilotriacetic acid resin as suggested by the manufacturer. Purified *X. campestris* pv. phaseoli OxyR was injected into rabbits for antibody production.

Lysate preparation and Western blot analyses. Cell lysates were prepared from fresh cell pellets by being resuspended in 50 mM potassium phosphate buffer (pH 7.0), followed by sonication for 2 min with cooling intervals. Cell debris was removed by centrifugation at 10,000 × *g* for 15 min, and clear lysates were either used immediately or stored frozen (7). Protein concentration was measured by dye binding method (4). For analysis of AhpC induction by Western blotting, 30 μg of protein from various samples was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [15% polyacrylamide]) gels and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and reacted with either anti-*Escherichia coli* AhpC antibody (a gift from G. Storz) or anti-*X. campestris* pv. phaseoli OxyR antibody. They were subsequently developed with a goat anti-rabbit antibody conjugated to alkaline phosphatase. For analysis of the dimeric form of AhpC, essentially the same protocol for gel electrophoresis and immunodetection was used except that dithiothreitol was omitted from the loading dye. The results of AhpC and OxyR Western analysis were quantitated with a Bio-Rad GS-700 densitometer.

Northern analyses. Total RNA isolation from *Xanthomonas* cultures, agarose formaldehyde gel electrophoresis, blotting, and hybridization conditions were as previously described (28). The coding regions of *ahpC* (a 567-bp *Bst*XI-*Bgl*II fragment), *ahpF* (a 575-bp *Pst*I fragment), *oxyR* (a 950-bp *Bam*HI-*Hind*III fragment from pQE-OXX), or *orfX* (a 180-bp *Bss*HII-*Spe*I fragment) were used for radioactive probes (Fig. 1).

RESULTS AND DISCUSSION

Transcription organization of *ahpC*, *ahpF*, *oxyR*, and *orfX*. The nucleotide sequences of pAhp4-1 and pOX show that *ahpC*, *ahpF*, *oxyR*, and *orfX* are arranged in a head-to-tail fashion (24). This unusual genome organization is highly conserved in *Xanthomonas* spp. and is likely to be important in the regulation of these genes. To elucidate the transcription organization of these genes, Northern blot analysis was performed with the coding regions of different genes as probes. The results are shown in Fig. 2. The *ahpC* probe hybridized to the 0.6-kb mRNA (Fig. 2, lane 1). This was consistent with the gene being transcribed as a monocistronic mRNA. In contrast, the *ahpF*, *oxyR*, and *orfX* probes each hybridized to mRNA approximately 3.0 kb in length (Fig. 2, lanes 2 to 4). The results supported the idea that these genes were arranged in an operon and transcribed as a polycistronic mRNA. In addition, no hybridization signals with the 3.0-kb *ahpF-oxyR-orfX* mRNA were detected when a region located 3′ outside the *orfX* coding region was used as a Northern probe (data not shown). Similarly, no hybridization with the 0.6-kb *ahpC* mRNA was detected when a region located 5′ outside *ahpC* was used as a probe. These controls confirmed the proposed transcription organization.

In *E. coli* and *Bacillus subtilis*, *ahpC* and *ahpF* are coregulated, and in the latter case, the genes are also arranged in an operon (1, 3, 36). In contrast, *Xanthomonas ahpC* was transcribed as a monocistronic mRNA. In other bacteria, *ahpC* is regulated by OxyR (13, 37). Thus, the separation of transcription regulation of genes coding for subunits of alkyl hydroperoxide reductase (*ahpC* and *ahpF*) from coregulation of *ahpF* with *oxyR* suggests novel complex interactions between these three genes. The physiological significance of close regulation of *ahpC*, *ahpF*, and *oxyR* in *Xanthomonas* is currently under investigation.

Expression analysis of *ahpC* in response to stress. It is generally accepted that physiological and/or environmental conditions which act as inducing signals for high expression of *ahpC* are likely to be important in bacterial oxidative stress response. We performed Western analyses to monitor the *ahpC* expression of *X. campestris* pv. phaseoli in response to various stress conditions.

First, we investigated the effects of superoxide anions and various peroxides on *ahpC* expression in *X. campestris* pv. phaseoli. AhpR can use a wide range of peroxide substrates (19, 31, 32), and this suggests that its expression may be regulated by many inducers. At appropriate concentrations, all peroxides (H_2O_2, tBOOH, and CuOOH) and superoxide generators (menadione [MD] and paraquat) tested were equally potent inducers of *ahpC*, causing four- to fivefold increases in steady-state levels of AhpC (Fig. 3A). In addition, we had tested the *ahpC* response to oxidative stress in four other *Xanthomonas* spp. and obtained a similar pattern of response to *X. campestris* pv. phaseoli (data not shown).

Expression analysis of *ahpC* showed interesting patterns. The induction of *ahpC* by peroxides was similar to that reported for other bacteria, except for *Staphylococcus aureus*, which has an oxidative stress-noninducible phenotype (2). The superoxide generators were potent inducers of *ahpC*. It is not possible to differentiate whether the superoxide anions themselves or their conversion via superoxide dismutase or sponta-

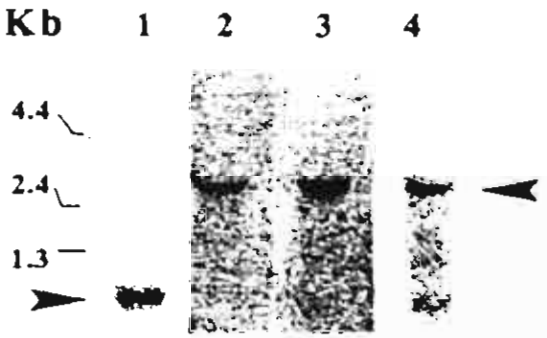

FIG. 2. Transcription organization of *ahpC* and the *ahpF-oxyR-orfX* operon. RNA isolation, electrophoresis, and hybridization were performed as previously described (11, 12). RNA was loaded at 5 μg in lane 1 and 25 μg in lanes 2, 3, and 4. The coding regions of radioactively labelled *ahpC* (lane 1), *ahpF* (lane 2), *oxyR* (lane 3), and *orfX* (lane 4) were used as probes (Fig. 1) for hybridization with Northern blots. The arrowhead to the left indicates monocistronic 600-bp *ahpC* mRNA, and the arrowhead to the right indicates the approximately 3.0-kb polycistronic *ahpF-oxyR-orfX* mRNA. Positions of RNA molecular weight markers (Bethesda Research Laboratories) are shown to the left.

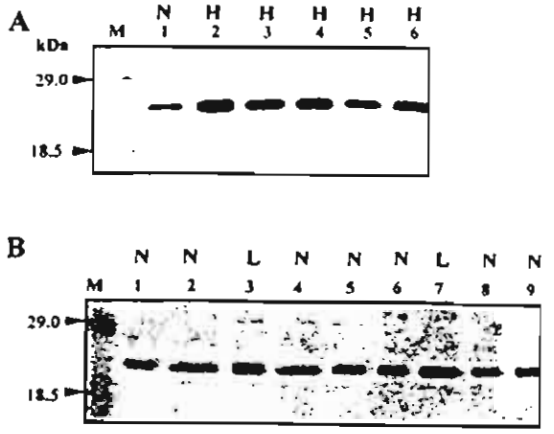

FIG. 3. Western analysis of AhpC levels in response to oxidants (A) and various stresses (B). (A) Total protein samples (30 μg) were separated on an SDS-PAGE gel, blotted, reacted with anti-*E. coli* AhpC antibody, and detected as described in Materials and Methods. Results are shown for uninduced cells (lane 1) and cells induced with 150 μM H_2O_2 (lane 2), 100 μM MD (lane 3), 100 μM paraquat (lane 4), 100 μM tBOOH (lane 5), and 100 μM cumene hydroperoxide (lane 6). (B) Sampling, gel electrophoresis, blotting, and immunodetection were performed as described for panel A. Lanes: 1, uninduced cells; 2 to 9, cells induced with SB medium at pH 5.0 (lane 2), SB medium at pH 8.5 (lane 3), 1.5 M NaCl (lane 4), 200 μM dipyridyl (lane 5), 200 μM diamide (lane 6), 200 μM NEM (lane 7), heat shock at 37°C (lane 8), and cold shock at 15°C (lane 9). N, noninduced; L, moderately induced (two- to threefold over the uninduced level); H, highly induced (increased four- to sixfold over the uninduced level).

neous dismutation to H_2O_2 is responsible for *ahpC* induction (15–17). Thus, the superoxide induction could be mediated via either superoxide (i.e., SoxRS) or peroxide (i.e., OxyR) sensor and activator systems. Alternatively, both systems could act synergistically to activate *ahpC*. In *E. coli* cells, which have both *oxyR* and *soxRS*, superoxide generators also induce *ahpC* and *ahpF* (16). In this system, OxyR and not SoxRS is responsible for the induction of *ahpC* and *ahpF* by superoxide generators. This favors the hypothesis that superoxide anions are being converted to H_2O_2 that in turn activates OxyR. However, the lack of *oxyR* and *soxR* mutants in *Xanthomonas* and closely related bacteria prevents a definitive answer regarding the mechanism of superoxide induction of *ahpC*. Only recently a *soxR* homolog from nonenteric bacteria was isolated from *Pseudomonas aeruginosa*, but its physiological roles have not been analyzed (23). The patterns of catalase (*katX* [28]), *ahpC*, and *oxyR* expression in response to oxidants in *X. campestris* pv. phaseoli showed many similarities and differences. All three genes were highly induced by superoxide generators, whereas peroxides were weak inducers of *katX* (8, 28) but potent inducers of *ahpC* and *oxyR*. Differences between the oxidant induction patterns of these genes imply that more than one mechanism may regulate these genes. A question remains of whether OxyR is acting as the sensor for different inducers and as a transcription regulator of *ahpC* in *X. campestris* pv. phaseoli. We are currently investigating the roles of OxyR in *ahpC* regulation.

Second, we examined the effects of various compounds that are known to induce oxidative stress on AhpC levels. The effects of the thiol reagents *N*-ethylmaleimide (NEM) and diamide were tested. In response to inducing concentrations of NEM, an accumulation of AhpC was observed (Fig. 3B, lane 7). Surprisingly, diamide had no effects on *ahpC* expression (Fig. 3B lane 6). This suggested that the two SH-depleting agents may operate by different mechanisms. The induction of *ahpC* by NEM is likely to be mediated by its ability to induce oxidative stress. Additionally, NEM can also directly inactivate AhpC and lead to further increase in oxidative stress and synthesis of AhpC (31).

Third, we examined *ahpC* expression in response to various stresses. The effects of temperature and osmotic and pH stresses on the steady-state levels of AhpC were investigated. Thermal stress as either heat (at 37 and 42°C) or cold (at 15 and 10°C) shock for 15 min or osmotic shock at 1.5 M NaCl for 15 min did not cause accumulation or reduction of AhpC (Fig. 3B). In contrast, pH stress did affect the expression of *ahpC*: exposure of *X. campestris* pv. phaseoli to an alkaline pH (8.5) produced a small (twofold) but consistent accumulation of AhpC (Fig. 3B, lane 3). Growth at acid pH (pH 5.0) did not affect the level of AhpC (Fig. 3B). *Xanthomonas* growth in SB medium did cause an increase in medium pH that reached its highest levels during the stationary phase (7). This is coincident with the highest resistance to organic hydroperoxides (40). Thus, alkaline pH induction of *ahpC* may partially contribute to the phenomenon.

Metals and *ahpC* expression. Metal ions play important roles in oxidative stress. They act as important cofactors for oxidants scavenging enzymes and/or regulatory proteins (9, 11, 14). Metal ions also catalyze formation of highly reactive oxygen radicals (17). In *X. campestris* pv. phaseoli, 100 μM $CdCl_2$ was a more potent inducer of *ahpC* than peroxides and superoxide generators (Fig. 3A and 4). We also tested the effect of other metal ions (i.e., cobalt, copper, manganese, nickel, and zinc [Fig. 4] and mercury [data not shown]) at 100 μM. None of these induced accumulation or caused any reduction in the steady-state levels of AhpC. Iron is known to induce oxidative stress. Moreover, iron deprivation induces synthesis of DirA, an AhpC homolog in *Corynebacterium diphtheriae* (39). On the contrary, *X. campestris* pv. phaseoli growth in media with excess iron (Fe^{3+} at 50 or 200 μM [Fig. 4]) or depleted of iron (in the presence of 200 μM dipyridyl [Fig. 3B, lane 5]) did not affect the levels of AhpC. *ahpC* induction by $CdCl_2$ is likely to be mediated by the well-known effects of the heavy metal ions to induce severe oxidative stress. Similarly, induction of an *ahpC* homolog in mice by oxidants, $CdCl_2$, and a thiol reagent has been reported (18).

Northern analysis of *ahpC*. To confirm that accumulation of AhpC induced by various stresses was due to increased transcription of *ahpC* and to assess levels of *ahpC* transcription induction in response to various inducers, a 367-bp sequence containing the coding region of *ahpC* was used to probe total RNA isolated from uninduced and induced cultures. The results are shown in Fig. 5. Uninduced expression levels of the gene were low but detectable. Addition of inducing concentrations of H_2O_2, tBOOH, MD, and $CdCl_2$ caused large increases

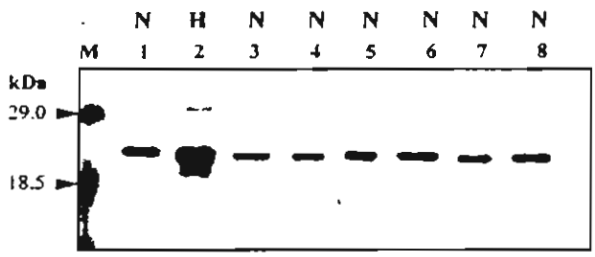

FIG. 4. Effects of metal ions on AhpC levels. Gel electrophoresis and immunological detection of AhpC were done as described in the legend to Fig. 3 and Materials and Methods. Unless otherwise stated, 50 μM metal ions was used as inducers. Lanes: 1, uninduced cells; 2 to 8, cells induced with $CdCl_2$ (lane 2), $CoCl_2$ (lane 3), $CuCl_2$ (lane 4), $FeCl_3$ (lane 5), 200 μM $FeCl_3$ (lane 6), $NiCl_2$ (lane 7), and $MnCl_2$ (lane 8). N, noninduced; H, highly induced (four- to sixfold over the uninduced level).

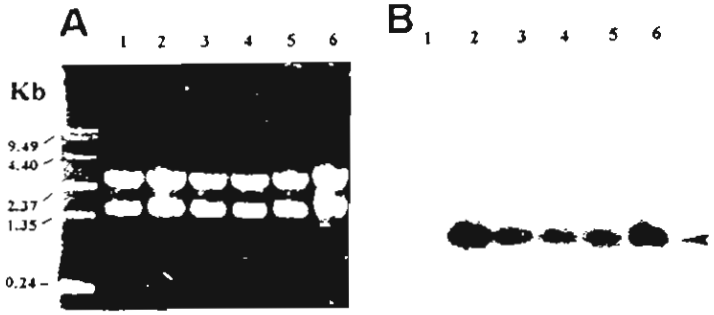

FIG. 5. Northern analysis of the effects of oxidants or $CdCl_2$ on *ahpC* mRNA levels. Total RNA samples were extracted from an uninduced *X. campestris* pv. phaseoli culture grown in SB medium (lane 1) and cultures induced with 50 μM $CdCl_2$ (lane 2), 200 μM H_2O_2 (lane 3), 100 μM MD (lane 4), 100 μM paraquat (lane 5), and 100 μM tBOOH (lane 6). Each lane contained 5 μg of RNA. (A) Ethidium bromide-stained gel showing the rRNA and RNA molecular weight markers (leftmost lane). (B) Hybridization signals of radioactive labelled *BstXI-BglII* 3o7-bp *ahpC* probe with Northern blot of gel in panel A. The arrowheads indicate the position of 600-bp hybridizing transcripts.

in steady-state levels of *ahpC* mRNA. Densitometer analysis of the *ahpC* hybridization results adjusted for the rRNA bands indicated that $CdCl_2$ induced 20% more *ahpC* mRNA than other inducers. Although all oxidants were equally potent inducers of *ahpC*, the increase in *ahpC* mRNA levels appeared to be more dramatic than the increase in AhpC detected by Western blotting. This could be due to high uninduced levels and stability of AhpC, which reduced the magnitude of the observed induction, although we could not eliminate the possibility that additional regulation at the levels of translation of *ahpC* could exist. Nevertheless, the results confirmed that the increase in AhpC levels in response to inducers resulted from increased *ahpC* expression. These inducers were likely to exert their effects at the transcriptional levels. This is similar to observations in enteric bacteria, in which exposure to oxidants lead to transcription activation of *ahpC* via a peroxide sensor-transcription activator OxyR protein.

Analysis of dimer and monomer forms of AhpC in response to stress. We had observed that in the uninduced sample, all of the AhpC was in the multiple dimeric forms (D1 and D2, Fig. 6). In all cases, exposure of *X. campestris* pv. phaseoli to *ahpC* inducers led to increased formation of the active dimeric form (D1, D2, and D3, Fig. 6) (6). However, the monomer became more apparent with $CdCl_2$ induction (Fig. 6, lane 2), which produced the highest level of AhpC. The conversion of monomeric AhpC to the dimeric form could be important in formation of active enzyme during high rates of enzyme synthesis.

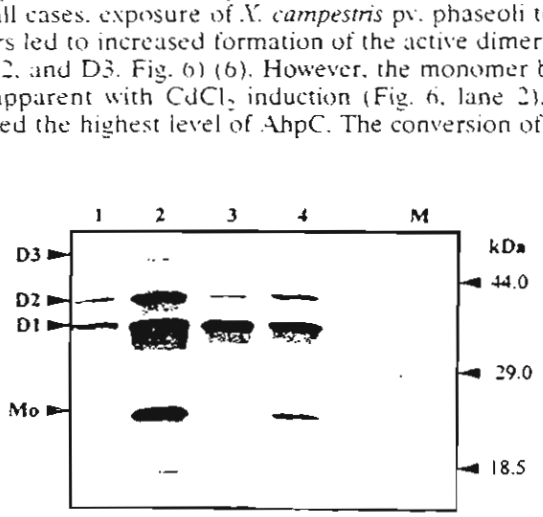

FIG. 6. Effects of oxidants and a heavy metal on AhpC dimerization. The effect of $CdCl_2$ (lane 2), MD (lane 3), and tBOOH (lane 4) on the levels of AhpC dimers (D1, D2, and D3) was investigated. The concentrations of various inducers were the same as in Fig. 3 and 4. The induction and Western blotting procedures were performed as described in Materials and Methods. Thirty-five micrograms of total protein was loaded into each lane, except in lane 1, in which 70 μg of protein from an uninduced sample was used. Mo, monomer

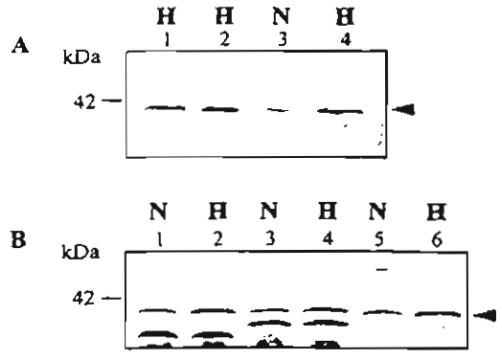

FIG. 7. Analysis of OxyR levels in response to oxidant treatments in *X. campestris* pv. phaseoli (A) and various *Xanthomonas* spp. (B). Sample preparation, gel electrophoresis, blotting, and immunodetection were performed as described in the legend to Fig. 3, except 50 μg of total protein plus anti-*Xanthomonas* OxyR antibody was used. (A) Lanes: 3, uninduced cells; 1, 2, and 4, cells induced with 100 μM MD (lane 1), H_2O_2 (lane 2), and tBOOH (lane 4). (B) Fifty micrograms of protein was prepared from uninduced cells (lanes 1, 3, and 5) and from cells induced with 100 μM tBOOH (lanes 2, 4, and 6). The conditions for electrophoresis, blotting, and antibody detection are as described for panel A. Lanes: 1 and 2, *X. campestris* pv. campestris; 3 and 4, *X. campestris* pv. malvacearum; 5 and 6, *X. oryzae* pv. oryzae. Additional bands detected were from nonspecific interactions. The position of OxyR is indicated by an arrowhead. N, noninduced; H, highly induced (increased four- to sixfold over the uninduced level).

Oxidant treatment leads to an increased OxyR concentration. In all bacterial systems thus far studied, OxyR responds to oxidative stress by changing from a reduced form to an oxidized form. Depending on the forms of the protein, OxyR can function either as a transcription repressor or as a transcription activator. OxyR is also self-regulated by acting as a repressor for its own gene transcription (20, 21, 37). The unusual arrangement of *oxyR* in *Xanthomonas* prompted us to investigate its response to oxidative stress (24). The level of OxyR was monitored with an anti-*X. campestris* pv. phaseoli OxyR antibody by Western analysis. The results are shown in Fig. 7. A three- to fourfold increase in the amount of OxyR was detected in *X. campestris* pv. phaseoli induced with H_2O_2, tBOOH, and MD. These oxidants produced similar induction levels. The increase in OxyR levels in response to oxidant treatments could be due either to increased protein stability or to increased expression of the gene. Subsequently, Northern analysis was performed with total RNA prepared from uninduced or MD- or tBOOH-induced *X. campestris* pv. phaseoli cultures with the *oxyR* coding region used as a probe. The results in Fig. 8 show that treatments with either tBOOH or MD lead to an increased level of *oxyR* transcripts. H_2O_2 produced induction levels similar to those of tBOOH (data not shown). The data indicated that oxidant treatments induced *oxyR* expression. Also, the oxidants were acting at the levels of transcription and not at the levels of protein stability. Contrary to the results of Western analysis of OxyR levels in response to oxidants, tBOOH induced higher levels of *oxyR* transcripts than MD (Fig. 7). We subsequently investigated the *oxyR* induction kinetic in response to H_2O_2, tBOOH, and MD. The results suggested that H_2O_2 and tBOOH produced a more rapid *oxyR* induction kinetic than MD. However, MD induction of *oxyR* lasts longer than that of peroxides (data not shown). The rapid *oxyR* induction kinetic induced by peroxides suggests that they could directly alter the redox state of the cells and result in immediate activation of *oxyR*, while the slower *oxyR* induction kinetic produced by MD implies that superoxide anions gen-

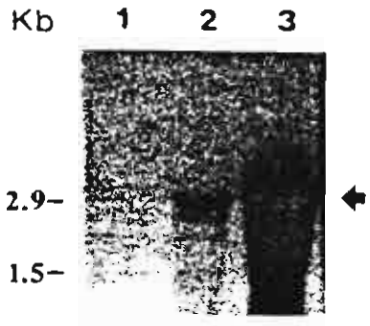

FIG. 8. Effects of oxidants on the levels of *oxyR* transcripts. Total RNA was tracted from uninduced cultures (lane 1) and from cultures induced with 100 M MD (lane 2) and 100 μM tBOOH (lane 3). Each lane contained 25 μg of tal RNA. Gel electrophoresis, blotting, and hybridization were performed as scribed in Materials and Methods. The filter was probed with the *oxyR* coding gion (Fig. 1). The arrow indicated approximately 3.0 kb of polycistronic *ahpF-yR-orfX* mRNA. The molecular weights of RNA markers are indicated to the left.

rated by MD may have to be converted to H_2O_2, either nzymatically via superoxide dismutase or nonenzymatically, nd H_2O_2 in turn induces *oxyR*. In addition, the rate of MD netabolism is considerably slower than the rate of peroxide netabolism. This could account for a slower but longer-lasting nduction of *oxyR* by MD. Nevertheless, we could not rule out hat superoxide anions may directly activate *oxyR* transcription ia a SoxR-like transcription activator but less efficiently than eroxides. These possibilities are being investigated.

Northern and Western analyses of *oxyR* and *ahpC* expression indicated that under both uninduced and induced conditions, *ahpC* was expressed at much higher levels than the *ahpF-oxyR-orfX* operon (Fig. 3 and 7). This is consistent with the proposed model that AhpF is only required in a catalytic amount to regenerate AhpC (32). Also, OxyR, being a transcriptional regulator, is required in a small amount, and an excessively high concentration of the protein may lead to unregulated gene expression, which could be harmful to the bacteria. We have shown that increased expression of *ahpC* alone conferred only partial protection against alkyl hydroperoxides (24), and cooperative expression of all three genes is essential to the overall response to peroxide stresses.

Questions remain regarding the mechanisms with which peroxides activate *oxyR* expression and the protein that mediates this response. OxyR is a member of a LysR family of transcription activators that autoregulate their expression (33). Preliminary experiments suggested that *X. campestris* pv. phaseoli OxyR also functions as a redox-sensitive transcription activator or repressor (27). It is tempting to suggest that *Xanthomonas* OxyR could act as both a peroxide sensor and a transcription activator of its own gene. Alternatively, other redox-sensitive transcription regulators may activate the *oxyR* operon. The lack of *oxyR* mutants prevents differentiation between these possibilities. We are constructing an *oxyR* mutant with which to investigate the possibility of autoregulation of the *oxyR* operon.

Regulation of *oxyR* is conserved in various *Xanthomonas* strains. We were interested in whether the unique pattern of *X. campestris* pv. phaseoli *oxyR* expression was conserved in other *Xanthomonas* spp. Western analysis with an anti-*X. campestris* pv. phaseoli OxyR antibody was performed with uninduced and MD-induced samples from *X. campestris* pv. campestris, *Xanthomonas oryzae* pv. oryzae, and *X. campestris* pv. malvacearum. The results are shown in Fig. 7B. In three strains tested, MD-induced samples showed an increased amount of OxyR. This indicates that in response to oxidative stress, *Xanthomonas* strains increase the synthesis and accumulation of OxyR, and this unique response is conserved. It would be interesting to see if this novel *oxyR* response to oxidative stress operates in other bacterial systems.

Summary. The transcription organization of *Xanthomonas ahpC* and *ahpF-oxyR-orfX* suggests the possibility of a new regulatory circuit between these genes that is different from those of other bacteria. Analysis of *ahpC* expression patterns showed that the gene was highly induced by peroxides, superoxides, and heavy metals and was moderately induced by alkaline pH. Surprisingly, increased expression of *oxyR* was detected in response to treatments with peroxides and superoxides. These interesting results add another level of complexity to the mechanism by which *oxyR* regulates the oxidative stress response. Various inducers were acting at the levels of transcription which led to increased amounts of *ahpC* and *ahpF-oxyR-orfX* mRNA. In *Xanthomonas*, exposure to oxidants not only changed the redox state of OxyR but also increased its cellular concentration. These observations could be generally important as an alternative strategy by which bacteria respond to oxidative stress. This information may facilitate elucidation of novel regulation of the oxidative stress response in other bacteria. Also, the ability to increase expression of genes involved in stress protection (i.e., catalase [28] and *ahpC*) and regulation (*oxyR*) in response to stress is likely to play a crucial physiological role in oxidant-induced adaptive and cross-protection responses in *Xanthomonas* (29, 40).

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Isolation and characterization of a multiple peroxide resistant mutant from *Xanthomonas campestris* pv. *phaseoli*

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Abstract

We have isolated a spontaneous multiple peroxide resistant *Xanthomonas campestris* pv. *phaseoli* mutant (*Xp*Hr). In the presence of peroxides, the mutant had a higher growth rate than the parent. It also had a greater than 100-fold increase in resistance levels to H_2O_2 killing but only slightly more resistance to tert-butyl hydroperoxide killing. Increases in activity were detected for the peroxide scavenging enzymes catalase (100-fold) and AhpC (over 30-fold). Also observed was cross-resistance to thermal killing; however, no cross-resistance to other oxidants or chemicals was found. Analysis of protein profiles revealed that proteins with molecular masses of 22 and 58 kDa were accumulated while proteins of 29, 33 and 41 kDa were depressed in the mutant. These results indicate that the mutant may have defect(s) in peroxide regulation, which resulted in high constitutive expression of peroxide scavenging enzymes. Nevertheless, the mutant retained growth phase dependent regulation of peroxide killing. The mutant should be useful in unravelling the nature of a complex peroxide stress regulon.

Keywords: H_2O_2; Organic peroxide resistance; Peroxide scavenging enzyme

1. Introduction

Xanthomonas belongs to an important family of bacterial phytopathogens. In response to microbial infection, the initial phase of the plant defense response involves increased production of reactive oxygen species, such as H_2O_2, lipid peroxides and superoxides, which can directly inhibit pathogen growth and also participate in signal transduction pathways to further activate host defense responses [1]. Reactive oxygen species (ROS) are also normal byproducts of aerobic life.

To establish infection bacterial pathogens must overcome the toxic effects of ROS. Bacteria have evolved highly complex oxidative stress protection mechanisms, which comprise genes for oxidant scavenging enzymes (e.g. alkyl hydroperoxide reductase, catalases, peroxidases and glutathione transferase), for enzymes involved in the synthesis of antioxidant molecules (e.g. glutathione reductase, glucose 6-phosphate dehydrogenase) and for enzymes involved in damage repair (e.g. exonuclease III, methionine sulfoxide reductase) [2]. Mutations in these genes

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Abbreviations: AhpC, alkyl hydroperoxide reductase subunit C; Td, doubling time; NEM, *N*-ethylmaleimide; ROS, reactive oxygen species; SOD, superoxide dismutase; tBOOH, tert-butyl hydroperoxide

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lead to increased sensitivity to peroxide stress [2]. Coordinated expression of these genes in response to oxidative stress is essential to bacterial survival and is governed, in part, by oxidative stress sensitive regulatory genes such as *oxyR* and *soxRS* [2]. Also, increased expression of the catalase gene confers higher growth rates in the presence of H_2O_2 [3].

As a first step towards the elucidation of the mechanism regulating the oxidative stress resistant response, this work describes the isolation and characterization of a peroxide resistant mutant.

2. Materials and methods

2.1. Isolation of H_2O_2 resistant mutants

The parental *Xp* strain was from our laboratory collection. Exponential phase cultures of *Xp* grown aerobically in SB medium at 28°C were spread directly on SB plates containing 1 mM H_2O_2 or 1 mM tert-butyl hydroperoxide (tBOOH) and incubated for 48 h. No spontaneous tBOOH resistant mutants were isolated. However, five H_2O_2 resistant mutants were isolated. All five mutants were grown non-selectively in SB for 10 generations and subsequently scored for H_2O_2 resistant phenotype. Only one mutant (designated *Xp*HR) retained the resistant phenotype after non-selective growth and it was selected for further investigation.

2.2. Effects of H_2O_2 on growth rate

The effects of H_2O_2 on growth rate of *Xp* or *Xp*HR were investigated by subculture of late exponential phase cells into fresh SB medium at 1:50 dilution. After 1 h 300 μM H_2O_2 was added to both cultures and growth was monitored spectrophotometrically at OD_{600}.

2.3. Cell survival

Quantitative determinations of surviving fractions of cells after treatment with oxidants were performed as described by Vattanaviboon et al. [4]. For reproducible results, it was essential to use cells from similar stages of growth since the resistance level to oxidants varied with the growth phase [4]. All experiments were performed at least three times and average values are shown. Killing zone experiments were performed three times as previously described [3] and the results shown are from three independently performed experiments.

2.4. Enzyme assays

Lysate preparations of exponential phase cells for enzyme assays, i.e. for catalase, superoxide dismutase (SOD), glucose 6-phosphate dehydrogenase, glutathione reductase and glutathione transferase, were carried out as previously described [5,6].

2.5. Immune analysis

For immune analysis of AhpC levels, conditions of SDS-PAGE, blotting to a nitrocellulose membrane, and immunodetection were as previously described [7], except that an anti-*E. coli* AhpC antibody was used as the primary antibody.

3. Results

3.1. Growth of XpHR in the presence of oxidants

The ability of *Xp* and *Xp*HR to grow in a minimal medium and in a complex SB medium with low concentrations of various oxidants was investigated. In the presence of 300 μM H_2O_2 parent *Xp* cells showed

Table 1
Comparison of resistance levels to killing concentration of various chemicals in *Xp* and *Xp*HR

	Diameter of killing zone (cm)	
	Xp	*Xp*HR
H_2O_2 (2 M)	3.2	1.5
tBOOH (1 M)	3.1	2.4
Paraquat (1 M)	2.6	2.5
Menadione (1 M)	2.7	2.7
NEM (0.1 M)	2.3	2.3
SDS (10%)	1.6	1.6
EDTA (0.5 M)	2.2	2.1
$CdCl_2$ (1 M)	4.3	4.6
$CoCl_2$ (1 M)	3.7	4.0

The values shown are averages from three independently performed experiments as described in Section 2.

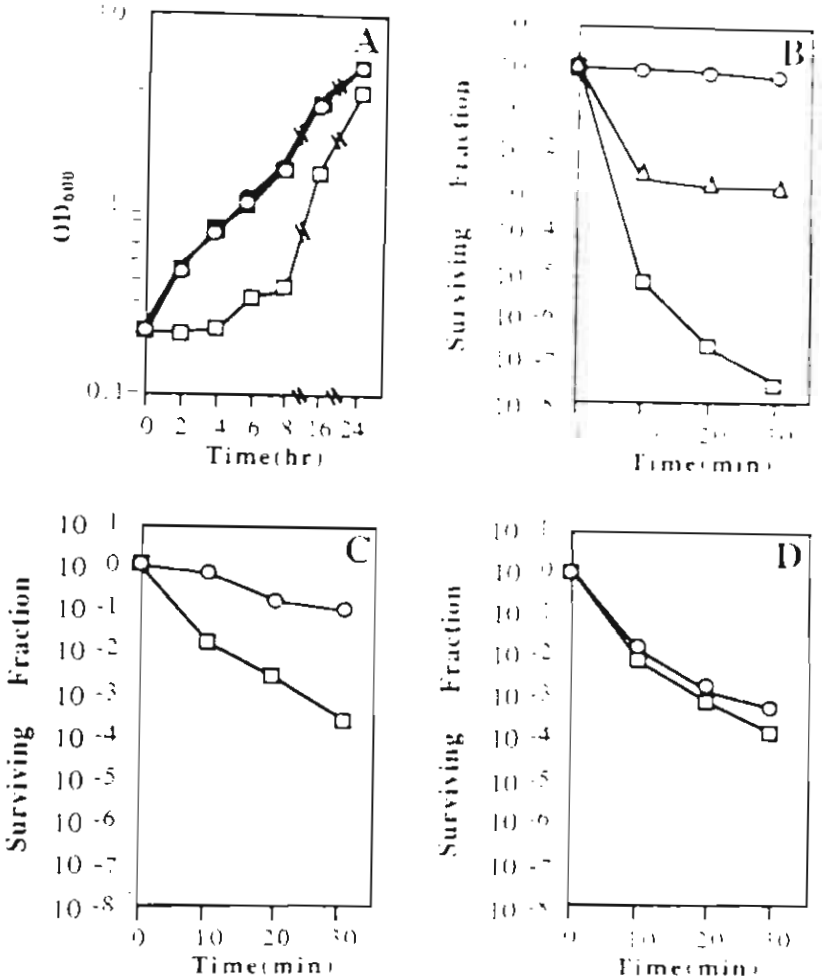

Fig. 1. The effects of either a low dose of H_2O_2 on growth or high doses of ... on survival of *Xp* and *XpHR*. A: ... μM H_2O_2 on *Xp* (■ SB or □ SB+H_2O_2) or *XpHR* (● SB or ○ SB+H_2O_2) grown with ... as described in Section 2. ... *XpHR* (○) were treated with killing concentrations of 20 mM or 500 mM H_2O_2 for ... B) ... mM tBOOH ... C) ... menadione (D) as described in Section 2.

marked growth inhibition with a doubling time (Td) of 240 min compared to 120 min in the absence of H_2O_2. By contrast, 300 μM H_2O_2 did not affect the *Xp*HR growth rate (Td 120 min) (Fig. 1A). The growth rate of *Xp*HR in the presence of 200 μM tBOOH (Td 160 min) was slower than that without tBOOH (Td 120 min) but it was significantly higher than the growth rate of wild-type cells in the presence of 200 μM tBOOH (Td 320 min). No differences in growth rates for the wild-type or the mutant were detected in SB medium containing 300 μM menadione or in a minimal medium (data not shown).

3.2. Effects of killing concentrations of oxidants on *XpHR* and wild-type *Xp*

*Xp*HR was isolated based upon its ability to grow in the presence of 1 mM H_2O_2. Here we investigated its sensitivity to high levels of peroxides and superoxides. *Xp*HR was highly resistant to H_2O_2 killing (Fig. 1B). A 30 min exposure of *Xp* to 20 mM H_2O_2 resulted in a 10^6 drop in survival while *Xp*HR was unaffected. Exposure of *Xp*HR to 500 mM H_2O_2 for 30 min caused a drop of ... survival (Fig. 1B). *Xp*HR had more resistance than the parent *Xp* strains to 300 mM tBOOH (Fig. 1C).

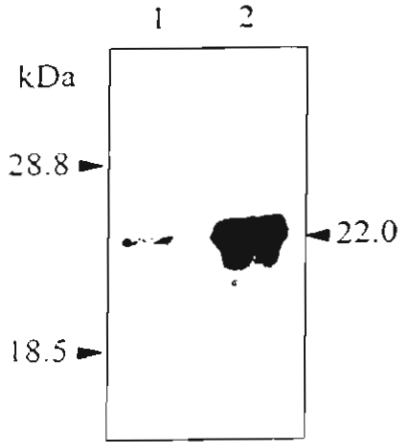

Fig. 2. Immunological determination of AhpC levels in wild-type and mutant *Xanthomonas*. Equal concentrations (30 μg) of total protein from *Xp* (lane 1) and *Xp*HR (lane 2) were loaded onto the gel which was blotted and probed with an anti-*E. coli* AhpC antibody as described in Section 2 [7].

However, the magnitude of the resistance to tBOOH was less than that to H_2O_2. There was no difference in resistance to menadione killing (Fig. 1D). Similar results were obtained using the disk diffusion growth inhibition assay. *Xp*HR had more resistance to peroxides (H_2O_2, tBOOH) but not to superoxide generators (menadione and paraquat) (Table 1).

3.3. The sensitivity of XpHR to other chemicals and stress

Extensive cross-regulation between different stress regulons has been demonstrated [2]. Resistance to one agent often leads to cross-protection to other unrelated agents [6]. In tests with various chemicals the mutant, *Xp*HR, showed no alteration in resistance levels (Table 1). However, it did have increased resistance to heat killing. Incubation at 45°C for 30 min caused a drop of 2×10^3 in *Xp* survivors but only 7×10^1 in *Xp*HR survivors. Unexpectedly, the mutant was slightly more sensitive than the wild-type to heavy metal salts (cadmium and cobalt) (Table 1).

3.4. Oxidant scavenging and oxidative stress related enzyme activities in XpHR

The peroxide resistance phenotype could be due to increased activity of oxidant scavenging enzymes and/or other oxidative stress protective enzymes (i.e. glucose 6-phosphate dehydrogenase, glutathione reductase, or SOD). Results from assays of these enzyme activities shown in Table 2. Catalase activity was about 100-fold higher in the mutant.

The activities of other scavenging enzymes for peroxide (peroxidase and glutathione transferase) or superoxide (SOD) remained similar in both *Xp* and *Xp*HR. Nor were there any differences in glutathione reductase and glucose 6-phosphate dehydrogenase activities (Table 2). The activity of alkyl hydroperoxide reductase, the major organic peroxide scavenging enzyme, could not be assayed directly. However, levels of AhpC (responsible for the reduction of organic hydroperoxides to the corresponding alcohols) could be measured using immunodetection with an anti-*E. coli* AhpC antibody. Densitometer analysis of Western immunoblots of AhpC showed that the mutant produced over 30-fold more AhpC than the wild-type (Fig. 2).

3.5. Protein profiles in XpHR and Xp

Because of the multiple alterations in resistance to various stresses in *Xp*HR, its protein profile was compared with that of *Xp*. The results are shown in Fig. 3. Two proteins of molecular mass 58 and

Table 2
Specific activities of various oxidative stress related enzymes in the wild-type and mutant

Strain	Kat		SOD		GOR		GST		G6PD	
	U	F	U	F	U	F	U	F	U	F
Xp	3.4	1.0	2.0	1.0	0.35	1.0	0.42	1.0	0.87	1.0
*Xp*HR	380	120	1.9	0.9	0.23	0.7	0.60	1.4	0.49	0.6

U, units/mg/ml protein; F, fold induction. Kat, catalase; SOD, superoxide dismutase; GOR, glutathione reductase; GST, glutathione transferase; G6PD, glucose 6-phosphate dehydrogenase. Enzyme assays were performed as described in Section 2. The values shown are averages from three experiments.

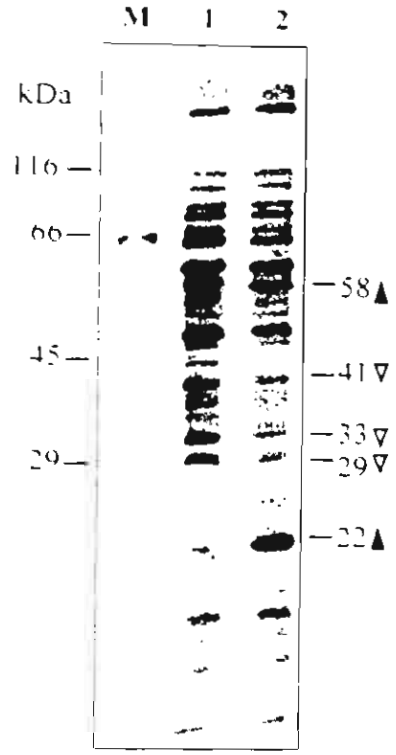

Fig. 3. Analysis of the protein profile of *Xp* and *Xp*HR. 60 μg of total protein from *Xp* (lane 1) and *Xp*HR (lane 2) were loaded on a 12% gel for SDS-PAGE. After electrophoresis, the gel was stained with Coomassie blue. Protein molecular mass markers (lane M) are shown on the left. The numbers on the right indicate molecular masses of the proteins, which had accumulated (▲) or were repressed (▽).

22 kDa showed high accumulation in *Xp*HR. The 22 kDa protein is likely to be AhpC (see Fig. 2). The identity of the 58 kDa protein is unknown. Its molecular mass was smaller than that expected for the monofunctional catalase of *Xanthomonas* [8]. Interestingly, the levels of 29, 33 and 41 kDa proteins were lower in the mutant compared to the parent strain.

3.6. *XpHR retained growth phase dependent resistance to peroxide killing*

We have shown that stationary phase cells are more resistant to peroxide and superoxide killing [4]. Treatment of parental *Xp* for 30 min with 20 mM H_2O_2 resulted in 10^0 and 10^2 reduction in survivors of exponential phase (Fig. 1B) and stationary phase cells respectively. Therefore, we investigated whether the mutant still retained growth phase dependent resistance to peroxide killing. Exponential and stationary phase *Xp*HR were treated with 500 mM for 30 min. This treatment resulted in a reduction of survivors by 10^2 for exponential phase cells and 10^1 for stationary phase cells. Treatment at a higher concentration of H_2O_2 (1000 mM) for 30 min of exponential phase cells resulted in a 10^6 reduction in the surviving fraction of parental *Xp* but a reduction of only 10^4 in the survivors for stationary phase cells of *Xp*HR.

4. Discussion

Results from the characterization of *Xp*HR supported the notion that it could be a regulatory mutant. The mutant may have defects in regulation of a peroxide stress regulon. This could have resulted in the high level, uncontrolled expression of peroxide scavenging enzymes, in the accumulation of some proteins and depletion of others. It is doubtful that these phenotypes could have arisen from gene amplification, since Southern analysis of *Xp* and *Xp*HR DNA probed with either catalase or *ahpC* gene probes showed no amplification or gross rearrangement of these genes (data not shown). It is also unlikely that multiple unlinked spontaneous mutations occurred, since increases in organic peroxides and heat resistance arose without selective pressure. Because the mutant retained growth phase dependent resistance to H_2O_2 killing, it is likely that regulation of this is independent of the regulation of peroxide scavenging enzymes. The *Xp*HR phenotypes showed similarity to phenotypes of a *B. subtilis* H_2O_2 resistance mutant, which has been shown to have defects in the *mrgA* gene, a transcription regulator of metals and oxidative stress response [8,9].

We have observed that heat shock does not induce catalase in *Xanthomonas* [5]. Thus, further characterization of the observation on cross-resistance to heat killing in the mutant might provide new clues to factors governing the co-regulation of oxidative stress and heat shock regulons.

In *Xp*HR, the levels of resistance to H_2O_2 killing correlated with catalase levels. The results are consistent with our findings that increased expression of

a catalase gene in *Xanthomonas* is sufficient to confer protection against H_2O_2 toxicity [3]. In the absence of a catalase negative mutant in *Xanthomonas*, these results support the notion that catalase is important in protecting *Xanthomonas* from H_2O_2 toxicity. Increased tBOOH resistance in *Xp*HR could be partially accounted for by the increased levels of AhpC. However, the increased AhpC levels were great compared to the small increase in resistance to tBOOH. This suggested that other factors such as AhpF levels (the other sub-unit of alkyl hydroperoxide reductase) might also contribute to the overall resistance to tBOOH. Co-regulation of these genes has been demonstrated in *B. subtilis* [10] but not in other bacteria.

Isolation of *Xp*HR is an important step in the elucidation of the highly complex regulation of the peroxide stress response. Since H_2O_2 and organic peroxides are microbicidal agents/growth retardants and also signal molecules in plant defense response, alterations in microbial catalase and/or AhpC regulation could have significant effects on the development and progression of plant disease.

Acknowledgments

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Identification and Characterization of a New Organic Hydroperoxide Resistance (*ohr*) Gene with a Novel Pattern of Oxidative Stress Regulation from *Xanthomonas campestris* pv. phaseoli

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We have isolated a new organic hydroperoxide resistance (*ohr*) gene from *Xanthomonas campestris* pv. phaseoli. This was done by complementation of an *Escherichia coli* alkyl hydroperoxide reductase mutant with an organic hydroperoxide-hypersensitive phenotype. *ohr* encodes a 14.5-kDa protein. Its amino acid sequence shows high homology with several proteins of unknown function. An *ohr* mutant was subsequently constructed, and it showed increased sensitivity to both growth-inhibitory and killing concentrations of organic hydroperoxides but not to either H_2O_2 or superoxide generators. No alterations in sensitivity to other oxidants or stresses were observed in the mutant. *ohr* had interesting expression patterns in response to low concentrations of oxidants. It was highly induced by organic hydroperoxides, weakly induced by H_2O_2, and not induced at all by a superoxide generator. The novel regulation pattern of *ohr* suggests the existence of a second organic hydroperoxide-inducible system that differs from the global peroxide regulator system, OxyR. Expression of *ohr* in various bacteria tested conferred increased resistance to *tert*-butyl hydroperoxide killing, but this was not so for wild-type *Xanthomonas* strains. The organic hydroperoxide hypersensitivity of *ohr* mutants could be fully complemented by expression of *ohr* or a combination of *ahpC* and *ahpF* and could be partially complemented by expression *ahpC* alone. The data suggested that Ohr was a new type of organic hydroperoxide detoxification protein.

Increased production of reactive oxygen species, including superoxides, H_2O_2, and organic hydroperoxides, is an important component of the plant defense response against microbial infection (23, 42) and is a consequence of normal aerobic metabolism (15, 16). Bacteria have evolved complex mechanisms to detoxify and repair damage caused by reactive oxygen species. For detoxification of superoxides and H_2O_2, the enzymes involved and the regulation of their genes are well studied (11, 12, 17). Much less is known regarding defense against organic peroxides.

Organic hydroperoxides are highly toxic molecules, partly due to their ability to generate free organic radicals, which can react with biological molecules and perpetuate free radical reactions (1, 19). Thus, genes involved in protection against organic peroxide toxicity are likely to play important roles in oxidative stress response and in host-pathogen interactions. Alkyl hydroperoxide reductase (AhpR) is the only major microbial enzyme that has been shown to be involved in converting organic hydroperoxides into the corresponding alcohols (3, 21, 40).

The AhpR mechanism of action is well studied. The enzyme consists of two subunits named AhpC and AhpF (4, 5, 21, 37, 38). The genes coding for these subunits are widely distributed (3, 4). Genetic analysis of several bacteria has shown that mutations in these genes lead to an organic hydroperoxide-hypersensitive phenotype, and this confirms their roles in protecting against organic hydroperoxides (2, 40, 45). Other bacterial enzymes, such as glutathione peroxidase (33), glutathione transferase (34), and peroxidases (12), can also use organic hydroperoxides as substrates with varying degrees of efficiency. However, these enzymes have not been well characterized biochemically and genetically. Also, their distribution in only certain groups of bacteria (33, 34) raises a question as to whether they play important physiological roles in the protection against organic hydroperoxide toxicity.

In this paper, we report the isolation and characterization of a possible new organic hydroperoxide detoxification enzyme with a novel regulatory pattern.

MATERIALS AND METHODS

Bacterial cultures and media. All *Xanthomonas* strains were grown aerobically at 28°C in SB medium as previously described (6, 35). To ensure the reproducibility of results, all experiments were performed on cultures at similar stages of growth. All *Escherichia coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37°C. Microaerobic growth conditions were achieved by placing the plates in an anaerobic jar with a *Campylobacter* gas pack (Oxoid) and incubating them at 28°C.

Nucleic acid extraction and analysis, cloning, and nucleotide sequencing. Restriction enzyme digestions were performed according to the manufacturers' recommendations. Molecular cloning, gel electrophoresis, and nucleic acid hybridization were performed as previously described (28). Nucleotide sequencing was done using ABI Prism dye terminator sequencing kits on an ABI 373 automated sequencer. *E. coli* and *Xanthomonas* were transformed by a chemical method (28) and by electroporation (30), respectively. Genomic DNA extraction from *Xanthomonas* spp. was done according to the method of Mongkolsuk et al. (30). Total RNA was isolated by a hot-phenol method (30). pUFR-ahpCF was generated by subcloning a DNA fragment containing *Xanthomonas campestris* pv. phaseoli *ahpCF* (24) into pUFR047.

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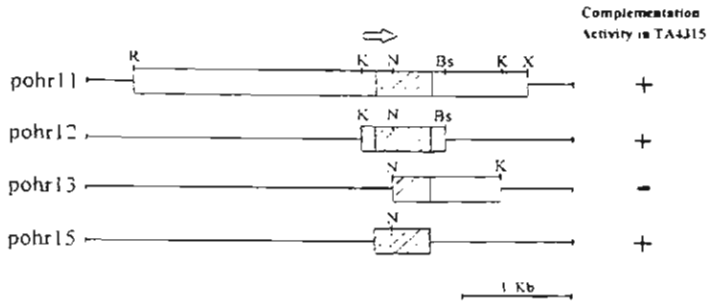

FIG. 1. Localization of *ohr*. The open boxes represent *X. campestris* pv. phaseoli genomic DNA cloned in the indicated plasmids, and the shaded boxes represent the *ohr* gene. The arrow indicates the direction of *ohr* transcription. Bs, *Bst*XI; K, *Kpn*I; N, *Not*I; R, *Eco*RI; X, *Xba*I; +, growth of TA4315 harboring the plasmid on LB-ampicillin plates containing 100 μM tBOOH; -, no growth.

Construction of an *ohr* mutant. An *ohr* mutant was created by marker exchange between a mutated copy of *ohr* and a functional genomic copy. Specifically, a *tet* gene from pSM-Tet (32) was digested with *Eco*RI and *Hin*dIII and cloned into similarly digested p18Not (10), resulting in pNot-Tet. A mutated *ohr* gene was then constructed by insertion of a *Not*I-digested *tet* gene from pNot-Tet into a unique *Not*I site located in the *ohr* coding region of pohr11 (Fig. 1). This resulted in a recombinant plasmid designated pohr-tet. *ohr* inactivation was confirmed by loss of the ability of pohr-tet to confer resistance to *tert*-butyl hydroperoxide (tBOOH) in *E. coli* TA4315 (*ΔahpCF* [40]). The plasmid was electroporated into *X. campestris* pv. phaseoli, and transformants were selected on SB plates containing 20 μg of tetracycline/ml. Tet^r colonies appeared after 48 h of incubation at 28°C. These colonies were picked and scored for Ap^r phenotype. Those transformants which were both Tet^r and Ap^s (a double crossover resulted in marker exchange) were selected for further characterization. Marker exchanges between the mutated *ohr* and the functional copy of the gene in putative mutants were confirmed by analysis of hybridization patterns of genomic DNA digested with restriction enzymes and probed with *ohr*.

Effects of oxidants on growth and killing. Effects of low concentrations of oxidants on *Xanthomonas* growth was tested with log-phase cells. Essentially, overnight cultures of late-log-phase cells were subcultured as a 5% inoculum into fresh SB medium and allowed to grow for 1 h. Oxidants were then added at appropriate concentrations, and the growth of both induced and uninduced cultures was subsequently monitored spectrophotometrically. Quantitative analysis of the killing effects of high concentrations of oxidants on various *Xanthomonas* strains was performed as previously described (30). Qualitative analysis of levels of resistance to various reagents was done by using a killing zone method (31). Essentially log-phase cells were mixed with SB top agar and poured onto SB plates. After the top agar had solidified, 6-mm-diameter discs containing appropriate concentrations of oxidants were placed on the cell lawn, and zones of growth inhibition were measured after 24 h of incubation at 28°C.

High-level expression and purification of Ohr for antibody production. High levels of *ohr* expression for Ohr purification were achieved by using a gene fusion expression vector system in *E. coli*. One oligonucleotide primer corresponding to the 5′ region of *ohr* from the second codon (5′ TACGAATTCATGGCCTCA CCC 3′) and a second primer corresponding to the *ohr* translation termination codon (5′ TCCAAGCTTGCATTACGCCA 3′) were used to amplify *ohr* from pohr15. A PCR product of 500 bp was then digested with *Eco*RI and *Hin*dIII, gel purified, and cloned into pMAL-C2 vector (New England Biolabs Inc.). This was expected to result in the frame fusion of *ohr* with maltose binding protein. Transformants with correct inserts were screened for high levels of expression of the fusion protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. A clone designated pMalohr showed high levels of expression of the fusion protein, and it was chosen for large-scale protein purification.

A 200-ml culture of pMalohr was grown and induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 1 h. The cells were subsequently pelleted, and the pellet was resuspended in 50 mM phosphate buffer, pH 7.0. The suspension was then sonicated for 20 min, with cooling intervals. Ohr fusion protein was purified from crude lysate by using amylose affinity columns according to the manufacturer's recommendations. The purified fusion protein was then cleaved with protease factor Xa, and Ohr was repurified from SDS-polyacrylamide gels. Purified Ohr was used to raise antibody in rabbits.

Immunodetection of Ohr. Crude lysates from *Xanthomonas* were prepared according to the method of Mongkolsuk et al. (30), and the total protein concentration was determined by the dye-binding method (2). SDS-polyacrylamide gel electrophoresis, blotting, blocking, and antibody reaction analysis were performed as previously described (31) except that an anti-Ohr antibody was used as a primary antibody at 1:3,000 dilution. Antibody reactions were detected by a goat anti-rabbit antibody conjugated to alkaline phosphatase (Promega) as recommended by the manufacturer.

Expression analysis. A 40-ml mid-log-phase *X. campestris* pv. phaseoli culture was equally divided among four flasks and grown for 1 h until the optical density at 600 nm reached 0.4. H_2O_2, tBOOH, or menadione (MD) at a 100 μM final concentration was added, and the cultures were grown for 1 h longer before both induced and uninduced cultures were harvested for Western analysis of Ohr. For analysis of *ohr* RNA a similar induction protocol was performed except that the induction time was reduced to 15 min. The cells were harvested as before, and total RNA was extracted by a hot-phenol method (30).

Nucleotide sequence accession number. The nucleotide sequence accession number for *ohr* in GenBank is AF036166.

RESULTS

Isolation and localization of *ohr*. Our objective was to isolate the *Xanthomonas* genes involved in organic peroxide protection by suppressing the organic hydroperoxide-hypersensitive phenotype of an *E. coli ahpCF* mutant. An aliquot of an *X. campestris* pv. phaseoli DNA library in pUC18 was electroporated into TA4315 (*ΔahpCF* [40]). Transformants were selected on LB-ampicillin agar containing 500 μM tBOOH and 1 mM IPTG. After 24 h of incubation, 12 colonies appeared on the plate. Plasmids were extracted from individual transformants and retransformed into TA4315, selecting for ampicillin resistance and scoring for concomitant tBOOH resistance. There were eight clones which retained tBOOH resistance. Restriction enzyme mapping and Southern analysis of plasmids purified from these colonies showed that they shared a common 1.2-kb *Kpn*I fragment that cross-hybridized (data not shown). The plasmid, designated pohr11, that contained the longest *X. campestris* pv. phaseoli DNA insert (3.8 kb) was selected for further characterization. Subcloning and deletions of pohr11 were performed to localize the gene responsible for tBOOH resistance. The results are shown in Fig. 1. The gene which was able to confer organic hydroperoxide resistance upon the *E. coli* mutant was located on the 890-bp *Kpn*I-*Bst*XI fragment (Fig. 1, pohr12).

The *Xanthomonas* DNA in pohr11 from *Kpn*I to *Bst*XI was completely sequenced (Fig. 2A). Analysis of the sequence revealed many open reading frames (ORFs) in the region that could have conferred tBOOH resistance on TA4315. An ORF (designated ORF-A) with a strong ribosome binding site 6 bp upstream of a translation initiation codon and with a coding capacity for 142 amino acids having a total predicted molecular mass of 14.5 kDa was a candidate for the tBOOH resistance gene. To confirm this, primers corresponding to DNA sequences 100 bp upstream from the translation initiation codon of ORF-A (5′ GAGAATTCCTTGGCGCGGGAT 3′) and 20 bases downstream of the stop codon of ORF-A (5′ GCATCA CGGCCTGGCCT 3′) were used to amplify *orf-A* from pohr11 in a PCR. The 560-bp PCR product was cloned into pBluescript KS, resulting in pohr15, which was transformed into TA4315. Transformants showed high levels of tBOOH resistance compared to that of TA4315 harboring the vector alone (Fig. 1). This confirmed that ORF-A was responsible for the organic hydroperoxide resistance phenotype, and it was therefore designated *ohr*.

The coding potential for *ohr* was confirmed by determination of pohr15-encoded proteins by using a coupled in vitro transcription-translation *E. coli* system (Promega). The results (Fig. 2B) showed that pohr15 encoded a polypeptide of 14.5 kDa, identical to the calculated molecular mass of Ohr.

Sequence analysis. The predicted Ohr amino acid sequence had the highest homology (63%) with an unknown protein from *Acinetobacter calcoaceticus* (36). Moderate homology (46%) with two unknown proteins (YkIA and YkzA) from *Bacillus subtilis* was observed. There was lower homology with OsmC (an osmotic inducible protein) from *E. coli* (31% [18]) and an ORF of unknown function from *Mycoplasma genitalium*

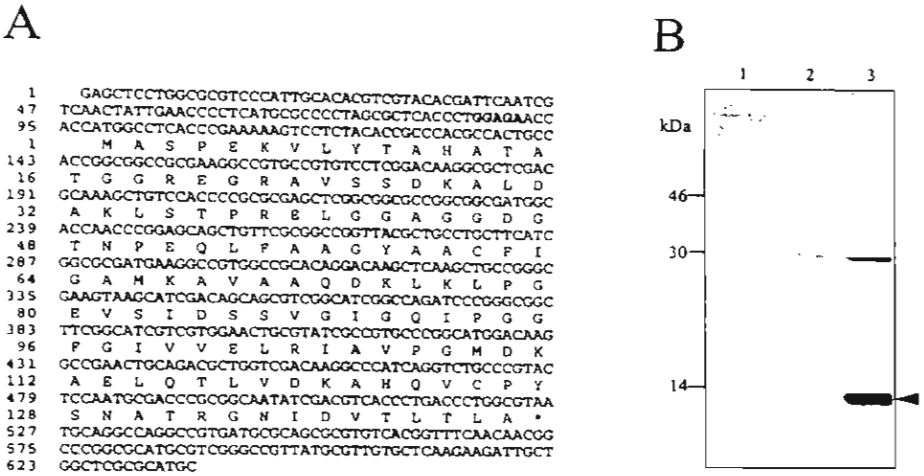

FIG. 2. Nucleotide sequence (A) and in vitro translation products (B) of *ohr*. (A) Nucleotide sequence and a predicted translation product of *ohr*. A putative bosome binding site for *ohr* is shown in boldface. (B) In vitro translation of plasmid-encoded proteins with *E. coli* S-30 extracts. A vector control or pohr15 was added o S-30 coupled in vitro transcription-translation lysates as described in Materials and Methods. The radioactively labeled translation products are shown. Lane 1, rotein molecular mass markers; lane 2, pUC18; lane 3, pohr15. The arrow indicates in vitro translation products of *ohr*. The second band at around 30 kDa is the roduct of the ampicillin resistance gene.

31% [14]). The multiple alignment of these ORFs is shown in Fig. 3. It was striking that all four proteins were similar in size and had two highly conserved redox-sensitive cysteine residues, suggesting they could be important in the structure and functions of Ohr.

Structural organization and distribution of *ohr*. The copy number of *ohr* in *X. campestris* pv. phaseoli was determined. *X. campestris* pv. phaseoli genomic DNA was individually digested with five restriction enzymes, separated, blotted, and subsequently probed with the coding region of *ohr*. The results showed hybridization patterns consistent with *ohr* being a single-copy gene (data not shown).

To determine the distribution of *ohr* in *Xanthomonas* and various other bacteria, both Southern and Western analysis were performed. Western blots prepared from total-protein lysates from six *Xanthomonas* species each showed a 14.5-kDa protein that specifically cross-reacted with an anti-Ohr antibody (Fig. 4A). In addition, genomic DNA from five *Xanthomonas* species was digested with a restriction enzyme, blotted, and probed with *ohr*. Under high-stringency washing conditions, positive hybridization signals were detected in all strains tested (Fig. 4B). By contrast, Southern blots prepared from genomic DNAs from *Pseudomonas aeruginosa* and *Burkholderia cepacia* did not cross-hybridize with *ohr* probes, even under low-stringency washing conditions. Furthermore, Western analysis of cell lysates from these bacteria did not show any proteins of a size similar to that of Ohr that specifically cross-reacted with the anti-Ohr antibody (data not shown).

Construction and physical characterization of a *Xanthomonas ohr* mutant. The role of *ohr* in protecting against organic hydroperoxide toxicity in *Xanthomonas* was evaluated by construction of an *ohr* inactivation mutant, as described in Materials and Methods. Essentially, a gene conferring Tet^r was inserted into the coding region of *ohr* and the recombinant

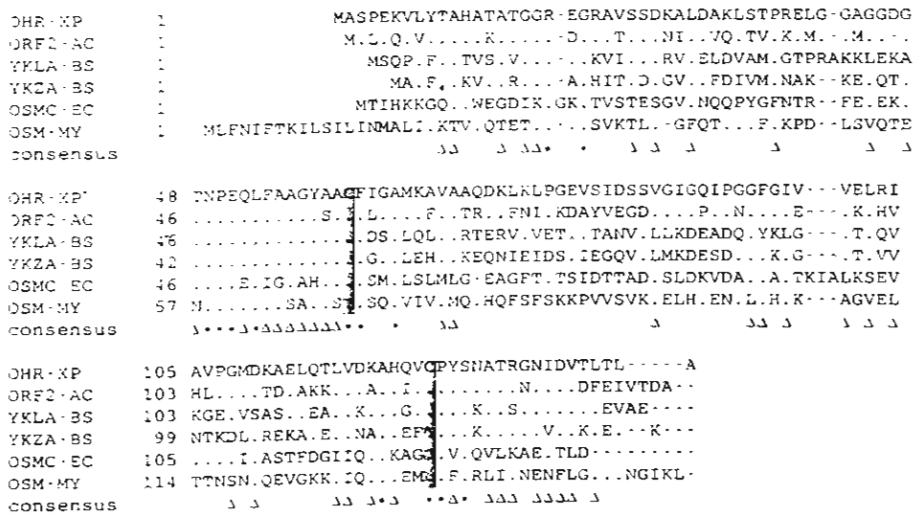

FIG. 3. Multiple alignment of *X. campestris* pv. phaseoli Ohr (OHR-XP [AF036166]), ORF2 of *Acinetobacter* (ORF2-AC [Y09102]), proteins from *B. subtilis* (YKLA-Bs [AJ002571] and YKZA-Bs [AJ002571]), OsmC from *E. coli* (OSMC-EC [X57433]), and OsmC-like protein from *M. genitalium* (OSM-MY [U39732]). Amino acid sequences were aligned by the Clustal W program (43). Periods represent amino acids identical to those found in Ohr, asterisks indicate identical amino acids in all six sequences, and triangles represent matches of at least four amino acids in the sequences. Hyphens indicate gaps in the sequences. Conserved cysteine residues are shaded.

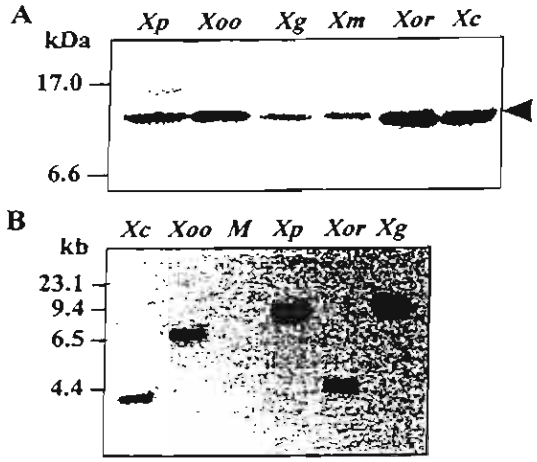

FIG. 4. Detection of Ohr and *ohr* in various *Xanthomonas* species. (A) Western analysis of Ohr in protein lysates from *X. campestris* pv. phaseoli (*Xp*), *Xanthomonas oryzae* pv. oryzae (*Xoo*), *Xanthomonas campestris* pv. glycine (*Xg*), *Xanthomonas campestris* pv. malvacearum (*Xm*), *Xanthomonas oryzae* pv. orizicolar (*Xor*), and *Xanthomonas campestris* pv. campestris (*Xc*). Fifty micrograms of protein was loaded into each lane. After electrophoresis, the gel was blotted onto a piece of polyvinylidene difluoride membrane that was reacted with an anti-Ohr antibody. Protein molecular mass markers are shown on the left. The arrow indicates the position of Ohr protein. (B) Southern analysis of total genomic DNA digested with *Eco*RI and probed with the coding region of *ohr*. Hybridization and high-stringency washing conditions were as previously described (30). Lane M contains DNA molecular size markers; the sizes are shown on the left.

plasmid was electroporated into *X. campestris* pv. phaseoli with selection for Tet^r transformants that also had an Ap^s phenotype. Subsequently, the levels of resistance to tBOOH of these 20 Tet^r Ap^s *X. campestris* pv. phaseoli transformants were tested by the killing zone method. The results showed that all putative *ohr* mutants were less resistant to tBOOH killing than parental *X. campestris* pv. phaseoli (data not shown).

An *ohr* mutant designated *Xp*18 was selected for detailed structural analysis. The results of Southern analysis of *Xp*18 DNA digested with restriction enzymes and probed with *ohr* are shown in Fig. 5. Hybridization of the *ohr* probe with *X. campestris* pv. phaseoli or *Xp*18 DNA digested with either *Bst*XI or *Cla*I showed that the positively hybridized bands in *Xp*18 were around 3 kb larger than a corresponding band detected in similarly digested *X. campestris* pv. phaseoli DNA (Fig. 5A). The size increase corresponded to the size of the inserted *tet* gene. There were no additional positively hybridized signals in digested *Xp*18 DNA. These results indicated that the mutated *ohr* had replaced the functional gene. Inactivation of *ohr* in *Xp*18 was also confirmed at the protein level by Western blot analysis with an anti-Ohr antibody. The results (Fig. 5B) showed that the Ohr protein was absent from *Xp*18.

Physiological and biochemical characterization of the *ohr* mutant. The availability of the *ohr* mutant allowed investigation of the physiological role of *ohr* in the *Xanthomonas* oxidative stress response. The effects of both oxidative and nonoxidative stress on the growth and survival of the mutant were evaluated.

In most bacteria, the mutation of genes involved in oxidative stress protection leads to a reduced aerobic growth rate and a lower plating efficiency (26). We tested these parameters first on the *ohr* mutant. The mutant showed an aerobic growth rate and plating efficiency similar to those of a wild-type strain on either rich or minimal media (data not shown). Next, the effect of low oxidant concentrations on the growth rates of *X. campestris* pv. phaseoli and *Xp*18 was investigated. No differences between the growth rates of these strains were observed in the presence of several concentrations of either H_2O_2 or MD (data not shown). However, a low concentration of tBOOH had a significant growth-inhibitory effect on the mutant. In the presence of 600 μM tBOOH, *Xp*18 had a doubling time exceeding 300 min, contrasting with a 140-min doubling time for *X. campestris* pv. phaseoli (Fig. 6A). Cumene hydroperoxide (CHP) produced a similar growth-inhibitory effect on the mutant (data not shown). We then tested the sensitivity of the mutant to killing concentrations of tBOOH, H_2O_2, and MD. The quantitative results are shown in Fig. 6. The mutant was more sensitive (over 100-fold) to tBOOH killing than the wild type, but there were no differences in sensitivity to either H_2O_2 or MD killing. Qualitative analysis of mutant and wild-type levels of resistance to killing concentrations of other oxidants, such as diamide, *N*-ethylmaleimide, paraquat, and chemical mutagens (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and methyl methanesulfonate) were performed by the killing zone method. No differences in sensitivity to these agents were detected. Similarly, levels of resistance to nonoxidative stress killing agents, such as heat or pH, were identical for the two strains (data not shown).

Mutation in genes involved in stress protection can often lead to compensatory increases in the expression of other functionally related genes (3, 39). Thus, basal levels of enzymes involved in peroxide detoxification (AhpC, catalase, glutathione transferase, and peroxidase) or in oxidative stress protection (glucose-6-phosphate dehydrogenase and superoxide dismutase) were determined in *Xp*18. The results showed that levels of these enzymes were not significantly different between the wild type and the mutant (data not shown).

***ohr* expression in response to stress.** Characterization of the *ohr* mutant suggested that *ohr* played an important role in the protection of *X. campestris* pv. phaseoli from organic peroxide toxicity. In *Xanthomonas*, as in other bacteria, exposure to low levels of oxidants leads to a severalfold increase in expression of peroxide stress protective enzymes, such as catalase and AhpR (7, 31). This inducible response likely plays an important role in protecting the bacterium against stress. This knowledge prompted an investigation into the regulation of *ohr* in response to various oxidants. The steady-state levels of Ohr in response to oxidant treatments was determined by Western analysis. The results in Fig. 7A show a fourfold increase in the amount of Ohr after treatment with inducing concentrations of tBOOH. By contrast, only a marginal increase in Ohr was

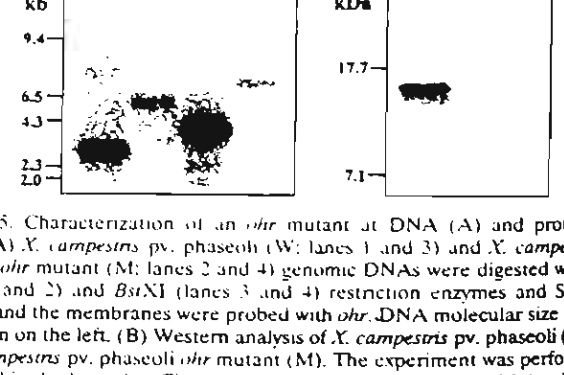

FIG. 5. Characterization of an *ohr* mutant at DNA (A) and protein (B) levels. (A) *X. campestris* pv. phaseoli (W; lanes 1 and 3) and *X. campestris* pv. phaseoli *ohr* mutant (M; lanes 2 and 4) genomic DNAs were digested with *Cla*I (lanes 1 and 2) and *Bst*XI (lanes 3 and 4) restriction enzymes and Southern blotted, and the membranes were probed with *ohr*. DNA molecular size markers are shown on the left. (B) Western analysis of *X. campestris* pv. phaseoli (W) and an *X. campestris* pv. phaseoli *ohr* mutant (M). The experiment was performed as described in the legend to Fig. 4A and Materials and Methods. Molecular mass markers are shown on the left.

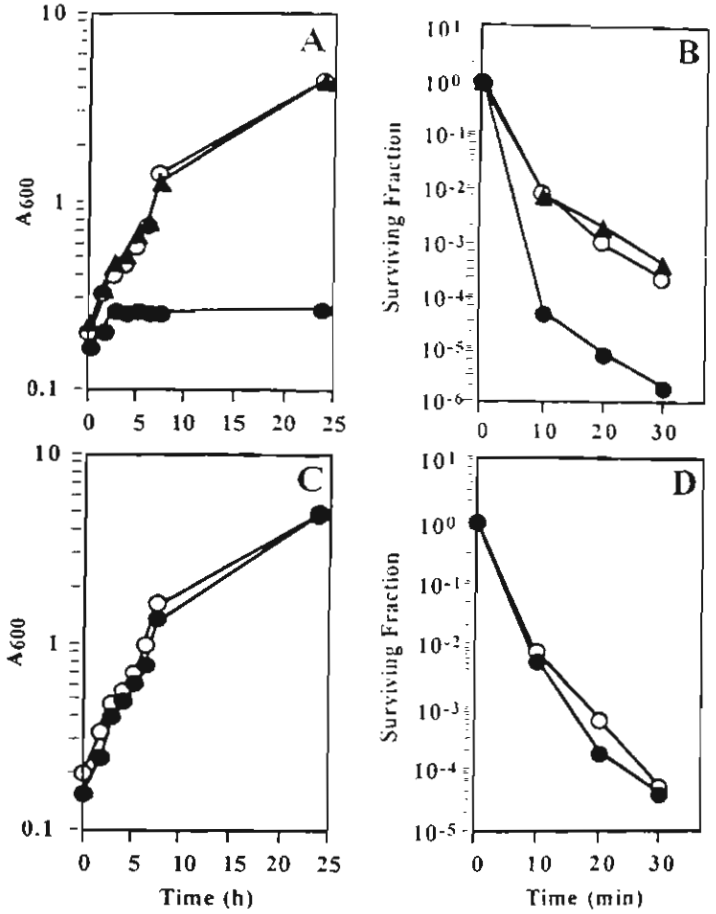

FIG. 6. Effects of peroxides on growth and survival of *X. campestris* pv. phaseoli and an *X. campestris* pv. phaseoli *ohr* mutant. The effects of low concentrations of either tBOOH (600 μM) (A) or H_2O_2 (200 μM) (C) on the growth, or of high concentrations of tBOOH (150 mM) (B) or H_2O_2 (30 mM) (D) on the survival, of *X. campestris* pv. phaseoli (○), *Xp18* (●), and *Xp18* containing pUFR-ohr (▲) were measured as described in Materials and Methods. The surviving fraction is defined as the number of living cells prior to a treatment divided by the number of living cells after a treatment. The experiments were done at least three times, and representative results are shown.

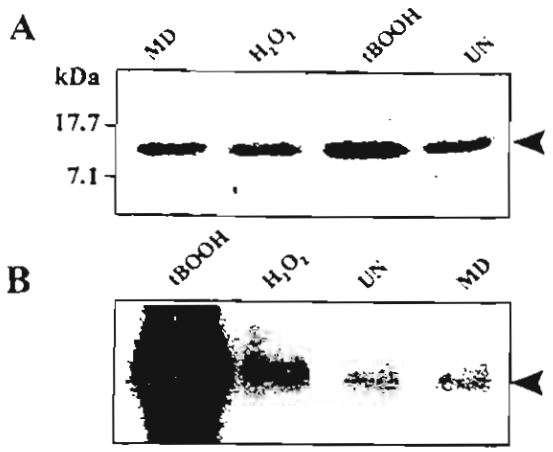

FIG. 7. Expression of *ohr* in response to various oxidants. (A) Western analysis of Ohr levels in *X. campestris* pv. phaseoli uninduced (UN) and induced with 100 μM MD, H_2O_2, or tBOOH was performed as described in Materials and Methods and in the legend to Fig. 4A. (B) Northern blot of total RNA isolated from *X. campestris* pv. phaseoli uninduced (UN) or induced with 100 μM of tBOOH, H_2O_2, or MD. Growth and induction conditions were the same as for panel A. Electrophoresis, blotting, hybridization, and washing were performed as previously described. The membrane was probed with radioactively labeled *ohr* probes. Ten micrograms of total RNA was loaded in each lane.

detected after exposure to H_2O_2 and none was detected after exposure to MD. To confirm that increased Ohr levels were due to increased *ohr* transcription, the amount of *ohr* mRNA was measured in a Northern experiment. Total RNA isolated from uninduced and tBOOH-, H_2O_2-, and MD-induced cultures was hybridized to *ohr* probes. The results are shown in Fig. 7B. Densitometer analysis of hybridization signals indicated that there was a greatly increased (15-fold) amount of *ohr* mRNA in response to tBOOH treatment. By contrast, there was only a minor increase (2.5-fold) after H_2O_2 treatments and no increase after MD treatment. The difference in *ohr* induction by tBOOH shown by protein and mRNA levels was likely due to the high stability of Ohr protein and its accumulation in the induced *X. campestris* pv. phaseoli.

Increased expression of *ohr* in *Xanthomonas* species. We have observed that in *Xanthomonas* increased expression of genes coding for oxidative stress protective enzymes can confer additional resistance to oxidants (24, 30). The levels of resistance of *X. campestris* pv. phaseoli harboring either pUFR-ohr or vector plasmids (9) to H_2O_2, tBOOH, and MD killing were determined. The results (Table 1) indicated that *X. campestris* pv. phaseoli and other *Xanthomonas* harboring the vector pUFR047 or pUFR-ohr had similar levels of resistance to killing concentrations of all three oxidants. Western analysis with an anti-Ohr antibody confirmed that lack of increased protection from tBOOH killing in strains harboring pUFR-ohr was not due to aberrant expression of *ohr* by the plasmid. In addition, the possibility that pUFR-ohr contained a defective copy of the gene was ruled out by the plasmid's ability to complement an *X. campestris* pv. phaseoli *ohr* tBOOH-hypersensitive mutant to a wild-type level of resistance (Table 2).

Heterologous expression of pUFR-ohr in various bacteria. Unexpectedly, increased expression of *ohr* from an expression vector did not confer additional tBOOH resistance on various *Xanthomonas* strains tested (Table 1). This effect of *ohr* expression on tBOOH resistance was tested in various other bacteria. The broad-host-range expression vector (9) containing *ohr* (pUFR-ohr) was electroporated into various bacteria. The tBOOH resistance of transformants harboring pUFR-ohr was then compared to that of cells harboring the

TABLE 1. Summary of levels of resistance of various bacteria to oxidants[a]

Species and strain	Zone of inhibition (mm)		
	CHP (200 mM)	tBOOH (500 mM)	H_2O_2 (500 mM)
X. campestris pv. phaseoli/pUFR047[b]	12	10	15
X. campestris pv. phaseoli/pUFR-ohr	12	11	15
X. campestris pv. campestris/pUFR047	12	15	12
X. campestris pv. campestris/pUFR-ohr	11	14	11
X. oryzae pv. oryzae/pUFR047	20	31	11
X. oryzae pv. oryzae/pUFR-ohr	20	32	11
E. coli TA4315/pUFR047	28	30	12
E. coli TA4315/pUFR-ohr	12	15	12
P. aeruginosa/pUFR047	14	34	12
P. aeruginosa/pUFR-ohr	11	27	12
A. tumefaciens/pUFR047	24	23	20
A. tumefaciens/pUFR-ohr	19	20	21

[a] Experiments were performed as described in Materials and Methods.
[b] pUFR047 is a broad-host-range expression vector (9).

TABLE 2. Summary of levels of resistance of wild-type *X. campestris* pv. phaseoli and an *ohr* mutant to oxidants[a]

Strain	Zone of inhibition (mm)			
	CHP (200 mM)	tBOOH (500 mM)	H_2O_2 (500 mM)	MD (500 mM)
X. campestris pv. phaseoli pUFR047[c]	12	10	15	10
*Xp*18 (*ohr* mutant) pUFR047	20	18	16	11
*Xp*18/pUFR-ohr	12	11	15	ND[b]
*Xp*18/pUFR-ahpCF	15	14	16	ND
*Xp*18/pahpC	17	16	15	ND
*Xp*18/pUFR-kat	20	19	12	ND

[a] Experiments were performed as described in Materials and Methods.
[b] ND, not done.
[c] pUFR047 is a broad-host-range expression vector (9).

vector alone by the killing zone method. The results shown in Table 1 clearly showed that expression of *ohr* in *E. coli* K-12, *P. aeruginosa*, and *Agrobacterium tumefaciens* conferred increased resistance to tBOOH killing.

Complementation analysis of the *ohr* mutant. We were interested in seeing whether increased expression of the genes involved in peroxide stress protection (those for catalase [*kat*] and alkyl hydroperoxide reductase [*ahpCF*]) or *ohr* could compensate for the mutant's hypersensitivity to tBOOH killing. For this purpose, plasmids containing *katX* (pUFR-kat [30]), *ahp* C (pahpC [24]), combined *ahp* C and F (pUFR-ahpCF), or *ohr* (pUFR-ohr) were electroporated into mutant *Xp*18 and levels of resistance to alkyl hydroperoxides were qualitatively determined by the killing zone method. The results are shown in Table 2. As expected, pUFR-ohr, complemented the mutant's increased sensitivity to alkyl hydroperoxide. In addition, increased expression of *ahpC* alone partially compensated for hypersensitivity to tBOOH killing, but the resistance achieved was still below that of the parental *X. campestris* pv. phaseoli strain. On the other hand, *Xp*18 harboring pahpCF showed resistance to tBOOH killing similar to that of wild-type *X. campestris* pv. phaseoli. Increased expression of *katX* provided no protective effect against tBOOH killing.

DISCUSSION

From *X. campestris* pv. phaseoli we have isolated a new gene (*ohr*) that is involved in organic hydroperoxide protection. An *ohr* mutant showed no significant growth defects under normal conditions, indicating that the gene was not essential. Important evidence from analysis of its mutant and heterologous expression suggests that Ohr plays a novel role in organic hydroperoxide metabolism. First, in many bacteria, the mutation of genes involved in oxidative stress protection always results in increased sensitivity to oxidative stress (3, 11, 12). Similarly, *ohr* mutants showed increased sensitivity to organic hydroperoxides. It is worth noting that the sensitivity of the *ohr* mutant was specific to organic hydroperoxides, unlike the mutants of other known peroxide protective (11, 12) and nonspecific DNA binding (29) genes, which often conferred increased sensitivity to several oxidants. The ability of *ohr* to complement the organic hydroperoxide hypersensitivity of the *Xp*18 mutant confirmed that the phenotype was due to a mutation in *ohr*. The biochemical action of Ohr is not known; its ability to increase tBOOH resistance in several unrelated bacterial species suggested that it might function directly in detoxification of organic hydroperoxides. However, we have not ruled out the possibility that Ohr is involved in the transport processes of organic molecules.

The highest degree of homology (63%) was found between Ohr and a protein of unknown function from *Acinetobacter* sp. (36) which is known to have metabolic pathways for *n*-alkane oxidation. In this bacterium, the first step in the oxidation pathway involves an attack by dioxygenase, which leads to the formation of *n*-alkyl hydroperoxides. These unstable intermediates are subsequently metabolized to various end products (13, 27). Thus, Ohr-like proteins in these bacteria could be involved either directly in the alkane metabolism pathway or indirectly, by providing protection against the toxic effects of alkyl hydroperoxides in a reaction similar to that of catalysis by AhpR. Some degree of homology (31%) with an unknown ORF from *Mycoplasma* was detected. Analysis of *M. genitalium* and *Mycoplasma pneumoniae* genome sequences surprisingly revealed a lack of known genes involved in peroxide metabolism (14, 20). This raises the question of how these bacteria protect themselves from peroxide toxicity. It is feasible that an Ohr-like protein in these bacteria could functionally substitute for AhpR and peroxidases in dealing with organic peroxides. The putative Ohr-like proteins had similar lengths and amino acid sequence motifs. This implied that they belonged to a new family of proteins involved in organic hydroperoxide metabolism.

Ohr or Ohr-like proteins as well as AhpR were found in *Xanthomonas*, *B. subtilis*, and *E. coli*. Both gene products seem to be involved in organic hydroperoxide detoxification. This notion was supported by the observation that expression of combined *ahpC* and *ahpF* complemented the tBOOH hypersensitivity of the *Xanthomonas ohr* mutant to wild-type levels. Similarly, *ohr* complemented the phenotype of an *ahpC-ahpF E. coli* mutant. Thus, there appeared to be a functional complementation between Ohr and AhpC.

Examination of the regulation of *ohr* expression in *Xanthomonas* indicated that there are at least two regulatory systems for organic hydroperoxide-inducible genes. We have shown that in one system in *Xanthomonas*, genes under *oxyR* regulation (i.e., the catalase gene and *ahpC*) can be induced by treatment with low concentrations of H_2O_2, organic hydroperoxides, or superoxide generators (7, 25, 31). The responses of *ohr* to peroxides and superoxide generators clearly differ, since they can be highly induced only by organic peroxides and only weakly by H_2O_2. In fact, the weak induction of *ohr* by H_2O_2 may be an indirect result of H_2O_2 reaction with membrane lipids that resulted in organic hydroperoxide production (22, 44). Thus, regulation of *ohr* responds to a narrower but overlapping range of inducing stresses than does the global peroxide regulator OxyR (8, 41).

Another difference between Ohr and AhpC could be related to cellular localization. Ohr has 31% homology with OsmC, a well-characterized periplasmic protein (18), while AhpC is a cytoplasmic protein. This might explain the unexpected observation that increased *ohr* expression in wild-type *Xanthomonas* did not confer resistance to higher levels of tBOOH. In wild-type *Xanthomonas*, transport of Ohr to the periplasmic space could be a rate-limiting step. If so, increased expression of *ohr* from an expression vector might not result in increased tBOOH resistance. We do not know whether OsmC is involved in organic peroxide metabolism. We are investigating this possibility.

The absence of Ohr-like protein sequences from many bacterial genome sequences in the database (e.g., those of *Haemophilus influenzae* and *Helicobacter pylori*) suggests that Ohr may perform special tasks in organic hydroperoxide detoxification for a subset of bacterial species. Indeed, these observa-

tions raise the possibility that Ohr and AhpC in *Xanthomonas* may have similar enzymatic functions but differ in regulation and cellular localization. The differences imply disparate physiological roles in spite of similar biochemical actions in organic hydroperoxide detoxification. Until the physiological substrates of Ohr and AhpC are known, a more definitive evaluation of their roles in organic peroxide detoxification cannot be made.

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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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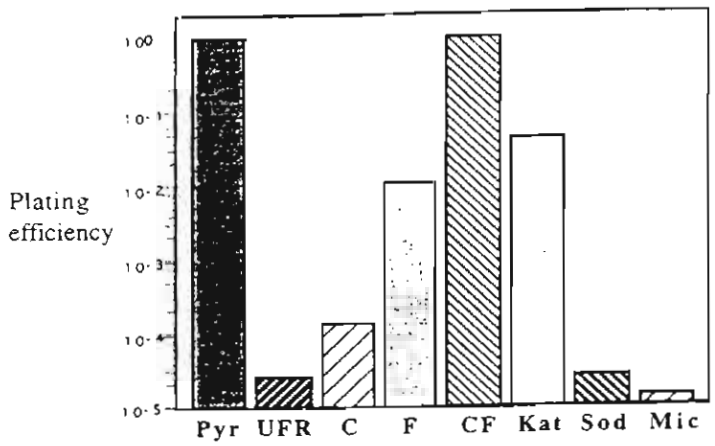

FIG. 1. Plating efficiency of an *oxyR* mutant harboring various expression plasmids containing genes involved in oxidative stress response or conditions that affected oxidative stress. In all experiments, a mid-log-phase *X. campestris* pv. phaseoli *oxyR* mutant grown in SB was serially diluted and 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 [35] in pUFR047); CF, pahpCF (*ahpC* and *ahpF* [15] in pUFR047); Kat, pkat (21); Sod, psod (*Xanthomonas sod* [28] coding region in pUFR047).

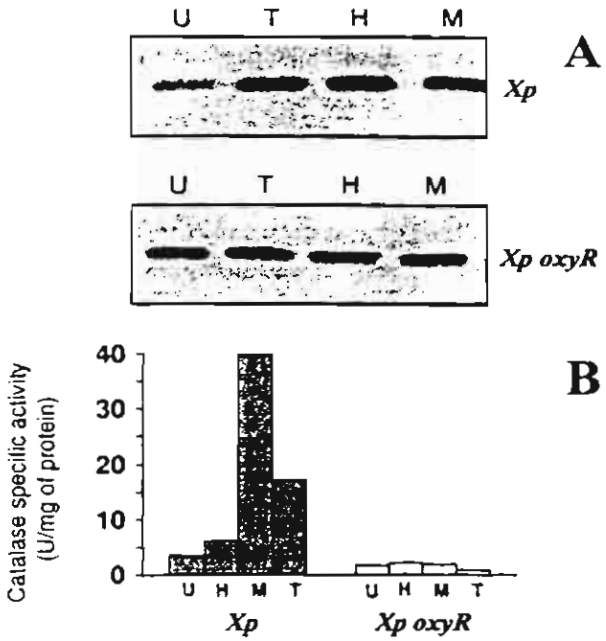

FIG. 2. Levels of AhpC and catalase activities in response to various oxidants in *X. campestris* pv. phaseoli (*Xp*) and an *X. campestris* pv. phaseoli *oxyR* mutant (*Xp oxyR*). Mid-log-phase *X. campestris* pv. phaseoli or an *X. campestris* pv. phaseoli *oxyR* mutant grown in SB was induced with 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

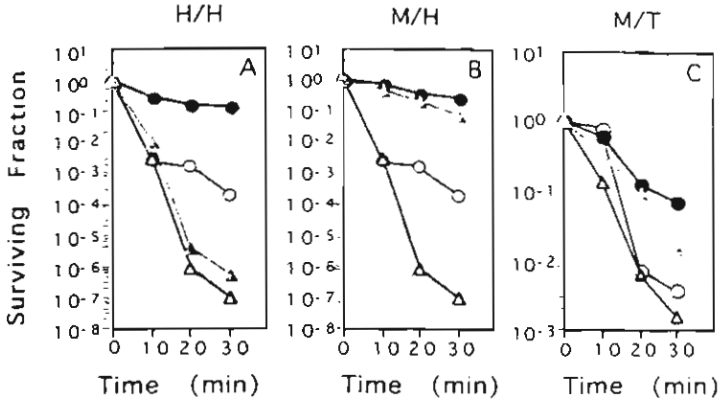

FIG. 3. Adaptive and cross-protective responses against peroxide killing in *X. campestris* pv. phaseoli and an *X. campestris* pv. phaseoli *oxyR* mutant. Log-phase uninduced *X. campestris* pv. phaseoli (○) and an *X. campestris* pv. phaseoli *oxyR* mutant (△) and oxidant-induced (30-min treatment 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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Evaluation of the role hydroxyl radicals and iron play in hydrogen peroxide killing of *Xanthomonas campestris* pv. *phaseoli*

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Abstract

Hydroxyl radical, a product of H_2O_2 metabolism in the Fenton reaction, is a major reactive oxygen species involved in H_2O_2 killing of *Xanthomonas campestris* pv. *phaseoli* (*Xp*). Compounds which absorbed hydroxyl radicals protected *Xanthomonas* from H_2O_2 killing, but not from killing by a superoxide generator or an organic peroxide. Addition of iron potentiated H_2O_2 killing. On the other hand, pre-treatment of *Xp* with an iron chelator showed no protective effects and H_2O_2 killing was actually enhanced. Unexpectedly, resuspension of *Xp* in either water or phosphate buffer induced high-level resistance to H_2O_2 killing. The unknown protection mechanism was independent of new protein synthesis.

Keywords: Hydroxyl radical; Iron; Peroxide killing

1. Introduction

H_2O_2 killing has been investigated in many enteric bacteria [1–3]. The ability of H_2O_2 to diffuse across the cell membrane contributes to its toxicity. H_2O_2 itself has a relatively low reactivity when compared to the hydroxyl radical (·OH), a highly reactive but short-lived product of its reaction with metal ions in the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$)[1]. ·OH can react with all biological macromolecules within its diffusion limit, and is known to cause mutagenesis and cell death [1,2]. In biological systems, there is no enzyme that specifically destroys ·OH. The most effective defense against ·OH-induced damage is to reduce the intracellular concentration of components in the Fenton reaction such as H_2O_2 and iron. This can be achieved either by enzymes which directly break down H_2O_2 such as catalases and peroxidases [3] or by sequestration of transition metals (i.e. Fe^{2+}) and repression of iron uptake [4].

Xanthomonas campestris pv. *phaseoli* (*Xp*) is a bacterial phytopathogen. During plant-microbe interactions, bacteria such as *Xanthomonas* are exposed to plant-generated H_2O_2 which is involved in signal transduction of the plant defense response as well as growth inhibition and killing of bacteria [5]. In addition, normal aerobic growth is known to produce significant quantities of H_2O_2 [6]. Bacteria have evolved a highly elaborate oxidative stress defense system [3]. We have observed that many as-

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pects of the *Xanthomonas* oxidative stress response differ from other bacteria, particularly the role of the peroxide global regulator, OxyR [7], and peroxide-scavenging enzymes [8,9]. Little is known regarding the mechanism of peroxide killing in bacterial phytopathogens. The ability to defend against peroxide killing is likely to be an important factor in determining the outcome of disease progression and development. This prompted us to investigate the effects of 'OH and iron on peroxide killing of *Xanthomonas*.

2. Materials and methods

2.1. Bacterial growth and experimental conditions

Xp from our laboratory collection [7] was grown aerobically in SB medium [7] at 28°C. Cells from the exponential phase (OD_{600} 0.5) were used in all experiments. Unless stated, indicated concentrations of oxidants were added to a 1-ml aliquot of *Xp* in SB [7]. At timed intervals, samples were removed and bacterial cells pelleted and washed once with fresh SB medium before being serially diluted in SB and plated at appropriate dilutions. The ability of 'OH scavengers to protect *Xp* from H_2O_2 killing was investigated by adding (final concentrations) either 1 M glycerol or 0.4 M dimethyl sulfoxide (DMSO) 10 min prior to addition of the killing concentrations of oxidants. Excess iron condition was achieved by adding ferrous chloride (5 μM final concentration) to SB medium prior to peroxide treatment. The effects of water and 50 mM phosphate buffer pH 7.5 on H_2O_2 killing were determined as described above except that *Xp* cells in SB medium were pelleted and washed once and resuspended in 1 ml of either water or phosphate buffer before being treated with H_2O_2. An iron chelator, 2,2′-dipyridyl, was added to *Xp* resuspended in water to give a final concentration of 1 mM 10 min before H_2O_2 treatment.

The surviving fraction is defined as the number of viable colonies after treatment divided by the number of colonies prior to the treatment [9]. All experiments were performed independently three times and representative results are shown.

3. Results and discussion

3.1. The effects of 'OH absorbers on peroxide killing

The role of 'OH radicals in peroxide killing of *Xp* was examined by adding various 'OH scavengers

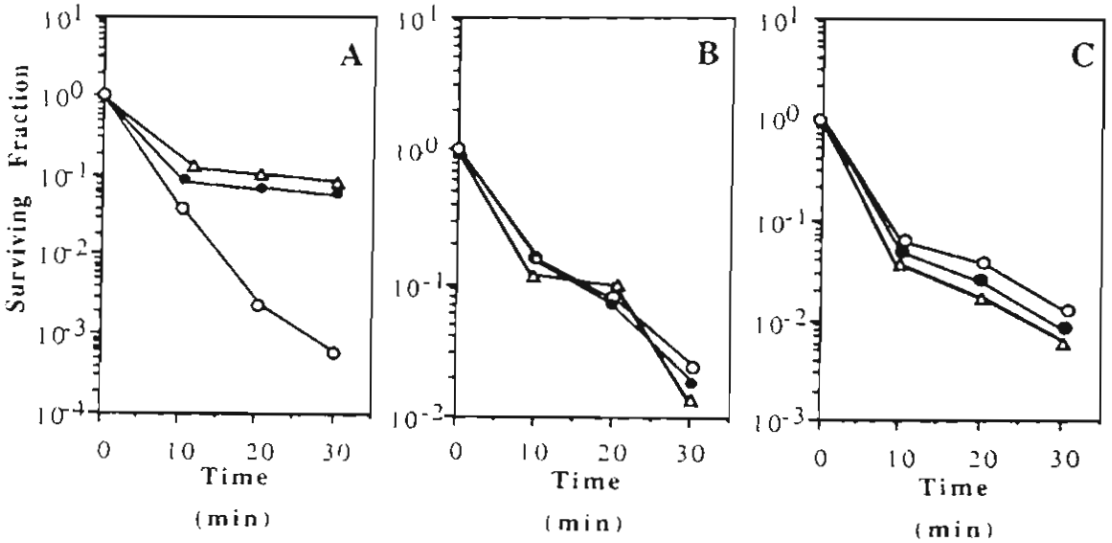

Fig. 1. The effects of 'OH radical scavengers on H_2O_2 killing of *Xp*. *Xp* was pre-treated with 'OH radical scavengers (1 M glycerol ● or 0.4 M DMSO △) prior to addition of killing concentrations of 10 mM H_2O_2 (A), 20 mM tBOOH (B) and 200 mM MD (C). The results were compared to untreated samples (○). Surviving fraction is as defined in Section 2.

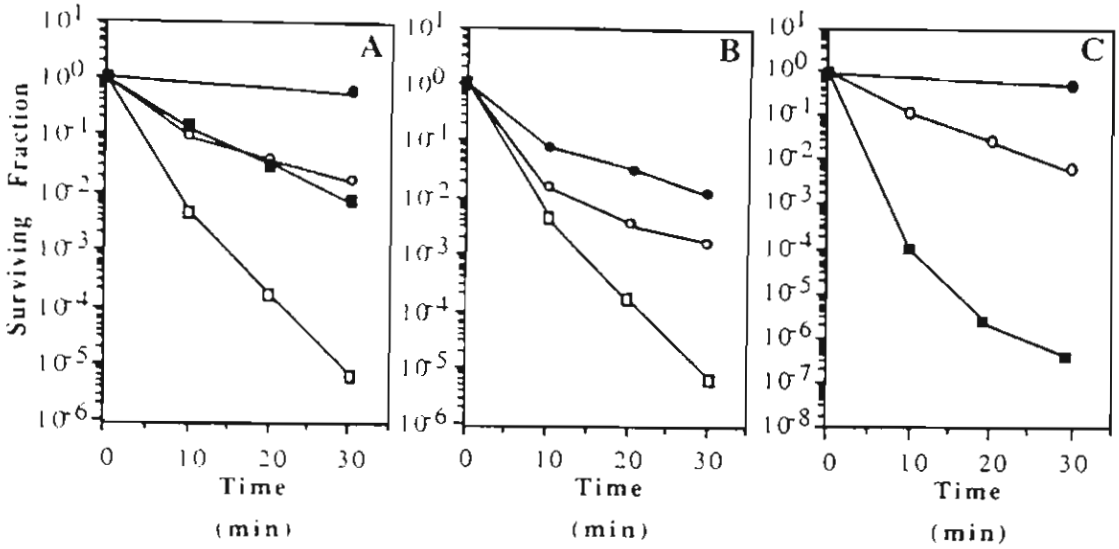

Fig. 2. The effects of excess ferrous ions or iron-depleted conditions on H_2O_2 killing. *Xp* growth, H_2O_2 killing conditions and viable cell count were performed as described in Section 2 and in Fig. 1, except in A where *Xp* was resuspended in either water (■) or water plus 5 μM ferrous chloride (□) 10 min prior to addition of H_2O_2. ○, *Xp* resuspended in H_2O plus 5 μM ferrous chloride or H_2O (●) without treatment with H_2O_2. B. *Xp* was resuspended in water plus 5 μM ferrous chloride (□), water plus 5 μM ferrous chloride and plus 1 M glycerol (○) and water plus 5 μM ferrous chloride and plus 1 mM 2,2'-dipyridyl (●) 10 min prior to addition of H_2O_2. C. *Xp* was resuspended in either water (○) or water and 1 mM 2,2'-dipyridyl (■) 10 min prior to addition of H_2O_2. ●, Untreated samples in either water or water plus 1 mM 2,2'-dipyridyl. Surviving fraction is as defined in Section 2.

(e.g. glycerol [10] or DMSO [11]) prior to the addition of killing concentrations of H_2O_2 or *tert*-butyl hydroperoxide (tBOOH) or menadione (MD). The results are shown in Fig. 1. *Xp* pre-treated with either DMSO or glycerol was 100-fold more resistant to H_2O_2 killing than untreated cells. Nevertheless, these $^{\cdot}OH$ scavengers did not completely block peroxide killing. The results indicate that $^{\cdot}OH$ radicals are the major, but not the only, reactive oxygen species responsible for H_2O_2 killing (Fig. 1). This is consistent with observations in other bacteria [1]. It is feasible that these $^{\cdot}OH$ scavengers could rapidly induce peroxide-scavenging enzymes which subsequently conferred protection against H_2O_2 killing. This assumption was tested and the results obtained ruled out the possibility. Both DMSO and glycerol at concentrations which conferred protection against H_2O_2 killing did not induce expression of peroxide protective enzymes (alkyl hydroperoxide reductase (AhpC), catalases or peroxidases) (data not shown). DMSO and glycerol did not protect against organic peroxide (tBOOH) killing. This could be due to their inability to absorb organic radicals generated from organic peroxides. Surprisingly, both $^{\cdot}OH$ scavengers did not confer protection against killing by a superoxide generator (MD) (Fig. 1B,C). MD killing of bacteria has been proposed to occur via several mechanisms involving the breakdown of intracellularly generated superoxide anions to H_2O_2 [12], reduction of intracellular iron by superoxide anions, or leaching of iron from iron-containing proteins [13]. These reactions generate important components of the Fenton reaction which lead to the production of $^{\cdot}OH$ radicals. The radicals then react with DNA and other macromolecules resulting in cell death. Additional evidence comes from the analysis of peroxide- and superoxide-scavenging enzymes and their regulatory mutants, both of which show increased sensitivity towards MD killing [3]. Nonetheless, our data suggest that MD killing of *Xp* could be mediated via unknown mechanisms in addition to the mechanism involving $^{\cdot}OH$ production proposed above. Moreover, high catalase activity in *Xanthomonas* confers protection against H_2O_2 but not against MD killing [8,14], suggesting that MD killing is not mediated via H_2O_2 production.

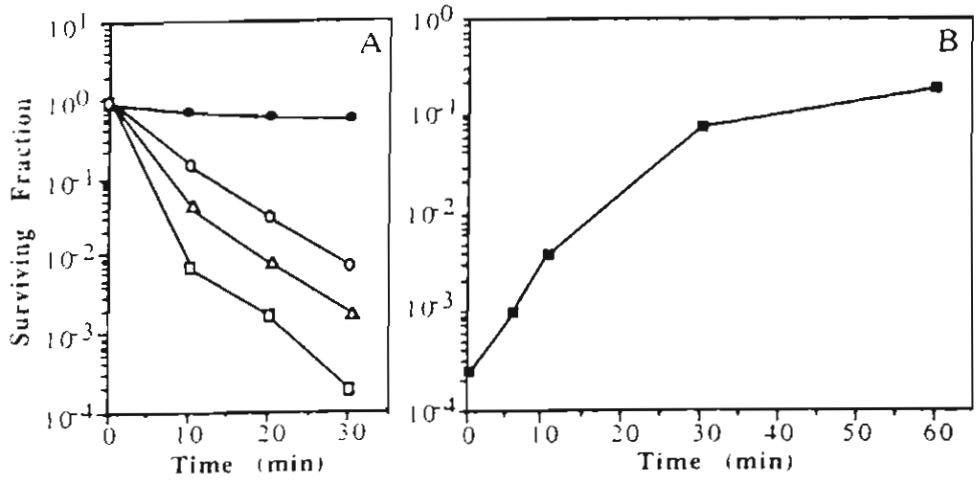

Fig. 3. Influence of SB, phosphate buffer and water on H_2O_2 killing of *Xp*. Bacterial growth was determined as described in Section 2. A: Aliquots of *Xp* were washed once and resuspended either in SB medium (○), 50 mM phosphate buffer (pH 7.5) (△) or distilled water (□) for 10 min prior to addition of 10 mM H_2O_2 or representative data from untreated samples of *Xp* in SB, phosphate or H_2O (●). B: *Xp* was washed and resuspended in water. At the times shown, samples were removed and treated with 10 mM H_2O_2. Surviving fraction is as defined in Section 2.

3.2. *Ferrous ion potentiates H_2O_2 killing*

Transition metals, especially ferrous ions, are important components in hydroxyl radical generation from H_2O_2 in the Fenton reaction. The effect of increased concentrations of ferrous ions on H_2O_2 killing of *Xp* was investigated. The results are shown in Fig. 2A,B. As expected, addition of 5 μM of a ferrous salt enhanced H_2O_2 killing. This effect can be partially reversed by addition of 1 mM 2,2′-dipyridyl or 1 M glycerol (Fig. 2B). This suggests that the enhancement of H_2O_2 killing by iron is due to increased hydroxyl radical production. However, we also observed that addition of micromolar concentrations of a ferrous salt had significant toxicity on *Xp*. The treatment resulted in a small but significant reduction in viable cell count in non-H_2O_2-treated samples (Fig. 2A,B). This effect is probably due to increased intracellular concentrations of ferrous ions reacting with H_2O_2 generated from normal metabolism [6] resulting in increased production of $^{\cdot}OH$ radicals which induced cell death. The data support the important role iron plays in the H_2O_2 killing of bacteria.

3.3. *The effects of buffers and an iron chelator on H_2O_2 killing*

A reduction of iron availability is expected to decrease the potency of H_2O_2 killing of cells by limiting production of $^{\cdot}OH$ radicals. This effect on H_2O_2 killing was tested in *Xp*. In SB medium, 1 mM 2,2′-dipyridyl (an iron chelator) had no effects on H_2O_2 killing (data not shown). Several other concentrations of 2,2′-dipyridyl were also tested and gave similar results (data not shown). This is probably due to the high iron content of complex SB medium. Experiments were repeated except that *Xp* cells grown in SB were washed once and resuspended in either water or 50 mM phosphate buffer pH 7.5. Unexpectedly, resuspension of *Xp* in water or phosphate buffer for 10 min prior to addition of killing concentrations of H_2O_2 induced high levels of protection against H_2O_2 when compared to cells resuspended in SB medium (Fig. 3A). This effect could arise from carbon starvation-induced protection against H_2O_2 killing [15]. This hypothesis was tested by repeating experiments in the presence of a protein synthesis inhibitor (150 μg ml^{-1} chloramphenicol) to inhibit gene expression. The results show that chloramphenicol had no effect on phosphate buffer- or water-induced resistance to H_2O_2 killing, suggesting

that induced gene expression was not involved (data not shown). It is possible that phosphate buffer and/or water could induce alterations in *Xp* membrane components that resulted in less H_2O_2 entering the cell. If this is the case then the observation could be a time-dependent event. The data in Fig. 3B show that increased resistance to H_2O_2 killing correlates with the length of time *Xp* was resuspended in water. Alternatively, components of SB medium (i.e. yeast extract, peptone) could react with H_2O_2 and generate more reactive oxygen species which potentiate the killing effects of H_2O_2. The mechanism of resistance is being investigated.

Additional experiments were performed to re-examine the effects of an iron chelator on H_2O_2 killing of *Xp* resuspended in water. The results are shown in Fig. 2C. Unexpectedly, treatment of *Xp* with 1 mM 2,2′-dipyridyl enhanced H_2O_2 killing. This is a contrast to a previous finding where 2,2′-dipyridyl protected *Escherichia coli* from H_2O_2 killing [16]. A possible explanation is that 2,2′-dipyridyl, a membrane-permeable iron chelator, could pass through the cell membrane and chelate intracellular iron. This assumption is supported by findings in Fig. 2B that the addition of excess iron could reverse the enhanced H_2O_2 killing effect induced by 2,2′-dipyridyl treatment. While addition of excess iron after treating cells with the iron chelator did not produce protective effects on H_2O_2 killing, the data suggest that enhanced sensitivity to H_2O_2 killing by 2,2′-dipyridyl treatment may result from inactivation of enzymes and/or regulatory proteins involved in oxidative stress protection (i.e. catalases, SoxRS [3]) which required iron as a co-factor. These enzymes in *Xp* could be more sensitive to inactivation by iron depletion conditions than *E. coli* counterparts and thus lead to increased H_2O_2 sensitivity. However, our preliminary investigation showed that total catalase activities in 2,2′-dipyridyl-treated and untreated cells were similar. The results implied that catalase inactivation was not the major mechanism responsible for H_2O_2 hypersensitivity in 2,2′-dipyridyl-treated cells.

In summary, we have shown that $^{\cdot}OH$ is the major reactive oxygen species responsible for the killing effects of H_2O_2. Unexpectedly, killing effects of MD appear not to be mediated via $^{\cdot}OH$ radical production and possibly involve a novel unknown mechanism in *Xp*. Iron concentrations appear to play crucial roles in enhancing H_2O_2 killing. This suggests that at the site of infection, concentrations of transition metals, plant-generated H_2O_2 and compounds produced by plants which can absorb $^{\cdot}OH$ could influence pathogen growth and survival, hence affecting the outcome of disease and its progression.

Acknowledgments

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Modulation of peroxide stress response by thiol reagents and the role of a redox sensor-transcription regulator, OxyR in mediating the response in *Xanthomonas*

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Abstract

Pretreatment of *Xanthomonas campestris* pv. *phaseoli* with low inducing concentrations of thiol reagents such as N-ethylmaleimide (NEM) and diamide induced resistance to H_2O_2 killing. In addition, these compounds were moderate inducers of peroxide detoxification enzymes such as catalase and alkyl hydroperoxide reductase. For both cases, thiol reagent induced responses required a functional redox sensor/transcription activator *oxyR* and were absent in an *oxyR* mutant. By contrast, NEM pretreatment enhanced the killing effects of organic peroxide. The observed *Xanthomonas* physiological responses to thiol reagent pretreatment, and subsequent challenge with peroxide stress, differed from other bacteria. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Induced protection; *oxyR*; Catalase; H_2O_2; Organic peroxide

1. Introduction

Xanthomonas is a soil bacterium and an important plant bacterial pathogen. In the environment, soil bacteria are exposed to numerous man made chemicals which are either deliberately released as pesticides or accidentally released in the form of industrial pollutants. Some of these chemicals can modify bacterial responses to environmental conditions either by introducing mutations or by altering physiological responses. These changes could lead to abnormal interactions between pathogens with their hosts, thus altering disease development and progression.

Oxidative stress is an important component of active plant defense against microbial invasion [1]. We have shown that *Xanthomonas* possesses inducible adaptive responses to oxidative stress killing [2,3]. Pre-exposure of the bacteria to low concentrations of an oxidant lead to induction of oxidative stress protective enzymes and protection from a subsequent exposure to killing concentrations of the oxidant [3]. In *Xanthomonas*, most oxidant inducible responses are mediated by a redox sensor and transcription regulator, OxyR [4]. OxyR is essential for

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[illegible] is illustrated in the [illegible] respective [illegible] oxidative [illegible]

[illegible] N-ethylmaleimide (NEM) and [illegible] react with sulfhydryl [illegible] found in peptides [illegible] cellular thiols. Depletion [illegible] affect the redox balance of [illegible] to cope with oxidative [illegible] pretreatment of *Xanthomonas* [illegible] affects peroxide stress response [illegible] alterations in peroxide [illegible] affect interactions of *Xanthomonas* [illegible] plant. Consequently, the effects [illegible] in peroxide killings and on peroxide [illegible] were investigated.

2. Materials and methods

2.1. Bacterial strains and oxidant treatments

Xanthomonas oryzae pv. *oryzae* (*Xoo*) or an *oxyR* mutant of *X. oryzae* pv. *oryzicola* (*Xoc*) were grown aerobically in SB medium at 28 °C. Log phase cells were used in all experiments. NEM and diamide were used at indicated concentrations which have been determined experimentally to induce good responses without severely [illegible]. Pretreatment of various *Xo* cultures with these oxidants and subsequent peroxide [illegible] at indicated peroxide concentrations was essentially described for wild-type [3] and *oxyR* mutant [4]. For reproducibility of results, cultures from a similar [illegible] with the number of colonies that survived peroxide [illegible] were counted after 48 h incubation. NEM [illegible] can be defined as the number of cells that survived the treatment divided by the number of cells left untreated by the treatment.

2.2. Preparation of cell extracts for immunoblot analyses

[illegible] were separated by resuspending [illegible] 50 mM phosphate buffer, pH 7.0, [illegible] 50 mM sodium fluoride (NaF) [illegible] sonicated intermittently on ice until [illegible] lysates were centrifuged at 4 °C. Clear lysates were used for catalase assay [6] or in Western immunoblot experiments performed as previously described against anti-*E. coli* AhpC [7] or anti-*Xanthomonas* Ohr [8] antibodies. Antibody reactions were developed using an alkaline phosphatase antibody detection kit from Promega.

All experiments were repeated three times and representative results are shown.

3. Results

3.1. NEM induced protection against H_2O_2 killing

NEM pretreatment of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) induces protection against H_2O_2 killing [5]. Recently, we have discovered significant differences between *Xoo* and *Xp* in many aspects of oxidative stress response. Hence, the effect of NEM pretreatment on H_2O_2 killing in *Xp* was investigated. The results in Fig. 1A show that NEM pretreatment induced high level resistance (100-fold more resistant than untreated cells) to H_2O_2 killing.

Analysis of an *Xp oxyR* mutant reveals that a functional *oxyR* is required for peroxide inducible adaptive response [4]. This is similar to observations in other bacteria. Nonetheless, *Xp* also possesses oxidant inducible resistance to peroxide killing that is dependent of *oxyR* [4]. We were curious if OxyR mediated NEM inducible protection against H_2O_2 killing. The NEM induction experiment was repeated using an *Xp oxyR* mutant [4]. The data in Fig. 1B clearly show that NEM did not induce resistance to H_2O_2 killing in an *Xp oxyR* mutant. On the contrary, NEM pretreatment slightly enhanced H_2O_2 killing of the mutant. Diamide pretreatment could also induce protection to H_2O_2 killing in *Xp* in an *oxyR* dependent fashion (Fig. 1A and B), although the induction only occurred at a diamide concentration 10-fold higher than that required for NEM induced resistance.

3.2. NEM induction of peroxide detoxification enzymes

To better understand the mechanism involved in NEM induced resistance against H_2O_2 killing, the effects of NEM on various peroxide metabolizing

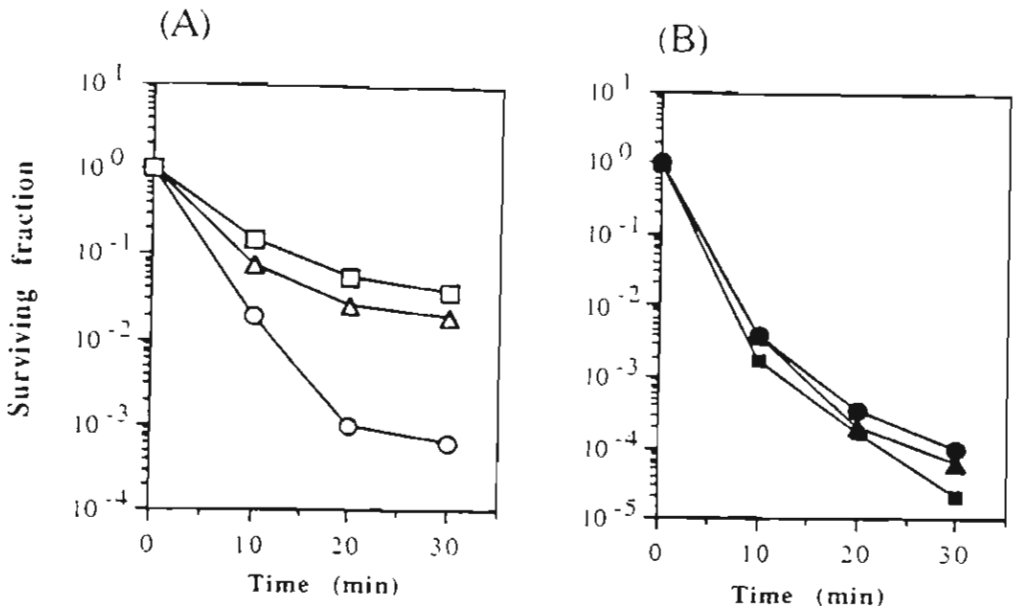

Fig. 1. The effects of NEM and diamide pretreatments on H_2O_2 killing in *Xp* and *Xp oxyR*. Uninduced *Xp* (A, □) or *Xp oxyR* (B, ●) grown in SB or similarly grown cultures but induced with 100 μM NEM in *Xp* (A, ○), *Xp oxyR* (B, ■) or 1 mM diamide in *Xp* (A, △), *Xp oxyR* (B, ▲) were treated with 20 mM H_2O_2 for *Xp* and 10 mM H_2O_2 for *Xp oxyR*. Samples were removed at indicated times and washed once with SB medium before plating on SB plates. Surviving fractions were determined after 48 h incubation and calculated according to Section 2.

enzymes were investigated. Catalase is an important enzyme for protection against H_2O_2 toxicity. In *Xoo*, a low concentration of NEM moderately induces catalase [9]. Similarly, results in Fig. 2A show that in *Xp* low concentrations of NEM (50–100 μM) moderately induce (2-fold) catalase. At higher NEM inducing concentrations (300 μM or greater) no increase in the magnitude of catalase induction was observed. Additionally, *Xp* rapidly lost viability (data not shown). Diamide pretreatment produced similar catalase induction at higher concentrations than those required for NEM. By contrast, there was a significant loss of viability after exposure of *Xp* to 5 mM diamide. In *Xp*, oxidant induction of catalase is mediated by OxyR [4]. NEM and diamide induction of catalase also require functional *oxyR* (Fig. 2A and B).

Alkyl hydroperoxide reductase (AhpR) is another peroxide metabolizing enzyme [7]. Purified AhpR can use both H_2O_2 and organic peroxide as substrates [10]. The enzyme consists of two subunits, a catalytic 22 kDa C subunit (AhpC) and a reductase 52 kDa F subunit. In *Xp*, *ahpC* is regulated by OxyR. High levels of *ahpC* expression confer increased resistance to organic peroxide killing [11]. The effects of inducing concentrations of NEM on *ahpC* expression were investigated by Western immuno blot analysis using an anti-*E. coli* AhpC antibody [7]. The results in Fig. 2C show that in wild-type *Xp*, a several-fold increase in the amount of AhpC was detected after NEM and diamide pretreatments. These increases were abolished in an *oxyR* mutant. We have recently shown that *Xp* possesses a novel gene, *ohr*, involved in protecting *Xp* from organic peroxide toxicity [8]. Analysis of *ohr* expression by Western immuno blot using an anti-Ohr antibody [8] showed, at all concentrations tested, that NEM pretreatment had no effect on *ohr* expression (Fig. 2D).

3.3. NEM pretreatment enhanced organic peroxide killing

In most bacteria, the *oxyR* dependent oxidant induced H_2O_2 resistance often results in concomitant protection against organic peroxide killing. Many aspects of inducible resistance to oxidant killing in *Xp* differ from other bacteria [2,4,9]. For example, pretreatment with low concentrations of a superox-

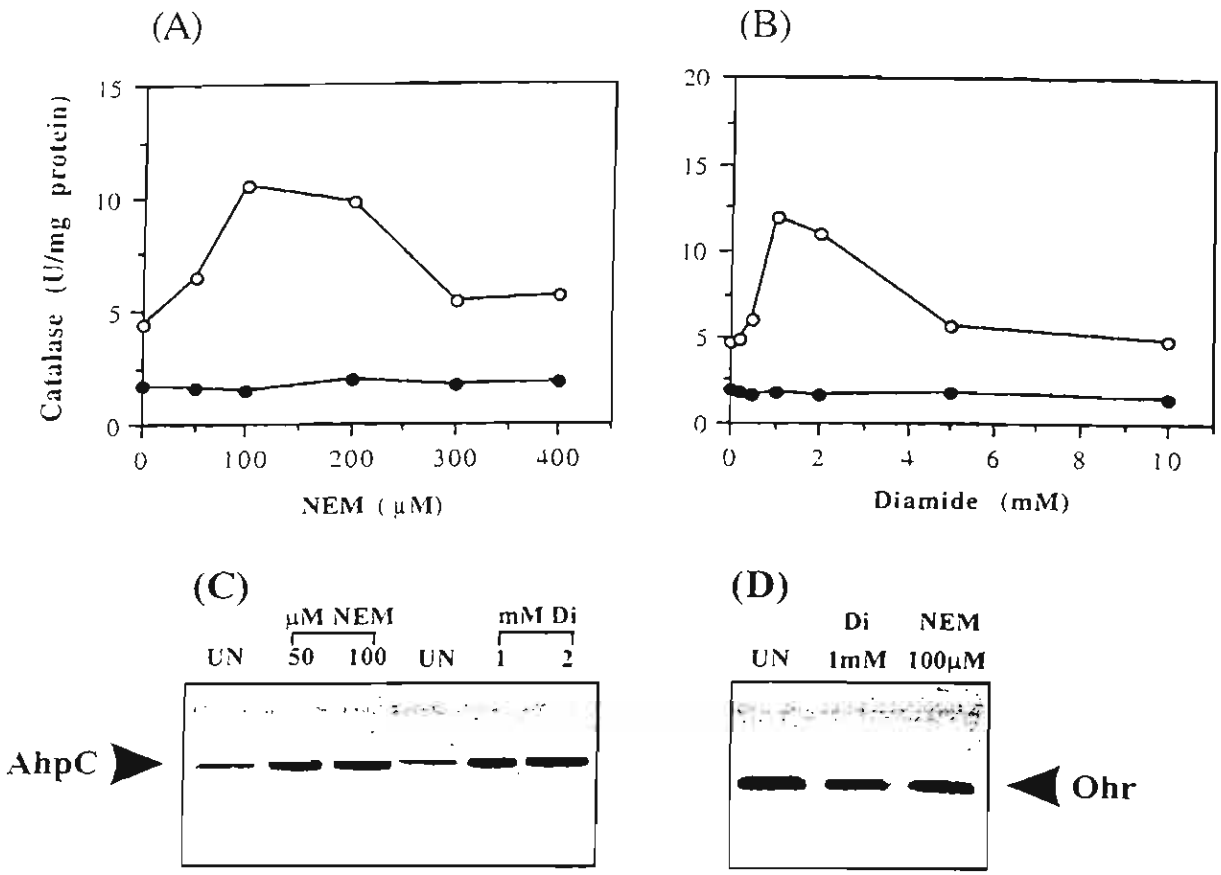

Fig. 2. The effects of either NEM or diamide pretreatment on catalase, AhpC and Ohr in *Xp* and *Xp oxyR*. *Xp* (○) or *Xp oxyR* (●) wer grown aerobically in SB medium. Indicated concentrations of either NEM (A) or diamide (B) were added to these cultures for 30 mi Sample collection, lysate preparation and catalase enzyme assays were done as described in Section 2. For Western immuno blot analys of AhpC or Ohr, 50 μg of total protein from lysates of *Xp* uninduced, NEM induced at 50, 100 μM, diamide induced at 1, 2 mM fc AhpC analysis and NEM induced at 100 μM and diamide induced at 1 mM for Ohr analysis were loaded, separated using PAGE ge and subsequently transferred to PVDF membranes. Immunodetection was carried out using an anti-AhpC antibody (C) [5] or an anti-Oh antibody (D) [8] with subsequent detection using a commercial alkaline phosphatase kit

ide generator, menadione, induces protection against both H_2O_2 and an organic peroxide (tBOOH) killing. Meanwhile, H_2O_2 or tBOOH pretreatments only induce protection against H_2O_2 but not tBOOH killing [4]. We wanted to determine if NEM could induce protection against tBOOH. Experiments were performed as described in Section 2. The results are shown in Fig. 3. Surprisingly, NEM pretreatment enhanced the killing effect of tBOOH. NEM treated cells were 10-fold more sensitive to a killing concentration of tBOOH than untreated cells. We also tested NEM pretreatment at several other concentrations prior to tBOOH killing. The levels of enhanced tBOOH killing showed a correlation with the inducing concentrations of NEM (data not shown). NEM did not induce protection to tBOOH killing for al concentrations tested.

4. Discussion

NEM and diamide induced resistance to H_2O killing in *Xp* is similar to observations in other bac teria [12,13]. Here, we further elucidated that NEM induced resistance required a functional *oxyR*. Con centrations of diamide (1 mM) significantly highe than NEM were required to induce H_2O_2 resistance This could be due to the ability of NEM to cova

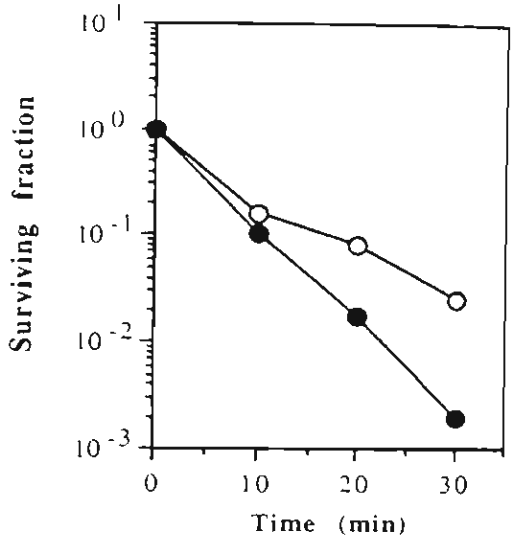

Fig. 3. NEM pretreatment enhanced tBOOH killing. *Xp* uninduced (○) or induced with 100 μM NEM (●) were treated with 20 mM tBOOH. At indicated time samples were removed and washed once with fresh medium, and plated on SB plates. Surviving fraction is as defined in Section 2.

lently modify SH groups, unlike diamide which forms non-covalent linkage with SH groups. The observation is consistent with an observation in *E. coli* that an OxyR regulated gene required 1 mM diamide to be induced [12]. Thiol reagent induced resistance to H_2O_2 killing is likely to be due to increased expression of OxyR regulated genes involved in peroxide detoxification, i.e. catalase and alkyl hydroperoxide reductase. This reasoning is supported by observations that in an *oxyR* mutant NEM or diamide could neither induce resistance to H_2O_2 killing nor peroxide metabolizing enzymes and also increased expression of catalase alone is sufficient to confer resistance to H_2O_2 killing in *Xanthomonas* [6], although it can not be ruled out that other OxyR regulated genes such as peroxide protective genes, i.e. *dps* [14] and/or macromolecule repair genes [15] are also contributing to peroxide resistance phenotype in NEM induced cells. These observations suggest that thiol reagents must activate OxyR by converting it to the oxidized form leading to increased expression of peroxide detoxification, peroxide protective and macromolecule repair genes. The ability to directly or indirectly sense thiol reagent is an additional role for OxyR. The question then remains of how NEM activates OxyR? There are several possibilities to explain the observation. Thiol reagents could alter cellular redox balance by reacting with free intracellular thiol such as glutathione [16]. This could result in OxyR being converted from reduced to oxidized form. Alternatively, these chemicals could directly interact with SH group of cysteine residues of reduced OxyR leading to a confirmational change in OxyR [11]. This could result in the formation of oxidized OxyR that in turn activates expression of catalase and *ahpC* genes [4]. Supporting evidence for both alternatives has been reported in *E. coli* that both reduced glutathione and diamide could convert OxyR from reduced to oxidized form in vitro [11]. Lastly, NEM is known to cause changes in intracellular pH [17]. This could lead to activation of OxyR by a similar mechanism where weak acids induce peroxide detoxification genes in an *oxyR* dependent manner [18]. These mechanisms are being investigated in *Xp*.

Unlike other bacteria, NEM pretreatment of *Xp* enhanced tBOOH killing. Yet the expression of *ahpC* was induced several-fold by the pretreatment. Apparently, this increased *ahpC* expression was not sufficient to confer protection against tBOOH killing contradicting a previous observation that in *Xp* increased *ahpC* expression alone conferred resistance to tBOOH killing [11]. We believe that NEM could directly interact with free SH groups of cysteine residues of AhpC and Ohr. The highly conserved cysteine residues in both proteins are essential for their catalytic activities [19]. Modifications of these cysteine residues possibly lead to inactivation of the enzymes and resulted in enhanced tBOOH sensitivity ([19] and an unpublished observation). This notion was supported by the observation that the levels of NEM enhanced tBOOH killing showed a direct correlation with concentrations of NEM.

Our data clearly show that thiol reagents could modulate *Xp* peroxide stress response. Thus, exposure to these compounds in the environment could have significant effects on the ability of bacterial pathogens to interact with their hosts and disease outcome.

Acknowledgements

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Construction and characterization of regulated L-arabinose-inducible broad host range expression vectors in *Xanthomonas*

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Abstract

Several versions of broad host range (BHR), L-arabinose-inducible expression vectors were constructed. These expression vectors were based on a high copy number BHR pBBR1MCS-4 replicon that could replicate in both enteric and non-enteric Gram-negative bacteria. Two versions of expression cassettes containing multiple cloning sites either with or without a ribosome binding site were placed under transcriptional control of the *Escherichia coli* BAD promoter and *araC* gene. Three versions of vectors containing ampicillin or kanamycin or tetracycline resistance genes as selectable markers were constructed. In all six new L-arabinose-inducible BHR expression vectors containing many unique cloning sites, selectable markers were made to facilitate cloning and expression of genes in various Gram-negative bacteria. A Tn*9* chloramphenicol acetyl transferase (*cat*) gene was cloned into an expression vector, resulting in pBBad18Acat that was used to establish optimal expression conditions (addition of 0.02% L-arabinose to mid-exponential phase cells for at least 1 h) in a *Xanthomonas campestris* pv. *phaseoli*. Comparison of the Cat enzyme activities between uninduced and a 180-min L-arabinose-induced culture showed a greater than 150-fold increased Cat specific activity. In addition, L-arabinose induction of exponential phase cells harboring pBBad18Acat gave a higher amount of Cat than similarly treated stationary phase cells. The usefulness of the expression vector was also demonstrated in both enteric and non-enteric Gram-negative bacteria. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved

Keywords: L-Arabinose induction; Multiple selectable marker; Non-enteric expression vector; *Escherichia coli* BAD promoter

1. Introduction

The ability to achieve a high level of expression of a cloned gene is very desirable for many reasons. Successful expression of a cloned gene facilitates protein purification, analysis of protein function and the study of complex physiological properties. However, some gene products are toxic at high levels. This could lead to undesirable effects such as cell death, growth inhibition, selection of mutations in the

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cloned gene and/or compensatory mutations in the host. Even in cases where products of cloned genes are not toxic, continuous high level expression of these genes could alter bacterial growth properties that resulted in reduction of cell mass. These undesirable effects could be minimized by using regulated expression systems where high levels of gene expression can be induced. Many of such systems have been developed for enteric bacteria [1,2]. One of the systems utilizes the *Escherichia coli* BAD promoter (P_{BAD}) and *araC* to achieve tight regulation and highly inducible expression of cloned genes [2]. In *E. coli*, in the absence of inducer (L-arabinose), AraC binds upstream of P_{BAD} to inhibit transcription. addition of L-arabinose leads to derepression of the promoter [2,3]. The *araC*-P_{BAD} system provides repression of transcription in the absence of the inducer and strong induction by L-arabinose. The system is widely used in the enteric bacteria but has not been used in other bacteria due to lack of a broad host range (BHR) replicon containing the expression system. It is only recently that *araC*-P_{BAD} on a BHR system has been reported [4]. Here, we described the construction and characterization of six BHR expression vectors containing two *araC*-P_{BAD} expression cassettes and a choice of three selectable markers. The induction kinetics and dose-response of P_{BAD} by L-arabinose were also characterized in a *Xanthomonas campestris* pv. *phaseoli* (*Xp*)

2. Materials and methods

2.1. Media, growth conditions and enzyme assay

Xanthomonas strains were grown aerobically in nutrient broth (NB) at 28°C. In all experiments, an overnight culture was used as a starting inoculum to give 0.1 optical density at 600 nm (OD_{600}). The culture was allowed to grow for 3 h or until the culture density had reached 0.5 OD_{600} [5]. Then, indicated concentrations of L-arabinose were added and growth continues for 1 h before cells were harvested. For investigation of L-arabinose induction of the chloramphenicol acetyl transferase gen (*cat*) during various growth phases, a exponential phase culture represented 3 h (OD_{600} 0.5) and a stationary phase culture represented 24 h sub-culture (OD_{600} 2.3). 0.02% L-arabinose was added to these cultures and growth was continued for 3 h before cells were harvested for lysate preparation. *E. coli* cells were grown in LB at 37°C. Lysate preparation and Cat enzyme assay were performed spectrophotometrically as described by Mongkolsuk et al. [6].

2.2. Gene transfer, molecular cloning and Western immunoblot analysis

E. coli (DH5α) was transformed using electroporation performed as previously described [5]. Electroporation was also used to transform plasmids into *Pseudomonas aeruginosa* and *Xp*. Briefly, exponential phase cells were collected and resuspended in the electroporation buffer and electroporation was performed using a Bio-Rad machine as previously described [5]. The following antibiotics at indicated concentrations were used to select transformants: *E. coli* (ampicillin 100 μg ml^{-1}), *P. aeruginosa* (carbenicillin 200 μg ml^{-1}) and *Xp* (carbenicillin 250 μg ml^{-1}). All molecular cloning and DNA manipulations were done according to Maniatis et al. [7].

Protein analysis was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Twenty-five μg total protein was loaded into each well and after electrophoresis, the gel was either stained with Coomassie blue dye or transferred to nitrocellulose membranes for Western immunoblot analysis. The membrane was subsequently reacted with a polyclonal anti-Tn*9* Cat antibody (5′-3′ company) using conditions previous described [5]. Antibody reactions were developed using an alkaline phosphatase detection kit, performed according to the manufacturer's (Promega) recommendations.

2.3. Construction of pGEMTet

A tetracycline resistance gene (Tet^R) cassette was constructed by polymerase chain reaction (PCR) of the gene from the plasmid pALTER-Ex2 (Promega). The following primers corresponding to 5′ (5′-AGCTTATCATCGGATAAC-3′) and 3′ (5′-GCCGCGGCTTCCATTCA-3′) of the Tet^R gene were used in PCR reactions under the following conditions: denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min for 35 cycles. The 1346-bp PCR products were cloned into

TA cloning vector pGEM-T Easy (Promega) and transformed into *E. coli* selecting for *Tet* resistance. The resultant new recombinant plasmid was designated pGEMTet. The vector has *Eco*RI sites flanking the *Tet* gene.

2.4. Construction of pBBad18Acat

The plasmid was constructed by cloning of a gel-purified *Sac*I-*Xho*I-digested 0.9-kb fragment containing the Tn*9 cat* from pSM-CAT2 [8] into *Sac*I-*Sal*I-digested pBBad18A. The recombinant plasmid designated pBBAD18Acat was transformed into *E. coli*, selecting for chloramphenicol resistance on LB plates containing 0.02% L-arabinose. The recombinant plasmid was characterized and then transformed into *Xp* and *P. aeruginosa* by electroporation [5].

3. Results and discussion

3.1. Construction of expression vectors

Our aim was to construct BHR expression vectors based on the pBBR1MCS-4 replicon [9], containing an *E. coli araC*-P_{BAD} expression system with different selectable markers. Two versions of expression cassettes containing *araC*-P_{BAD} with (pBAD22) or without (pBAD18) a ribosome binding site and multiple cloning sites were used in the construction. pBAD18 and pBAD 22 were double-digested with *Cla*I and *Sca*I and gap-filled with Klenow DNA polymerase. The 2.18-kb fragments containing the *araC*-P_{BAD} expression cassette were gel-purified. These fragments were ligated into the 4.08-kb fragment of pBBR1MCS-4 digested with *Eco*ICRI-*Sca*I. The resulting recombinant plasmids designated pBBad18A and pBBad22A were transformed into *E. coli* selecting for Xp resistance. The usefulness of the expression vectors was expanded by constructing new vectors with two new selectable markers conferring kanamycin or tetracycline resistance. The 1.35-kb Tet^R gene from pGEMTet was digested with *Eco*RI and gap-filled. The 1.4-kb kanamycin resistance gene from pKRP11 [10] was digested with *Hind*III and gap-filled. Both cassettes were gel-purified and ligated into pBBad18A and pBBad-22A digested at a *Sca*I site. Recombinant plasmids were transformed into *E. coli* selecting for either tetracycline or kanamycin resistance. The new plasmids were designated pBBad18T, pBBad22T (conferring tetracycline resistance) and pBBad18K, pBBad22K (conferring kanamycin resistance). These plasmids were subsequently characterized at restriction enzyme levels. The construction of all BHR expression vectors is summarized in Fig. 1. The functionality of all expression vectors, pBBad18A, pBBad18K, pBBad18T, pBBad22A, pBBad22K and pBBad22T, was tested by cloning a Tn*9 cat* into these vectors and transformants were checked for L-arabinose-inducible expression of the cloned gene (data not shown). In addition, all vectors were transformed into *Xp* and plasmids re-isolated and analyzed on an agarose gel. This was to confirm that these vectors could replicate as plasmids in non-enteric bacteria (data not shown). The six expression vectors have many unique restriction enzyme sites located in the multiple cloning site to facilitate gene cloning (Fig. 1). The pBBR1MCS-4 replicon has been shown to function in a diversed group of Gram-negative bacteria such as *Agrobacterium*, many enteric bacteria, *Pseudomonas*, *Rhizobium* and *Xanthomonas* [9]. Mobilization (mob) function on the plasmid allows for conjugal transfer of the plasmid from *E. coli* to a variety of Gram-negative bacteria via the RK2 conjugal transfer function [9]. These facts, coupled with availability of a variety of vector selectable markers, increased the usefulness of these expression vectors.

3.2. Establishing optimal inducing conditions for Xanthomonas

The functionality of the expression vector and optimal L-arabinose inducing conditions were determined by monitoring Cat levels in *Xp* harboring pBBad18Acat. First, the optimum inducing concentrations of L-arabinose were tested. The results in Fig. 2A show that a significant induction of *cat* was detected at L-arabinose concentrations of 0.005% or higher. When the concentration of the inducer was less than 0.001%, no induction was detected. Addition of L-arabinose at greater concentrations than 0.02% did not result in a significant increase in *cat* induction (Fig. 3). Next, the induction kinetics were investigated. 0.02% L-arabinose was added to the culture. At indicated times, cells were

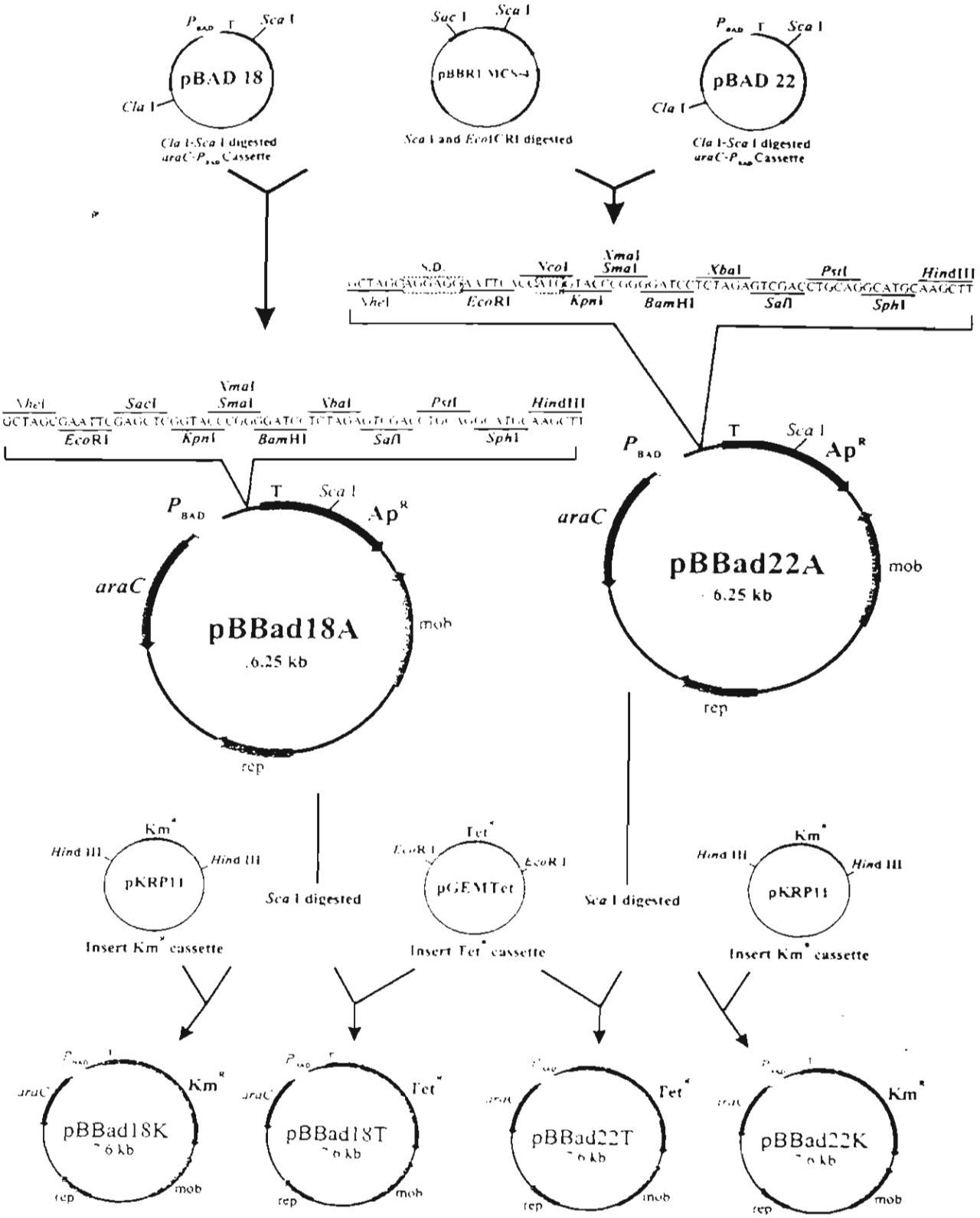
pBAD 18
Cla I
Sca I
Cla I-Sca I digested
araC-P BAD Cassette
Sac I
Sca I
pBBR1 MCS-4
Sca I and EcoICRI digested
pBAD 22
Cla I
Sca I
Cla I-Sca I digested
araC-P BAD Cassette
S.D.
XbaI
PstI
HindIII
XhoI
EcoRI
KpnI
BamHI
SalI
SphI
SacI
SmaI
araC
pBBad18A
6.25 kb
mob
rep
pBBad22A
6.25 kb
Km
pKRP11
Insert Km cassette
Sca I digested
Tet
pGEMTet
Insert Tet cassette
pBBad18K
pBBad18T
pBBad22T
pBBad22K

Fig. 1. Summary of construction and maps of BBR, L-arabinose-inducible expression vectors. Unique restriction enzyme sites in the multiple cloning sites are in bold. mob, mobilization functions; rep, *trans* acting replicator protein; T, transcription terminator. (pBT 1). Details of plasmids construction were described in Section 3.

removed and the amount of Cat determined. The results are shown in Fig. 2B. At 5 min after addition of L-arabinose, a small increase in the amount of Cat was detectable. When the induction time increased from 5 to 30, 60, 120 and 180 min, a corresponding increase in the amount of Cat protein was detected. This increase in the amount of Cat protein correlated with the Cat enzyme activity. Uninduced cells had a Cat specific activity of 0.03 U mg^{-1} protein comparing to 4.63 U mg^{-1} protein in a culture that was induced for 180 min. This represented a 154-fold induction in Cat specific activity. However, the Cat specific activity of a 3-h-induced sample was only 2-fold greater than Cat activity in 1-h-induced sample. Addition of glucose (0.5%) to growth medium did not significantly repress the uninduced Cat levels but instead reduced the derepression of the promoter by L-arabinose over 50-fold (data not shown). This is similar to observations in *Agrobacterium* [4]. Thus, optimal inducing conditions of P_{BAD} for *Xp* should be the addition of 0.02% L-arabinose for at least 1 h.

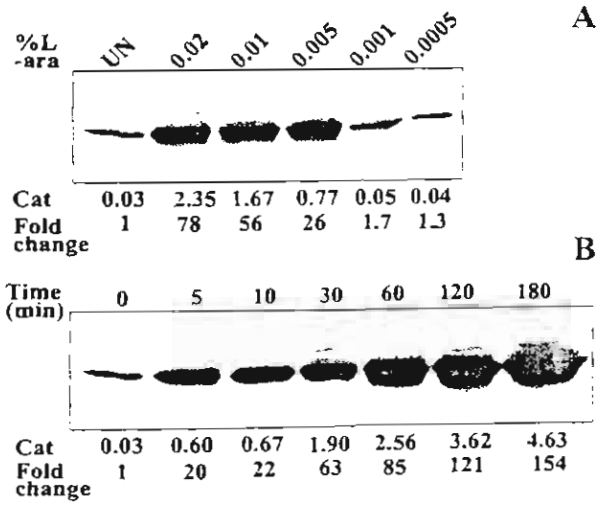

Fig. 2. A dose-response and time course analysis of L-arabinose induction of *cat* expression. In A, a dose-response of L-arabinose and induction of *cat* expression. A *Xp* harboring pBBad18Acat was split into six flasks and various concentrations of L-arabinose (%L-ara) were added. After 1-h incubations, cells were collected and lysates prepared for Cat Western immunoblot analysis and enzyme assays. UN represents an uninduced culture. In B, a time course analysis of L-arabinose induction of *cat* expression. At time 0, 0.02% L-arabinose was added to an uninduced culture of *Xp* harboring pBBad18Acat. At indicated times, samples were withdrawn and lysates prepared for Cat Western immunoblot analysis and enzyme assays. In both A and B cultures conditions, Western immunoblot analysis and Cat enzyme assays were performed as described in Section 2.

3.3. Growth phase dependent L-arabinose induction

High level production of a recombinant protein depends upon many factors. Two of these factors are cell mass and the level of cloned gene expression. Ideally, if high level gene expression could be induced during the early stationary phase of growth, this would maximize the product yield. The ability of L-arabinose to induce P_{BAD} during different stages of growth has not been investigated. Experiments were done to test L-arabinose induction of a cloned *cat* gene in *Xp* harboring pBBad18Acat at different growth phases. The results are shown in Fig. 3. Densitometer analysis indicates that addition of L-arabinose to exponential phase cells resulted in an over 20-fold increase in Cat. Moreover, Western immunoblot analysis showed that a large quantity of Cat was produced. At the stationary phase, the basal Cat level was reduced 4-fold. Addition of L-arabinose

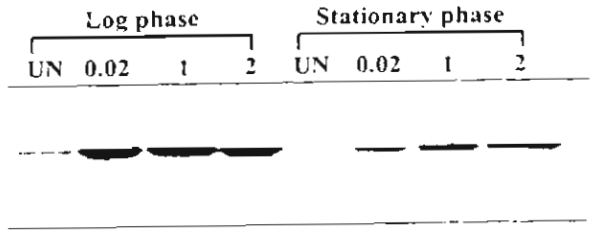

Fig. 3. Induction of *cat* expression by L-arabinose at the exponential and stationary phase of growth. A culture of *Xp* harboring pBBad18Acat grown in NB was removed at 5 h (OD_{600} 0.61) for a exponential phase culture and 24 h (OD_{600} 2.5) for a stationary phase culture. 0.02, 1 and 2% L-arabinose were added to these cultures and growth continued for 1 h. UN represent cultures with no L-arabinose added. Cells were harvested and lysates prepared for Cat Western immunoblot analysis as described in Section 2.

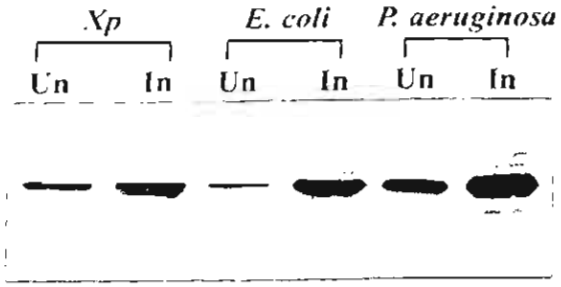

Fig. 4. Induction of *cat* expression in various bacteria. Mid-exponential cultures of *E. coli*, *P. aeruginosa* and *Xp* were induced with 0.02% L-arabinose for 1 h. Lysates were prepared from induced (In) and uninduced (Un) cultures for Cat Western immunoblot analysis as described in Section 2.

significant induced expression of *cat* (10-fold) but this level was 5-fold less than the level attained in similarly induced exponential phase cells. To rule out that transport of L-arabinose could be inefficient during the stationary phase of growth, 50- and 100-fold higher concentrations of L-arabinose were added (1 and 2%). These treatments resulted in a less than 0.5-fold increase in the levels of Cat comparing to the levels attained at 0.02% L-arabinose (Fig. 3). We do not know the mechanism that causes a reduced basal and induced level of expression of a cloned gene at the stationary phase. Since *E. coli* P_{BAD} is a heterologous promoter in *Xp*, reduction in the level of the sigma factor that recognized P_{BAD} at the stationary phase of growth could lead to the observed effects on gene expression. The mechanism is being investigated. The data clearly show that the growth phase of a culture is an important parameter for obtaining a maximum yield from expression of a cloned gene.

3.4. L-Arabinose induction of a cloned gene in various bacteria

The ability of L-arabinose to induce expression of a cloned *cat* gene in the expression vector was investigated in *E. coli*, *P. aeruginosa* and *Xp*. The results are shown in Fig. 4. Clearly, L-arabinose induced a high level expression of *cat* gene in all three bacteria. A greater than 10-fold induction was observed in *E. coli* and *Xp*, respectively. In *P. aeruginosa*, at least a 5-fold induction was observed. However, it is noteworthy that the uninduced Cat levels varied significantly. In *E. coli*, very little Cat was detected in the uninduced culture, indicating a good repression of the expression system. However, in *Xp* and *P. aeruginosa*, significant levels of Cat in uninduced cultures were detected. In these two bacteria, the regulation of P_{BAD} is not as tight as in *E. coli*. The induced Cat levels were similar in all bacteria tested. Full activation of P_{BAD} requires CRP protein interaction [2,3,10]. The data suggest that heterologous CRP proteins in *P. aeruginosa* and *Xp* could function on *E. coli* P_{BAD}. The L-arabinose-inducible expression system is proved to be useful for regulating expression of a cloned gene in various bacteria.

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Expression analysis and characterization of the mutant of a growth-phase- and starvation-regulated monofunctional catalase gene from *Xanthomonas campestris* pv. *phaseoli*

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Abstract

Analysis of the *Xanthomonas campestris* pv. *phaseoli* (*Xp*) catalase profile using an activity gel revealed at least two distinct monofunctional catalase isozymes denoted Kat1 and Kat2. Kat1 was expressed throughout growth, whereas Kat2 was expressed only during the stationary phase of growth. The nucleotide sequence of a previously isolated monofunctional catalase gene, *Xp katE*, was determined. The deduced amino acid sequence of *Xp* KatE showed a high percentage identity to an atypical group of monofunctional catalases that includes the well-characterized *E.coli katE*. Expression of *Xp katE* was growth phase-dependent but was not inducible by oxidants. In addition, growth of *Xp* in a carbon-starvation medium induced expression of the gene. An *Xp katE* mutant was constructed, and analysis of its catalase enzyme pattern showed that *Xp katE* coded for the Kat2 isozyme. *Xp katE* mutant had resistance levels similar to the parental strain against peroxide and superoxide killing at both exponential and stationary phases of growth. Interestingly, the level of total catalase activity in the mutant was similar to that of the parental strain even in stationary phase. These results suggest the existence of a novel compensatory mechanism for the activity of *Xp* catalase isozymes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Catalase mutant; Compensatory; *katE* gene; Stationary phase of growth

1. Introduction

Xanthomonas is an important genus of plant pathogenic bacteria. The bacteria are Gram-negative and are obligate aerobes. Aerobic organisms are commonly exposed to variety of reactive oxygen species (ROS) that arise from either normal aerobic metabolism or from external sources. Increased production and accumulation of ROS also are a part of the first line of defense against invasive microbes in plants (Keppler et al., 1989). H_2O_2 is released as a part of the active plant defense response against microbial invasion. It inhibits bacterial growth and is bactericidal. Pathogenic bacteria must overcome H_2O_2 toxicity to multiply and colonize plant tissue, and catalases are enzymes employed for its detoxification. They convert H_2O_2 to oxygen and water. The typical catalases of both eukaryotes and prokaryotes are heme-containing proteins that share a high degree of amino acid sequence identity (Loewen, 1997). Many prokaryotes have multiple monofunctional catalase isozymes under differential regulation (Miller et al., 1997). Some bacteria also possess bifunctional catalase-peroxidase enzymes (Triggs-Raine et al., 1988; Hochman and Goldberg, 1991; Loewen, 1997).

In *Xp*, the protection against H_2O_2 damage depends on both physiological factors and catalase levels. Stationary phase cells are highly resistant to H_2O_2 and superoxide anion (Vattanaviboon et al., 1995). Exposure of *Xanthomonas* to sub-lethal concentrations of oxidants induces catalases, which give an increased resistance to otherwise lethal doses of H_2O_2 (Chamnongpol et al., 1995b). A monofunctional catalase gene (*katY*) isolated

Abbreviations: Ap, ampicillin; Gm, gentamicin; H_2O_2, hydrogen peroxide; Kat, catalase; kb, kilobase(s); MD, menadione; PB, sodium phosphate buffer; ^{R}, resistance; ROS, reactive oxygen species; ^{S}, sensitive; tBOOH, tert-butylhydroperoxide; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*; *Xp*, *Xanthomonas campestris* pv. *phaseoli*.

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from *X. oryzae* pv. *oryzae* (*Xoo*) (Mongkolsuk et al., 1996) has expression induced by oxidants, especially superoxide anion (Mongkolsuk et al., 1996). In this report, we show that *Xp* possesses at least two distinct monofunctional catalase isozymes. The *katE* gene for one of these isozymes was characterized, and a mutant lacking a functional form of the gene was constructed to examine its physiological role.

2. Materials and methods

2.1. Bacterial strains, growth and transformation

E. coli and *Xanthomonas* strains were grown aerobically at 37 and 28°C, in Luria–Bertani (LB) and Silva–Buddenhagen (SB) media (Chamnongpol et al., 1995a), respectively. The carbon starvation medium was a M9 salt without carbon sources. The carbon starvation experiment was performed by growing *Xp* in SB until the culture reached log phase of growth (0.3 A_{600}). Then, cells were pelleted, and SB removed, washed once and resuspended in M9 medium. Half of the cells were aliquoted into a carbon-starvation medium, while the remaining cells were placed into a new SB medium. These cultures were allowed to grow for an additional 3 h before cells were harvested for lysate preparation. Oxidant induction of catalase in stationary phase cells was accomplished by adding 100 μM of H_2O_2, tBOOH or MD for 30 min prior to lysate preparation and catalase assay.

2.2. Catalase assay and activity gel staining

Preparation of cell lysates, determination of protein concentration and catalase enzyme assay were done as previously described (Mongkolsuk et al., 1996). Visualization of catalase isozymes on native PAGE gels was performed by the method of Kim et al. (1994) with some modifications as followed. After gel electrophoresis of 80 μg of protein loaded in each lane, gels were soaked in 50 mM sodium phosphate buffer (PB), pH 7.0, containing 50 μg ml of horse-radish peroxidase (Sigma, USA) for 45 min at room temperature. They were then soaked in PB containing 5 mM H_2O_2 for 10 min. After briefly washing twice with distilled water, they were immediately stained with freshly prepared PB containing 0.5 mg ml diamino benzidine until the background become dark brown. Catalase activity appeared as colorless bands. Peroxidase activity staining was carried out as previously described (Wayne and Diaz, 1986).

2.3. Qualitative and quantitative determination of oxidant resistance

Resistance to oxidant killing of log phase cells or stationary phase cells was quantitatively determined. *Xp* and an *Xp katE* (*Xp*13) mutant were grown aerobically in SB at 28°C. Log phase cells (5 h, 0.5 A_{600}) or stationary-phase cells (24 h, 3.5 A_{600}) were used in oxidant killing experiments. Oxidant treatments were performed as described by Vattanaviboon and Mongkolsuk (1998). Surviving cells were counted after 48 h incubation at 28°C. Surviving fractions are defined as the number of culturable cells after the treatment divided by the number of viable cells before treatment (Vattanaviboon et al., 1995). All experiments were independently performed three times, and typical values are shown.

2.4. Construction of an Xp katE mutant

The strategy for construction of *katE* mutants is shown in Fig. 2A. A 3.3 kb DNA fragment containing *katE* coding region from *Kpn*I–*Eco*RV of pXPkat5 (Loprasert et al. 1996) was cloned into pBluescript KS at *Kpn*I–*Eco*RV sites to create a plasmid pKSkatE. Inactivation of the *katE* gene was achieved by insertion of a *Sma*I-digested gentamicin resistance gene from pUCGM (Schweizer, 1993) into an unique *Hin*cII site in the middle of the *katE* coding region. The resultant recombinant plasmid (called pKSkatEGm) was electroporated into *Xp* followed by selection for Gm^R and scoring for Ap^S phenotypes as previously described (Mongkolsuk et al., 1998). Putative exchange of the mutated *katE* and its functional counterpart gave rise to the Gm^RAp^S phenotype. These colonies were further analyzed by Southern hybridization against a *katE* probe (*Sph*I fragment of *katE* coding region) and by catalase activity gels to confirm that the mutated *katE* had replaced the functional gene.

2.5. Molecular cloning and nucleotide sequencing

The plasmid pUFRkatE was constructed by subcloning of the 3.3 kb *Kpn*I–*Xba*I fragment from pKSkatE into *Kpn*I–*Xba*I-digested pUFR047 (DeFeyter et al., 1990). pXPkat5 (Loprasert et al., 1996) was sequenced in both orientations by the primer walking technique with ABI Prism kits on an ABI 373 automated DNA sequencer. Routinely, *E. coli* was transformed by a chemical method, while *Xanthomonas* was transformed by electroporation under conditions previously described (Mongkolsuk et al., 1996). The nucleotide and deduced amino acid sequences of *Xp katE* have been deposited in the GenBank database under the Accession No. AF170449

3. Results and discussion

3.1. Multiple monofunctional catalase isozymes in Xp

Many bacteria have multiple catalase isozymes. In most cases, the functions of the different isozymes are

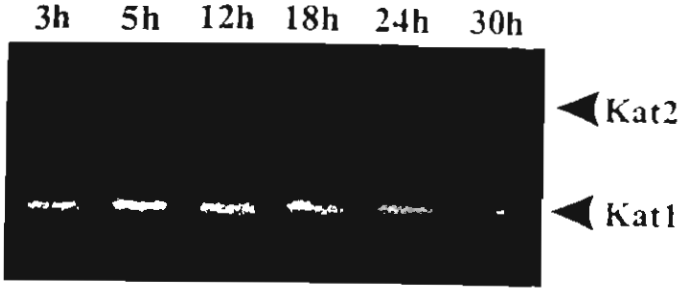

Fig. 1. Multiple catalase isozymes in *Xp* at various stages of growth. *Xp* was grown aerobically in SB at 28°C. At the indicated time, cells were harvested, and lysates were prepared as described in Sections 2.1 and 2.2. Equal amounts of protein (80 μg) were applied to wells of a 7.5% native PAGE and subsequently stained for catalase activity. Lane 1, early exponential (3 h); 2, mid exponential (5 h); 3, early stationary (12 h); 4, stationary (18 h); 5, late stationary (24 h); and 6, late stationary (30 h).

unknown, and this is true for *Xanthomonas*. Thus, *Xp* cell lysates from different stages of growth were stained for catalase activity using activity gels. The results are shown in Fig. 1. During the exponential phase of growth, an isozyme, designated Kat1, was detected and was present throughout all stages of growth thereafter, although there was a small reduction in the level of Kat1 activity at the stationary phase of growth. A second higher molecular mass catalase isozyme, designated Kat2, was detected as cells entered and during the stationary phase of growth. The levels of Kat2 continued to increase in the stationary phase, whereas the levels of Kat1 declined. No additional catalase isozymes were detected even at the late stationary phase. Densitometer analysis of catalase activity gels revealed that Kat1 was the major catalase of *Xp* during exponential phase of growth and that Kat1 and Kat2 accounted for 60 and 40%, respectively, of total catalase activity at the stationary phase. Additional experiments (data not shown), using native PAGE gels and peroxidase activity stain, showed that neither Kat1 nor Kat2 possessed peroxidase activity (Wayne and Diaz, 1986). The results confirmed that *Xp* produced at least two monofunctional catalase isozymes (Kat1 and Kat2).

3.2. Characterization of the *katE* gene

We have previously reported the isolation of a monofunctional catalase gene (*katE*) from *Xp* (Loprasert et al., 1996). Southern blot analysis of restriction-enzyme-digested *Xp* genomic DNA patterns probed with a *kat* probe indicated that *katE* was a single-copy gene (data not shown). A 4.2 kb DNA fragment containing *katE* was sequenced. DNA sequence analysis showed an open reading frame (ORF) of 2106 bp that had a coding potential for a 702-amino-acid protein with a molecular mass of 76 kDa. The sequence was used to search against databases where a high similarity was found to monofunctional catalases from bacteria and eukaryotes. There are two subgroups of bacterial monofunctional catalases. Catalases in Group I have molecular masses around 55–65 kDa, while those in Group II have molecular masses around 80–84 kDa. A putative size around 80 kDa suggested that *Xp* KatE belonged to Group II in the phylogenetic tree described by Klotz et al. (1997). Multiple alignment of catalase amino acid sequences was done using the Clustal W program (Thompson et al., 1994). As expected, the deduced amino acid sequence of *Xp* KatE showed a high identity (90%) to *Xoo* KatX. The percentage identity was around 50% when *Xp* KatE was compared with *E. coli* KatE and *Pseudomonas putida* CatC. *Xp* KatE also showed a high percentage identity to catalases from eukaryotes including maize (42%, X12538), bovine (43%, P00432) and human (42%, X04076) (data not shown).

E. coli KatE is an atypical catalase because it lacks an NADPH binding site and contains heme d instead of heme b (Loewen 1997). Amino acid residues involved in the catalase active site and heme binding site of *E. coli* KatE are all conserved in *Xp* KatE (data not shown). The binding of NADPH to catalase has been demonstrated for bovine liver catalase (BLC) (Kirkman and Gaetani, 1984). The key residues proposed to be important for BLC interaction with NADPH were not present in either *E. coli* KatE or *Xp* KatE. Thus, it is likely that *Xp* KatE does not bind NADPH.

3.3. Construction of an *Xp katE* mutant

Xp katE mutants were constructed (as described in Section 2 and depicted in Fig. 2A) to investigate the physiological role of *katE* and to determine whether *Xp* had multiple monofunctional catalase genes. Results from a Southern blot analysis using a *kat* probe with presumptive *katE* mutants of Gm^R and Xp^- phenotypes are shown in Fig. 2B. *Sph*I-digested DNA from one of the *katE* mutants designated *Xp*13 showed a positively hybridized fragment of 2.7 kb, which corresponded to 1.8 kb in *Xp* plus the inserted gentamicin resistance gene (0.9 kb) in the mutant. This confirmed that the nonfunctional, mutated copy of *katE* had replaced the functional gene copy. *Xp*13 is the first catalase mutant constructed in *Xanthomonas*.

3.4. *katE* coding for the Kat2 isozyme and regulation of its expression

To study the effects of *katE* disruption on catalase isozyme patterns, gels of cell lysates prepared from a stationary-phase culture of the *Xp*13 mutant and the *Xp* parental strain were stained for catalase activity (Fig. 2C). Only the Kat1 isozyme was detectable in *Xp*13 stationary-phase cell lysates. Kat2 in *Xp*13 was restored by expression of a functional *katE* on a broad-host-range plasmid vector, pUFRkatE. Thus, we con-

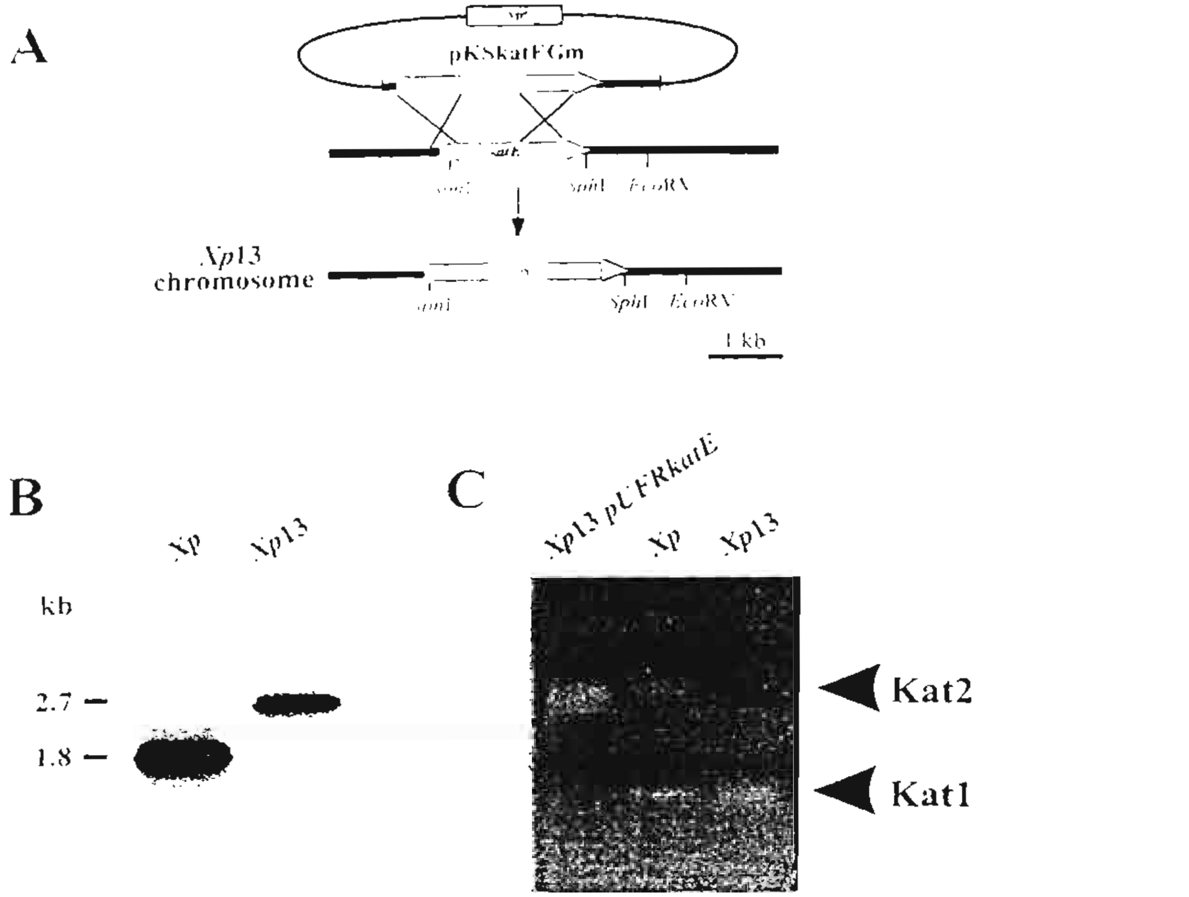

Fig. 2. Strategy for homologous marker exchange mutagenesis to construct a *Xp katE* mutant (*Xp*13). (A) Gm^R, gentamicin resistance; Ap^R, ampicillin resistance. (B) Southern blot analysis of a *Xp katE* mutant. Genomic DNA (5 μg) of *Xp* wild type (*Xp*) and *Xp katE* (*Xp*13) were digested with *Sph*I and applied to a 1.0% agarose gel. The blotted membranes were hybridized with a ^{32}P-labelled 1.8 kb *Sph*I fragment of the *katE* coding region (lane: 1, and 2). (C) Catalase isozymes in *Xp* wild type (lane 2), *Xp*13 (lane 3) and *Xp*13-pUFRkatE (lane 1). Experiments were performed as described in the Fig. 1 legend, but only late stationary-phase cells (30 h) were used, and in *Xp*13-pUFRkatE, only 10 μg of protein were loaded.

cluded that *katE* coded for Kat2 monofunctional catalase isozyme and that the two monofunctional catalase isozymes in *Xp* were produced by distinct genes. Thus, hereafter, the Kat2 designation is changed to KatE. Moreover, *katE* expression was growth-phase-dependent (Fig. 3) similar to reports in several other bacteria (Mulvey et al., 1990; Miller et al., 1997). The expression of *katE* during stationary phase of growth could be due to depletion of nutrients. This idea was tested by comparing the level of KatE in log-phase cells grown in rich medium (SB) and a carbon starvation medium (M9 salts minus a carbon source). The results are shown in Fig. 3. KatE was not detected in log-phase cells grown in a rich medium. Nonetheless, carbon starvation of *Xp* for 3 h induced *katE* expression. This suggests that nutrient limitation during the stationary phase could be the inducing signal. Similar observations of stationary-phase-dependent and carbon-starvation-induced expression of *kat* genes have been reported in *E. coli* and *B. subtilis*. In these bacteria, *kat* genes are regulated by stationary-phase sigma factors, *rpoS* (Mulvey et al., 1990; Hengge-Aronis, 1993) and *rpoB* (Engelmann et al.,

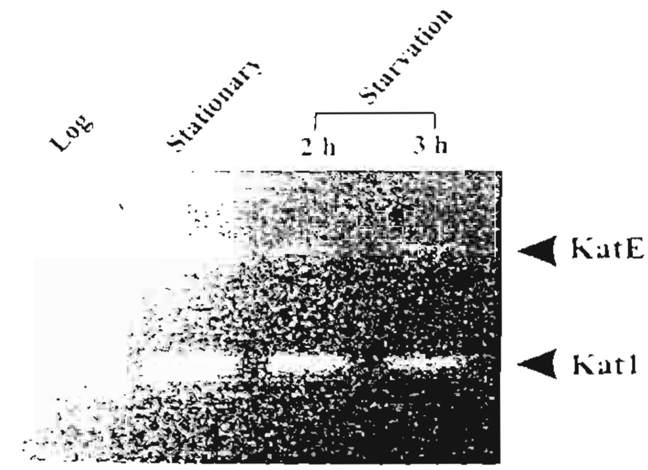

Fig. 3. Catalase activity gel of carbon-starvation-induced *katE* expression. *Xp* culture conditions, lysate prepration and catalase activity gel were performed as described in the Fig. 1 legend and Sections 2.1 and 2.2. Samples were prepared from log-(Log), stationary-(Stationary) phases of growth and 2, 3 h after carbon starvation of log-phase cells (Starvation).

1995), respectively. Although, a *rpoS* homologue has not been identified in *Xanthomonas*, the expression pattern would be consistent with *rpoS* regulation.

We have previously investigated oxidant regulation of catalase enzymes in *Xp* (Chamnongpol et al., 1995a,b; Loprasert et al., 1996) and shown that exposure to low levels of oxidants leads to over 10-fold increases in total catalase activity. This induction depends on a transcription regulator and the peroxide sensor, OxyR (Mongkolsuk et al., 1998). In *Xoo*, oxidant induction of *katX* has also been observed (Mongkolsuk et al., 1996). Here, we investigated the role of oxidants in *katE* induction during exponential and stationary phases of growth. We found, using activity-stained gels, that the KatE activity did not change significantly after 100 μM H_2O_2 or 100 μM MD (a superoxide generator) treatments at any stages of growth (data not shown), even though the total catalase activities did increase during the exponential phase and stationary phase respectively (Table 1). The total catalase activity also similarly increased in the *Xp*13 *katE* minus mutant in response to H_2O_2 or MD at the exponential and stationary phases of growth (Table 1). Thus, *katE* does not seem to respond to oxidant induction, but a direct assay is required for conformation.

It is remarkable that *Xp katE* and *Xoo katX*, two highly conserved genes (over 90% homology) from closely related organisms, should have such different patterns of regulation. *Xp katE* is growth-phase- and starvation-regulated but not oxidant-inducible, while *Xoo katX* is not growth-phase-regulated but is oxidant-inducible (Mongkolsuk et al., 1996). The physiological significance of disparate regulation of these genes is being investigated.

Next, the effect of the *katE* mutation on catalase activity was investigated. Unlike many other bacteria, catalase levels of *Xanthomonas* decrease as cells enter the stationary phase of growth (Chamnongpol et al., 1995a,b). KatE accounted for 40% of total catalase activity during the stationary phase of growth. Unexpectedly *Xp* and the *Xp*13 mutant had similar catalase specific activities at both the exponential (5.0 and 5.1 U/mg protein) and stationary phase of growth (3.9 and 3.7 U/mg protein). Thus, a loss of KatE did not cause a reduction in total catalase activity. Analysis of the catalase profile of the mutant from the stationary phase of growth showed a compensatory increase in KatI isozyme levels (Fig. 2C). These data imply the presence of a novel compensatory mechanism for catalase isozymes. Such a compensatory response would be very important in determining the effect of gene mutation on cell physiology, and an understanding of this novel process might prove useful to other bacterial systems. The regulator mediating this response is being investigated. Alternatively, the catalase activity during stationary phase could be limited by the amount of another co-factor such as heme. The catalase activity observed in the mutant could represent the maximum value of activity due to co-factor limitations.

3.5. Physiological characterization of the Xp13 katE mutant

The physiological roles of various catalase isozymes is unclear, and the availability of an *Xp katE* mutant should help to clarify the situation. Inactivation of oxidative stress protective genes often results in altered aerobic growth (Mongkolsuk et al., 1998). Thus, we investigated the growth of the mutant and found that it had similar aerobic growth rates to the parental strain in both complex and minimal media (data not shown). The role of *katE* in protecting *Xp* against H_2O_2 and other oxidants during the stationary phase of growth was determined. *Xp* from log- and stationary phases of growth were treated with killing concentrations, 10 and 50 mM, respectively, of H_2O_2. *Xp*13 was as susceptible to H_2O_2 killing as the parental strain at both log- and stationary phases of growth (Fig. 4A and B). Also, *Xp*13 resistance to 30 mM tBOOH or 200 mM MD was not significantly different at any stage of growth (data

Table 1
Induction of catalase specific activity in *Xp* and *Xp*13

	Exponential phase		Stationary phase	
	Catalase U[a] mg protein	Fold induced	Catalase U[a] mg protein	Fold induced
Xp				
Uninduced	5.0 ± 1.1	-	3.9 ± 1.0	-
+ 100μM H_2O_2	13.9 ± 1.4	2.8	6.6 ± 1.2	1.7
+ 100μM MD	42.2 ± 3.6	8.4	8.3 ± 1.0	2.1
*Xp*13 (*Xp katE*)				
Uninduced	5.1 ± 1.2	-	3.7 ± 1.1	-
+ 100μM H_2O_2	12.3 ± 1.5	2.4	6.5 ± 1.3	1.8
+ 100μM MD	40.3 ± 4.1	7.9	8.5 ± 1.2	2.3

[a] One unit of catalase is defined as the amount of enzyme required to hydrolyse 1 μmol of H_2O_2 per min at 25°C, pH 7.0. Data represent mean ± S.D. of three independent experiments.

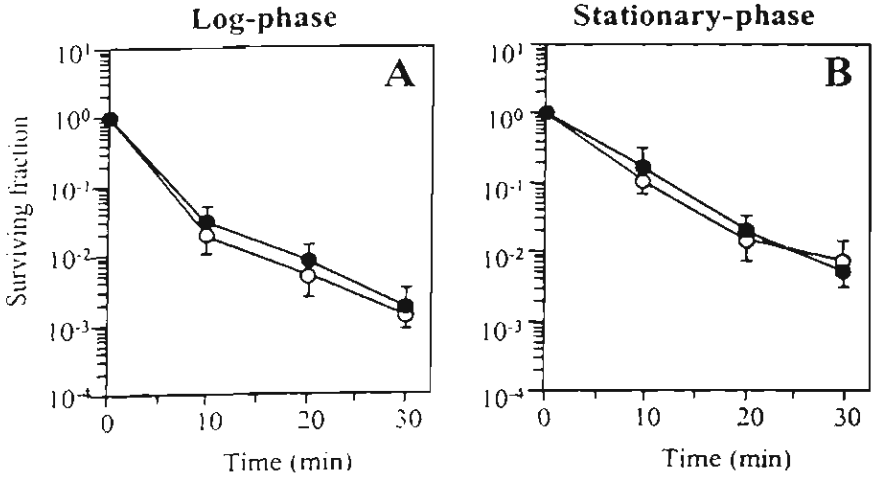

Fig. 4. Survival curves for *Xp* wild type and the *Xp katE* mutant (*Xp* 13) against H_2O_2 killing. log phase cells (**A**) or stationary phase cells (**B**) of wild type (○) and *Xp* 13 mutant (●) cultured in SB medium were treated with 10 or 50 mM H_2O_2, respectively.

not shown). Thus, disruption of *katE* did not have any adverse effects under oxidative stress conditions. These results were consistent with our (Vattanaviboon et al., 1995) and other (Kolter et al., 1993) previous observations that stationary phase bacteria are highly resistant to oxidants including H_2O_2, organic peroxide and superoxide. This resistance does not correlate with levels of various oxidative stress detoxification enzymes (Vattanaviboon et al., 1995), so other factors such as alterations in membrane structure and non-specific DNA binding proteins may be more important for the response (Kolter et al., 1993). Furthermore, *Xp katE* showed a compensatory increase in total catalase activity. This mechanism would minimize any effects of the *katE* mutation on H_2O_2-resistance levels. None the less, the compensatory increase in Kat1 levels in the *Xp katE* mutant suggested that it might be necessary for *Xp* to maintain the catalase activity during stationary phase. We are investigating the physiological significance of this catalase compensatory reaction.

4. Conclusions

1. *Xp* produced at least two different monofunctional catalase isozymes (Kat1 and Kat2) from two distinct genes. The expression of Kat1 was detected throughout growth, while Kat2 was expressed only at the stationary phase.
2. Analysis of the *Xp* KatE amino acid sequence showed that it belonged to the NADPH-independent, Group II catalases. It shared a close homology with *E. coli* KatE at the active- and heme-binding sites and lacked an NADPH-binding site.
3. *katE* showed growth phase-dependent- and starvation-induced expression, but it was not induced by oxidants.
4. The total catalase specific activity of the *Xp katE* mutant was similar to the parental strain, indicating a novel mechanism for catalase compensation.
5. The mutant did not show any increase in susceptibility to killing concentrations of H_2O_2, tBOOH, and MD when compared to a parental strain at exponential and stationary phases of growth.

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

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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

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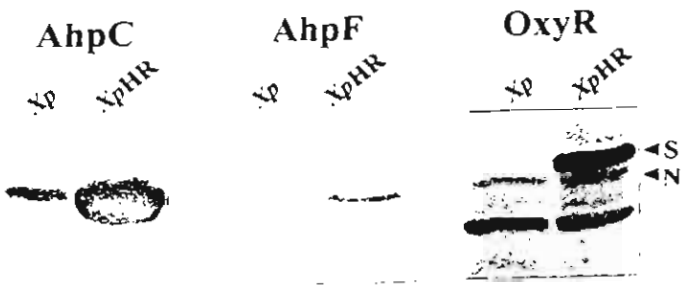

FIG. 1. Western analysis of AhpC, AhpF, and OxyR in *Xp*HR and the parental strain, *X. campestris* pv. phaseoli (*Xp*) and *Xp*HR were grown aerobically to mid-log phase in Silva Buddenhagen (SB) medium at 28°C. Cell lysate preparation, gel electrophoresis, blotting to nitrocellulose membranes, and 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-

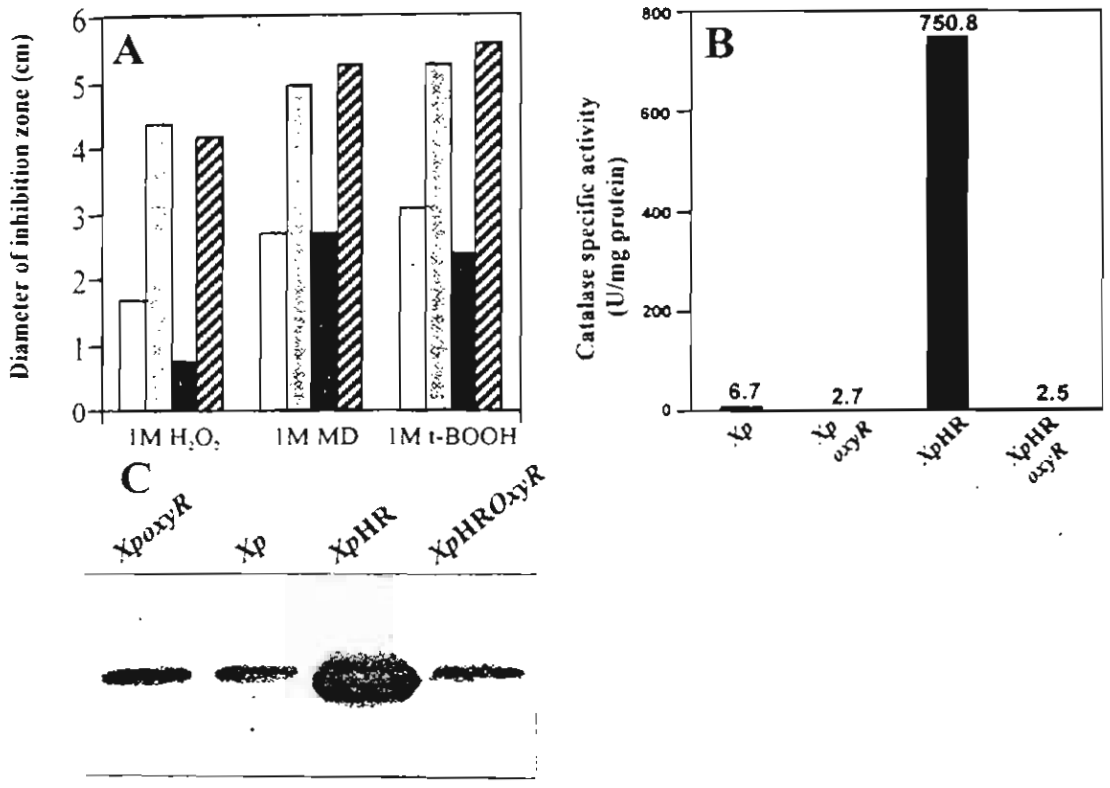

FIG. 2. Resistance to oxidant killing and levels of peroxide-scavenging enzymes in *Xp*HR and its parental strain. (A) Qualitative determination of levels of resistance to killing concentrations of H_2O_2, menadione (MD), and *tert*-butyl hydroperoxide (t-BOOH) in *X. campestris* pv. phaseoli (*Xp*; □), *X. campestris* pv. phaseoli *oxyR* (*X. 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).

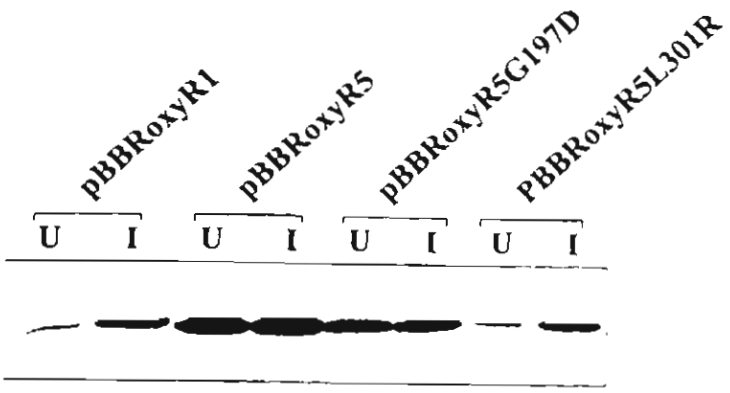

FIG. 3. Effects of various mutations in *oxyR* on levels of AhpC during uninduced and menadione-induced growth. Western analysis of AhpC levels in *X. campestris* pv. phaseoli *oxyR* harboring various *oxyR* genes on an expression vector from a parental strain (pBBRoxyR1), the *Xp*HR mutant (pBBR*oxyR5*), and the gene with one amino acid changed (pBBRoxyR5G197D and pBBRoxyR5L301R). 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 pBBRoxyR5G197D 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*.

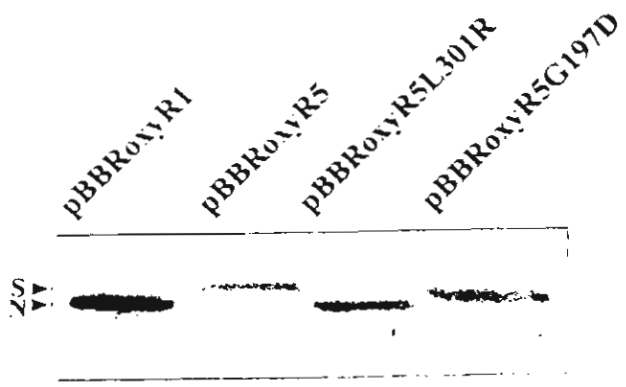

FIG. 4. Effects of mutations in *oxyR* on protein mobility. Cell growth, lysate preparation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western analysis of OxyR were performed as described previously (17) and in Fig. 1. Proteins from lysates (20 μg) were loaded into each lane. N and 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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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 H_2O_2 resulted in 100-fold protection compared to that for cells treated with the individual oxidants. Treatment of *X. campestris* with a combination of H_2O_2 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 H_2O_2 and *tert*-butyl hydroperoxide plus H_2O_2. These data suggest that H_2O_2 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 H_2O_2-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 (H_2O_2, 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_{620} of 0.1. Bacterial growth was monitored spectrophotometrically at A_{620}. Both log-phase (A_{620} of 0.5 after 4 h) and stationary-phase (A_{620} 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 H_2O_2 (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 H_2O_2, and 20 mM H_2O_2 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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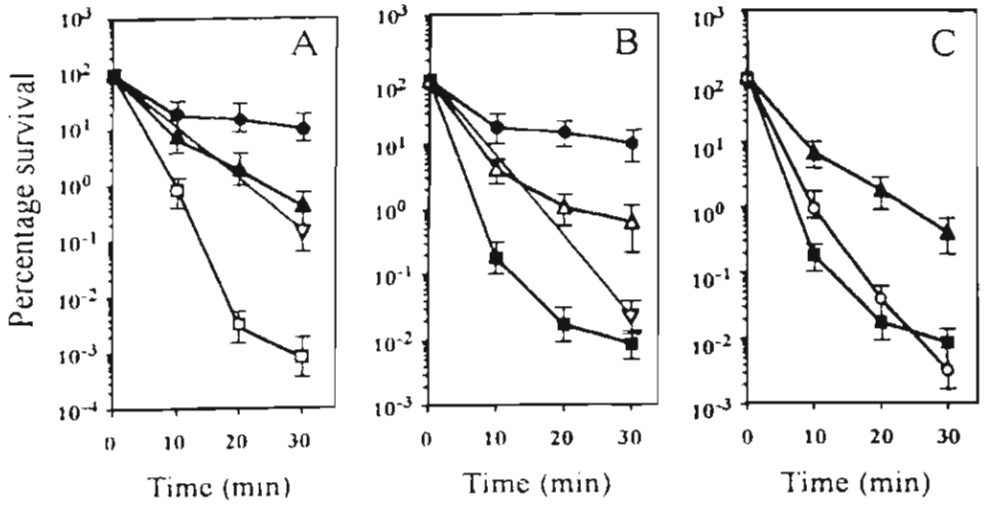

FIG. 1. Killing of *X. campestris* pv. phaseoli by multiple oxidants. *X. campestris* culture growth and oxidant treatment were performed as described in Materials and Methods. (A) *X. campestris* cultures were exposed to 40 mM MD (●), 15 mM tBOOH (▲), 40 mM MD plus 15 mM tBOOH (□), and 40 mM MD plus 15 mM tBOOH under anaerobic conditions (▽). (B) *X. campestris* cultures were exposed to 40 mM MD (●), 20 mM H_2O_2 (■), 40 mM MD plus 20 mM H_2O_2 (□), and 40 mM MD plus 20 mM H_2O_2 under anaerobic conditions (▽). (C) *X. campestris* cultures were exposed to 15 mM tBOOH (▲), 20 mM H_2O_2 (■), and 15 mM tBOOH plus 20 mM H_2O_2 (□). 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), H_2O_2, and combinations of these oxidants (MD plus tBOOH, MD plus H_2O_2, and tBOOH plus H_2O_2). The results are shown in Fig. 1. *X. campestris* was resistant to MD killing, but was highly and moderately sensitive to H_2O_2 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 H_2O_2 gave a 100-fold increase in protection compared to killing by H_2O_2 alone (Fig. 1B). This increased protection, resulting from MD-plus-H_2O_2 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-H_2O_2 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 H_2O_2 (4, 11), suggesting that intracellular superoxide anions are converted to H_2O_2 by either enzymatic or nonenzymatic reactions, and that H_2O_2 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 H_2O_2 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 H_2O_2 killing (16). Thus, it is likely that MD-induced resistance to H_2O_2 killing was responsible for the observed increased resistance to MD-plus-H_2O_2 killing. This idea is supported by the data presented in Fig. 2A showing that high levels of catalase conferred protection against MD-plus-H_2O_2 killing.

Treatment of the bacteria with a combination of tBOOH plus H_2O_2 neither enhanced nor protected them from the lethal effects of these agents. Although high concentrations of H_2O_2 are known to cause formation of organic peroxide (8), no additive lethal effects arising from tBOOH-plus-H_2O_2

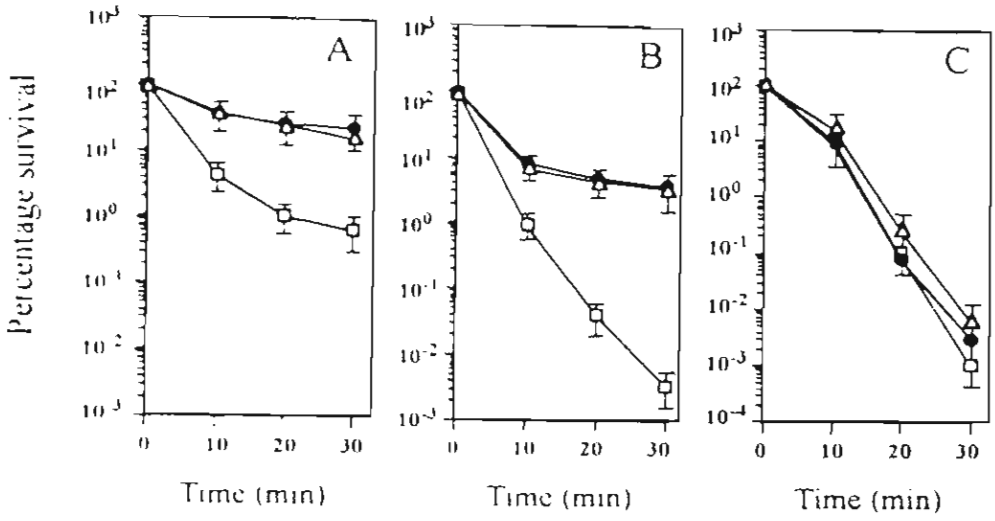

FIG. 2. Effects of high levels of catalase on oxidant killing. *X. campestris* pv. phaseoli cells harboring pUFR047 (□), pUFRkat (△) (15), and pUFRoxyR (●) (14) were grown as described in Materials and Methods and treated with the indicated concentrations of oxidants. 40 mM MD plus 20 mM H_2O_2 (A), 15 mM tBOOH plus 20 mM H_2O_2 (B), [illegible] mM MD [illegible] 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 H_2O_2 treatment alone (Fig. 1C). This suggests that H_2O_2 was responsible for killing the bacteria. The results in Fig. 2B show that high levels of catalase conferred protection against the tBOOH-plus-H_2O_2 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 H_2O_2 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-H_2O_2 and tBOOH-plus-H_2O_2 treatments was investigated. *X. campestris* pv. phaseoli harboring the plasmid pUFRkat (15) or the vector alone (pUFR047) had catalase-specific activities of 148 and 0.5 U/mg of protein, respectively. These cells were treated with MD plus H_2O_2 and tBOOH plus H_2O_2. The results (Fig. 2A and B) show that *X. campestris* harboring pUFRkat were more than 100-fold more resistant to MD-plus-H_2O_2 and tBOOH-plus-H_2O_2 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 H_2O_2 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-H_2O_2 and tBOOH-plus-H_2O_2 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 μM) 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 H_2O_2-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

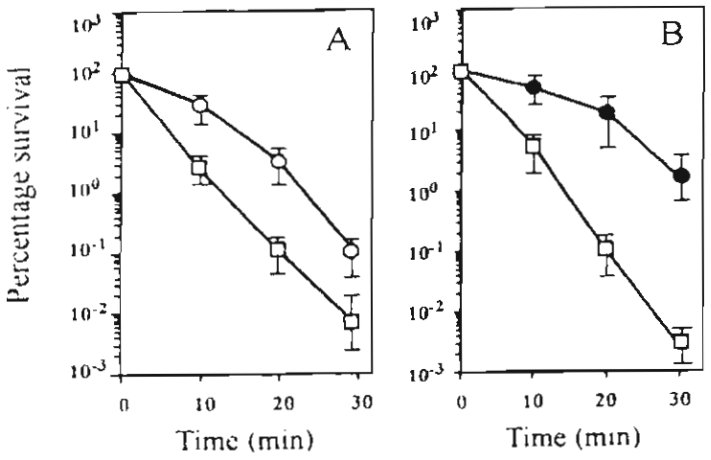

FIG. 3. MD-plus-tBOOH killing of cells. (A) Log-phase cells (□) or stationary-phase cells (○) of *X. campestris* pv. phaseoli were treated with 40 mM MD plus 15 mM tBOOH, as described in Materials and Methods. (B) *X. campestris* pv. phaseoli (□) and a spontaneous H_2O_2-resistant mutant, *X. campestris* 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 H_2O_2 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 H_2O_2, 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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Molecular and physiological analysis of an OxyR-regulated *ahpC* promoter in *Xanthomonas campestris* pv. *phaseoli*

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Summary

In *Xanthomonas campestris* pv. *phaseoli*, a gene for the alkyl hydroperoxide reductase subunit C (*ahpC*) had unique patterns of regulation by various forms of OxyR. Reduced OxyR repressed expression of the gene, whereas oxidized OxyR activated its expression. This dual regulation of *ahpC* is unique and unlike all other OxyR-regulated genes. The *ahpC* transcription start site was determined. Analysis of the region upstream of the site revealed promoter sequences that had high homology to the *Xanthomonas* consensus promoter sequence. Data from gel shift experiments indicated that both reduced and oxidized OxyR could bind to the *ahpC* regulatory region. Moreover, the reduced and the oxidized forms of OxyR gave different DNase I footprint patterns, indicating that they bound to different sites. The oxidized OxyR binding site overlapped the −35 region of the *ahpC* promoter by a few bases. This position is consistent with the role of the protein in activating transcription of the gene. Binding of reduced OxyR to the *ahpC* promoter showed an extended DNase I footprint and DNase I hypersensitive sites, suggesting that binding of the protein caused a shift in the binding site and bending of the target DNA. In addition, binding of reduced OxyR completely blocked the −35 region of the *ahpC* promoter and prevented binding of RNA polymerase, leading to repression of the gene. Monitoring of the *ahpC* promoter activity *in vivo* confirmed the location of the oxidized OxyR binding site required for activation of the promoter. A mutant that separated OxyR regulation from basal *ahpC* promoter activity was constructed. The mutant was unable to respond to oxidants by increasing *ahpC* expression. Physiologically, it had a slower aerobic growth rate and was more sensitive to organic peroxide killing. This indicated that oxidant induction of *ahpC* has important physiological roles in normal growth and during oxidative stress.

Introduction

Xanthomonas campestris pv. *phaseoli* is an important aerobic bacterial plant pathogen. During plant–microbe interactions, bacteria are exposed to reactive oxygen species (ROS) including H_2O_2, organic peroxides and superoxide anions synthesized by plants as an active defence response to inhibit bacterial proliferation (Levine *et al*., 1994; Baker and Orlandi, 1995). Moreover, normal aerobic metabolism generates a considerable quantity of these ROS (Gonzalez-Flecha and Demple, 1997). *Xanthomonas* species have evolved complex strategies to protect themselves from ROS (Loprasert *et al*., 1996).

The ability to detoxify organic peroxides is important to cell survival. Organic peroxides participate in free radical reactions that result in increased production of the toxic radicals. Alkyl hydroperoxide reductase (AhpR) is the best-characterized organic peroxide detoxification enzyme in bacteria (Demple, 1991; Storz and Imlay, 1999). The enzyme consists of two subunits, the 22 kDa catalytic C subunit that reduces organic peroxides to corresponding alcohols (Poole, 1996), and the 56 kDa reductase F subunit that is involved in regeneration of oxidized AhpC using either NADPH or NADH as cofactors (Poole and Ellis, 1996). Purified AhpR can also use H_2O_2 as a substrate (Niimura *et al*., 1995; Poole, 1996). *ahpC* is highly conserved and can be found in organisms from bacteria to man (Chae *et al*., 1994). This suggests that it serves important physiological roles. Mutations in *ahpC* result in increased sensitivity to organic peroxide toxicity and in an altered overall oxidative stress response (Storz *et al*., 1989; Antelmann *et al*., 1996; Bsat *et al*., 1996; Rocha and Smith, 1999).

In many bacteria, understanding the regulation of *ahpC* has been of major interest as alterations in *ahpC*

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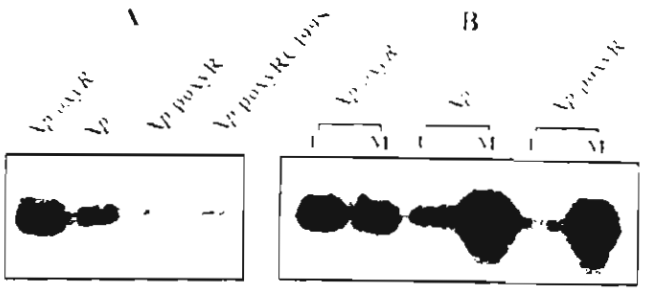


Fig. 1. Northern analysis of the effect of OxyR on *ahpC* expression. Total RNA was isolated from *X. campestris* pv. *phaseoli* XO1 (*oxyR* mutant (*Xo oxyR*), *X. campestris* pv. *phaseoli* harbouring poxyR wild-type *oxyR* on pBBR1MCS-4 (*Xp poxyR*) or poxyRC199S on pBBR1MCS-4 (*Xp poxyRC199S*) grown aerobically in SB either uninduced (U) or induced with 100 μM menadione (M). These RNA samples were separated on 1% agarose gels and probed with *ahpC* as described in *Experimental procedures*. The autoradiographs show hybridization of *ahpC* probe with uninduced RNA samples (A) and both uninduced and induced RNA samples (B).

expression have profound effects on bacterial physiology. For example, in *Mycobacterium tuberculosis*, *ahpC* promoter mutations confer resistance to the antibiotic isoniazid (Sherman *et al.*, 1996; Wilson and Collins, 1996; Heym *et al.*, 1997). In *Xanthomonas*, a multiple peroxide resistance mutant has an unregulated high-level expression of *ahpC* (Fuangthong and Mongkolsuk, 1997). In all bacterial systems studied thus far, *ahpC* expression is oxidative stress inducible in a manner dependent on either OxyR, a peroxide sensor and transcription regulator in Gram-negative bacteria, or PerR, a peroxide stress regulator in Gram-positive bacteria (Storz *et al.*, 1990; Bsat *et al.*, 1996; Dhandayuthapani *et al.*, 1997; Mongkolsuk *et al.*, 1998a; Storz and Imlay, 1999). OxyR can exist in two forms; in uninduced cells, OxyR exists in a reduced form. Upon exposure to peroxide, OxyR becomes oxidized by the formation of a disulphide bond at the highly conserved C199–C208 cysteine residues (Zheng *et al.*, 1998). In general, oxidized OxyR activates *ahpC* expression, whereas reduced OxyR does not interact with the gene promoter region (Toledano *et al.*, 1994). Detailed analysis of molecular interactions between OxyR and *ahpC* promoters have been performed in a few cases (Toledano *et al.*, 1994; Hattman and Sun, 1997). Results from these studies have shown variation in OxyR binding sites and in their effect on gene expression.

In *Xanthomonas*, *ahpC* is transcribed as a monocistronic mRNA. The gene is located upstream of the *ahpF–oxyR* operon (Loprasert *et al.*, 1997). *ahpC* expression is inducible by oxidants in an OxyR-dependent manner (Mongkolsuk *et al.*, 1997; 1998a). Here, we describe the effects of different redox states of OxyR on *ahpC* expression. Molecular interactions between different forms of OxyR and the *ahpC* promoter were also characterized. In addition, we examined the physiological

Results and discussion

OxyR regulation of *ahpC* expression

X. campestris *ahpC* was [illegible] compared with other OxyR-regulated genes in bacteria. Oxidant induction of the gene [illegible] that *oxyR* Mongkolsuk [illegible] we have observed that the [illegible] *oxyR* mutant are higher than the [illegible] parental strain (Mongkolsuk *et al.*, 1998a). This suggested that reduced and oxidized OxyR might differently from regulate *ahpC*. Experiments were performed to test this hypothesis. First, we compared *ahpC* mRNA in an *oxyR* mutant with that uninduced levels in its parental *X. campestris* pv. *phaseoli* strain (Fig. 1A). This finding is in agreement with previous Western analysis of AhpC in an *oxyR* mutant (Mongkolsuk *et al.*, 1998a). In normal growing cells, OxyR exists in reduced form (Zheng *et al.*, 1998). The data implied that *ahpC* expression was repressed by reduced OxyR in the parental strain and that inactivation of *oxyR* led to relief of this repression. This observation was extended by determining *ahpC* mRNA levels in an *oxyR* mutant harbouring either poxyR (Mongkolsuk *et al.*, 1998a), which produced high levels of the reduced OxyR, or poxyRC199S, which had the critical redox active C199 residue mutated to S199. This mutation locks OxyR in the reduced form (Kullik *et al.*, 1995). The results shown in Fig. 1A showed a threefold reduction in the amount of *ahpC* mRNA detected in cells harbouring these plasmids when compared with the uninduced level in the *X. campestris* pv. *phaseoli* strain. These observations confirmed that reduced OxyR repressed *ahpC* expression.

The *oxyR*-dependent oxidant induction of *ahpC* suggests that oxidized OxyR activates expression of the gene. The idea was tested using an inducing concentration of menadione (MD) to treat *X. campestris* pv. *phaseoli* the strain harbouring poxyR and the *oxyR* mutant. In oxidant treatment of these cells, MD was used instead of H_2O_2 because *Xanthomonas* harbouring various poxyR plasmids produced high levels of catalase that rapidly degraded H_2O_2. Northern analysis of *ahpC* mRNA levels of these cells (data not shown) revealed that 100 μM MD induced *ahpC* expression in *X. campestris* pv. *phaseoli* and in the strain harbouring poxyR (Fig. 1B), to high levels. Expression of the *ahpC* mRNA was induced to similarly high levels in the two strains, although the uninduced level of the gene was much lower in *X. campestris* pv. *phaseoli* poxyR. This suggested that

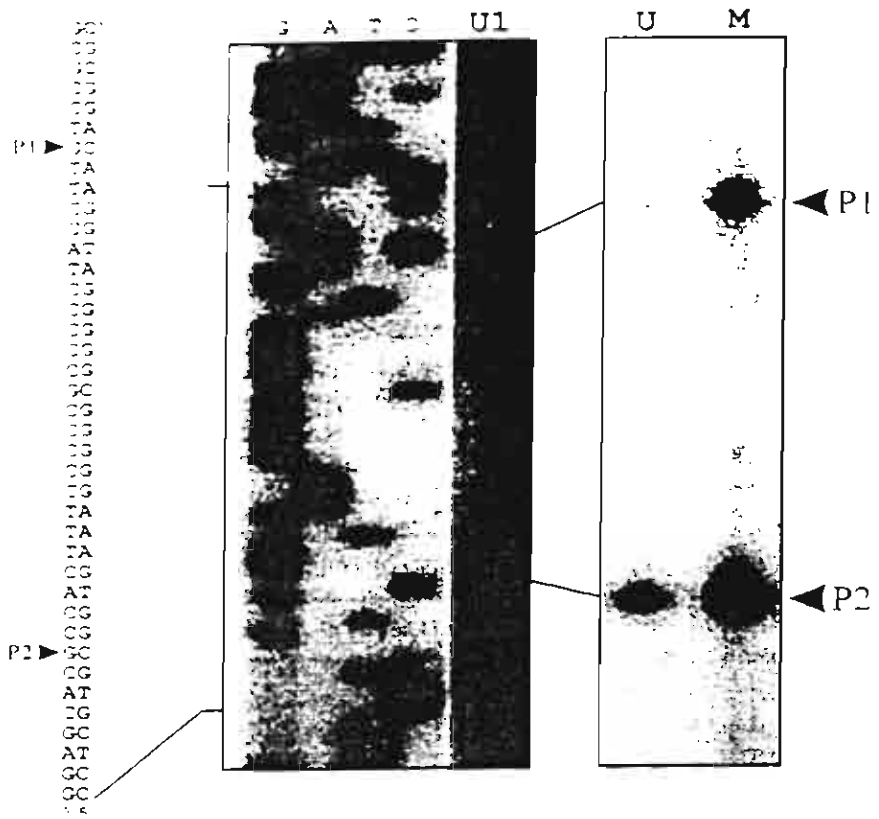


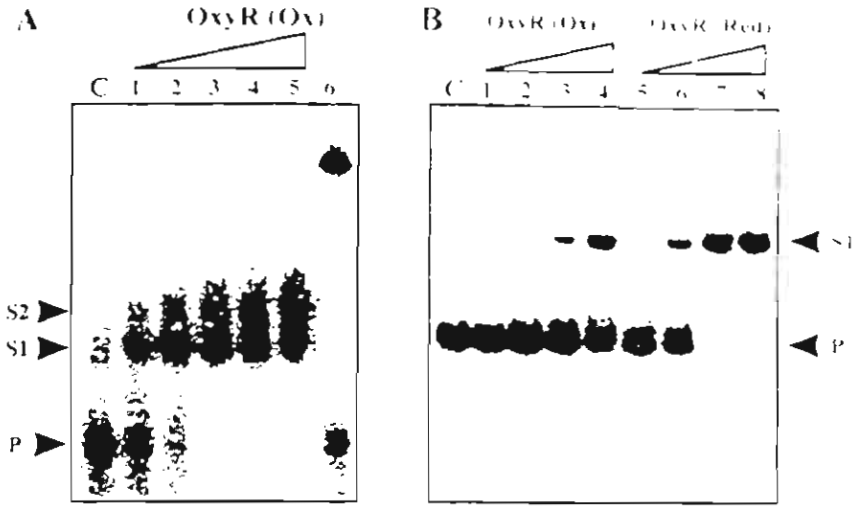
Fig. 2. Primer extension analysis to locate transcription start sites of *ahpC*. Primer extension was performed using RNA isolated from uninduced samples (U) or samples induced with 100 μM menadione (M) as described in *Experimental procedures*. G, A, T and C were sequence ladders. P1 and P2 indicate the position of primer extension products. Lane U1 was a longer exposure of lane U. The position of transcription start sites with respect to the *ahpC* sequence is shown on the left.

ahpC was expressed close to the maximum level after MD treatment in both strains. As expected, MD induction was dependent on functional *oxyR* and was absent in the *oxyR* mutant (Mongkolsuk *et al.*, 1998a). *ahpC* mRNA levels detected in the *oxyR* mutant represented unrepressed levels but in wild-type cells treated with oxidant, *ahpC* expression was activated further. This indicates that *ahpC* could be regulated either negatively or positively depending on the redox state of OxyR. This is unique to *Xanthomonas ahpC*. In all other OxyR-regulated genes in various bacteria, different forms of OxyR either repressed or activated gene expression, but not both from the same promoter (Toledano *et al.*, 1994; Dhandayuthapani *et al.*, 1997; Hattman and Sun, 1997).

Identification of ahpC transcription start sites

To understand the mechanisms of OxyR regulation of *ahpC*, it was essential to determine the location of the *ahpC* promoter. Primer extension experiments were performed to locate *ahpC* transcription start sites. The results presented in Fig. 2 show two major primer extension products designated P1 and P2. These transcription start sites corresponded to positions 40 bp for P1 and 15 bp for P2 upstream of the *ahpC* translation initiation codon (ATG) (Fig. 5). The *Xanthomonas* consensus promoter sequences are TTGTNN at the −35 region and T/GATNAA/T at the −10 region, although the distance between these two sequence motifs varied from 16 bp to 24 bp (Katzen *et al.*, 1996). Examination of the upstream region of the weaker transcription start site (P1) showed sequences TTGAGG and TACCAT at the −35 and −10 regions of P1, respectively, and they were separated by 17 bp. These two regions of the P1 promoter show five out of six nucleotides that matched the promoter consensus sequences and have perfect distance between the two conserved regions. The data suggest that P1 probably acts as a strong promoter *in vivo*. On the other hand, P1 accounted for less than 30% of the primer extension products. Inspection of the region upstream of the start of the major primer extension product (P2) revealed no promoter-like sequences that had homology to *Xanthomonas* promoter consensus sequences. We suggest that the P2 product probably corresponds to premature termination of reverse transcriptase caused by the presence of poly(C) residues (13 C out of 18 residues) (Fig. 5) upstream of the P2 transcription start site.

Next, we examined whether the increased amounts of *ahpC* mRNA in response to the oxidant treatments shown in Fig. 1B resulted from increased transcription initiation of the gene. Primer extension experiments were performed on RNA samples extracted from uninduced and MD-induced *Xanthomonas* cultures (Fig. 2). In the



uninduced sample, there was much less primer extension product compared with the MD-induced sample (Fig. 2). This suggested that increased transcription initiation was responsible for the induction of *ahpC* expression. Moreover, the observation was consistent with the model in which oxidized OxyR activates gene transcription by recruiting RNA polymerase to the promoter region (Toledano *et al.*, 1994).

Different forms of OxyR had differed binding affinity to the ahpC *promoter region*

To understand better the regulation of *ahpC* by various forms of OxyR, the protein–DNA binding gel shift technique was used to investigate binding of OxyR to the *ahpC* promoter. OxyR was purified as described in *Experimental procedures*. The results of a gel shift experiment are shown in Fig. 3A. The addition of increasing amounts of purified OxyR to end-labelled DNA fragments containing the *ahpC* promoter (from −112 to −93) resulted in the retardation of fragment migration in native polyacrylamide gels. Two slower migrating DNA bands designated S1 and S2 were detected when OxyR was added to the binding reactions (Fig. 3A). The faster migrating band S1 was detected when low concentrations of OxyR were added to binding reactions. As OxyR concentrations increased, a second slower migrating DNA fragment (S2) was apparent. The addition of a 100-fold greater concentration of a non-specific protein such as bovine serum albumin (BSA) did not produce any DNA fragment mobility shift (data not shown). However, adding an anti-OxyR polyclonal antibody to the binding reaction after the addition of OxyR resulted in supershifting of the protein–DNA complex (Fig. 3A). These data confirmed that the observed gel retardation of DNA fragments resulted from OxyR binding to the *ahpC* promoter. Although [illegible] might bind to the *ahpC* promoter [illegible] could be responsible for the observed S2 band [illegible] shift experiment (Fig. 3A).

We wished to determine and to compare the affinity of the oxidized and the reduced forms of OxyR for the *ahpC* promoter using gel shift assays. Dithiothreitol (DTT, 200 mM) was added to the reaction to reduce OxyR. The results in Fig. 3B show that both oxidized and reduced OxyR could bind to the DNA fragment containing the *ahpC* promoter. As the binding reactions were carried out at low OxyR concentrations, the second S2 species of retarded DNA fragment was not detected. Densitometer analysis of the ratio of bound versus unbound DNA fragments indicated that reduced OxyR had about 20% higher affinity for the *ahpC* promoter than oxidized OxyR. This small difference in affinity was observed consistently even when different batches of OxyR and labelled *ahpC* promoter fragments were used. This supports the observations from Fig. 1 that only oxidized OxyR in uninduced cells would bind to the *ahpC* promoter and inhibit its transcription. Unlike other bacterial species, *Xanthomonas* to oxidants induces synthesis and accumulation of oxidized OxyR. Moreover, [illegible] Although oxidized OxyR has lower affinity for the *ahpC* promoter, this does not affect [illegible] promoter by the protein caused by [illegible] centration of oxidized OxyR as a result [illegible] treatment.

Determination of reduced and oxidized OxyR binding sites

Xanthomonas ahpC promoter is unique with regard to differential regulation by reduced and oxidized forms of OxyR. This allowed for an examination of how different forms of OxyR interacted with [illegible]

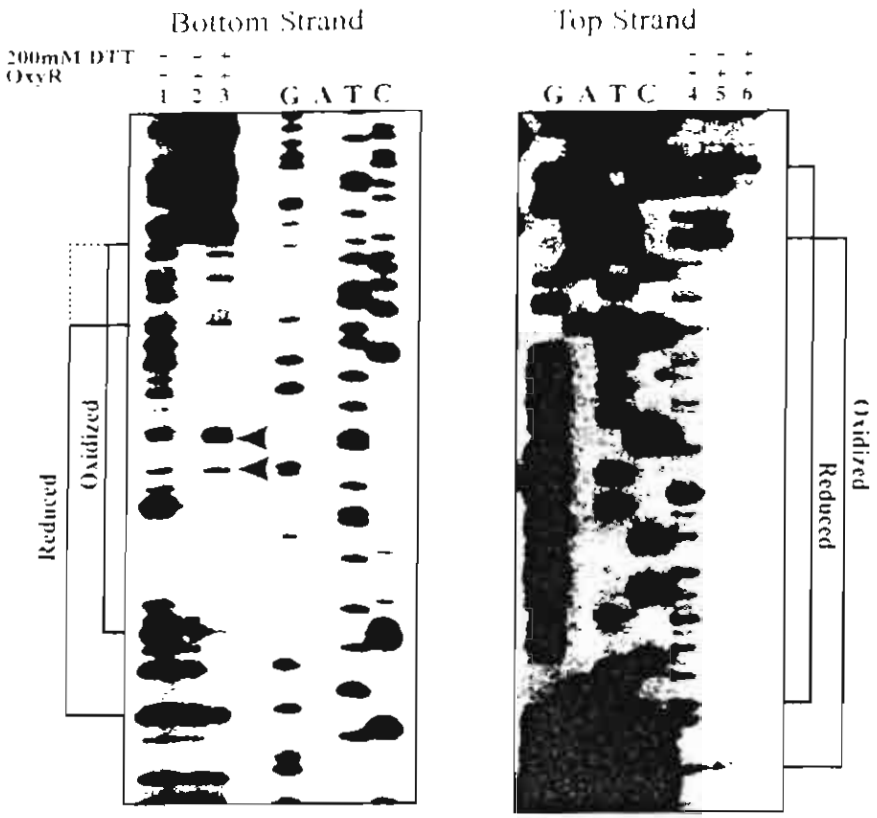


Fig. 4. DNase I protection assay to locate OxyR binding sites. Experiments were performed as described in *Experimental procedures*. Lanes 1–6 were DNase I-digested probes for top and bottom strands of the *anpC* promoter, with or without OxyR and DTT. G, A, T and C, sequence ladder. Solid brackets indicates regions protected by OxyR. Dotted line indicates region of partial protection by OxyR. Arrows show DNase I hypersensitive sites.

achieve differential effects on a single promoter. DNase I footprinting experiments were performed to locate OxyR binding sites (Fig. 4). Clearly, binding of the reduced and oxidized OxyR to both top and bottom strands gave distinct footprint patterns. On the bottom strand, binding of oxidized OxyR protected a region from −80C to −34G, whereas binding of reduced OxyR showed an extended footprint covering the region −67A to −24T. In addition, a partial DNase I protected region from −80C to −67A was also detected (Figs 4 and 5). Major and minor DNase I hypersensitive sites in the middle of the reduced OxyR protected region at −52A, −53T and −48C, respectively, were also observed (Fig. 4). On the top strands, binding of reduced or oxidized OxyR protected DNA from −78A to −28T and from −73A to −15C respectively.

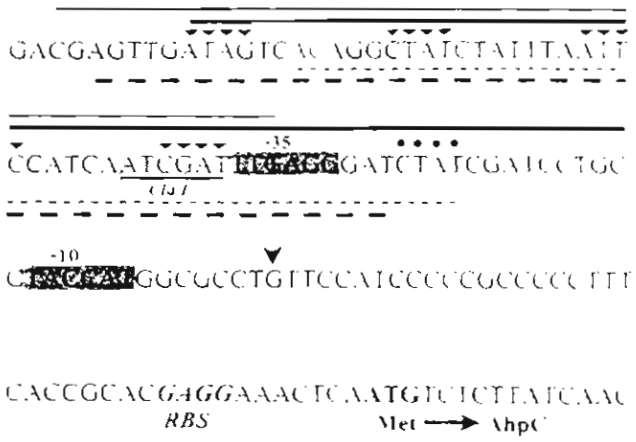


Fig. 5. Nucleotide sequence of the regulatory regions of *anpC*. The positions of the *anpC* promoter (−10 and −35 regions are shaded), regions protected by oxidized (——— top strand and ——— bottom strand) and reduced (— — — top strand and - - - - - bottom strand) OxyR, conserved motifs for OxyR binding site (▼▼▼), proposed fifth motif involved in reduced OxyR binding (●●●), a transcription start site (▼), a ribosome binding site (RBS), the translation initiation codon of *anpC* and the *Cla*I site used for making deletions and the *anpCE* mutant are shown.

Major and minor DNase I hypersensitive sites in the middle of the reduced OxyR protected region at −52A, −53T and −48C, respectively, were also observed (Fig. 4). On the top strands, binding of reduced or oxidized OxyR protected DNA from −78A to −28T and from −73A to −15C respectively.

The data are consistent with the idea that oxidized OxyR binds to four successive major grooves on one face of the helix, whereas reduced OxyR binds to a pair of major grooves separated by one helical turn (Toledano *et al.*, 1994). Binding of reduced OxyR introduces bending in the target DNA that results in DNase I hypersensitive sites. These observations are similar to DNase I footprints of reduced OxyR high-affinity binding sites in two other promoters, *Escherichia coli oxyR* (Toledano *et al.*, 1994) and the Mu phage *mom* operon (Sun and Hattman, 1996; Hattman and Sun, 1997), in which the protein represses expression from both promoters.

OxyR has extended binding sites, and the proposed consensus binding motif for *E. coli* has twofold dyad symmetry with the following sequence: ATAGntnnnanC-TATnnnnnnnATAGntnnnanCTAT (Tartaglia *et al.*, 1992;

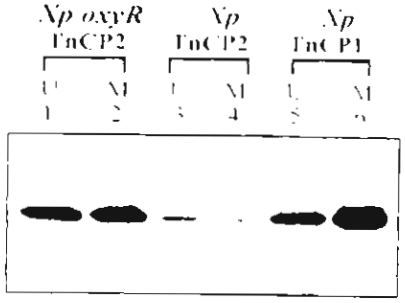


Fig. 6. Monitoring of *ahpC* promoter activities in *X. campestris* pv *phaseoli* strains containing TnCP1 and TnCP2. Transcription activity of *ahpC* promoter constructs was determined by Western analysis of Cat from the reporter gene. Log phase uninduced (U) and menadione (M)-induced cultures of *X. campestris* pv. *phaseoli* strains containing TnCP1 and TnCP2 were used for lysate preparation. Protein (30 μg) was loaded into each lane. After gel electrophoresis, the separated proteins were blotted to a nitrocellulose membrane and subsequently reacted against an anti-Cat antibody and an alkaline phosphatase-conjugated second anti-rabbit antibody.

Toledano *et al.*, 1994). Examination of the putative oxidized OxyR binding site (ATAGnxnnnanCTATnnnnnnnATxxnxnnnanCxAT) upstream of the *Xanthomonas ahpC* promoter showed a sequence motif that had 14 out of 20 bases matched to the consensus OxyR binding site (Fig. 5). In addition, it also contained the highly conserved LysR-binding motif, T-N$_{11}$-A (Schell, 1993). The proposed location of the binding site is in agreement with the footprinting data. The degree of homology of the *Xanthomonas ahpC* promoter to the consensus sequence is similar to other binding sites of OxyR-regulated genes in *E. coli* (Toledano *et al.*, 1994). Also, the *Xanthomonas* OxyR binding site has closer homology to the *E. coli* consensus sequence than to the proposed shorter binding site for *Mycobacterium* spp. (Dhandayuthapani *et al.*, 1997). The *E. coli* reduced OxyR binding is extended by one helical turn, and this is responsible for extended footprints. This suggests that a fifth contact point is essential for the reduced OxyR binding (Toledano *et al.*, 1994). We searched for a motif in the fifth region of the *ahpC* promoter and identified a CTAT motif 9 bases from the oxidized OxyR binding motif. Examination of two other high-affinity reduced OxyR binding sites showed that, 9 bases from the oxidized OxyR binding sites, there are motifs CxAT for *nom* and CxAx for *oxyR*. The location of this motif corresponds to the fifth motif of the OxyR binding site of the *E. coli oxyR* promoter. The deletion analysis of the OxyR binding site of the *E. coli oxyR* promoter has shown that removal of the fifth region affected reduced OxyR binding (Toledano *et al.*, 1994).

The region of partial DNase I protection and two DNase I hypersensitive sites caused by binding of the reduced OxyR to the region are unusual. Closer examination of the protected region revealed an alternative OxyR binding site. The alternative binding site (ATxxnntnnnxnCxATnnnnnnnATxxnxnnnanCTAT) matched at 13 out of 20 bases of the consensus OxyR binding site (Fig. 5). This site was protected from DNase I digestion by reduced OxyR, but not by oxidized OxyR (Fig. 4). It is possible that *in vitro* reduced OxyR could bind to both sites, leading to two DNase I hypersensitive sites and an area of partial DNase I protection. We are examining whether the alternative OxyR binding site functions *in vivo*. Regardless, binding of reduced OxyR to either site completely blocked the −35 region of the *ahpC* promoter leading to repression of the gene.

In vivo *promoter analysis*

The ability of a DNA fragment containing the *ahpC* promoter and OxyR binding sites to direct oxidant-inducible expression of a reporter *cat* gene *in vivo* was tested. The experiment was performed to confirm data from primer extension and DNase I footprinting experiments, which located regions important for OxyR-dependent, oxidant-inducible expression of *ahpC*. DNA fragments containing the *ahpC* promoter either with (pUTTnCP1) or without (pUTTnCP2) OxyR binding sites were transcriptionally fused to a promoterless *cat* gene and subsequently cloned into a mini-Tn*5* vector (De Lorenzo and Timmis, 1994). pUTTnCP1 and pUTTnCP2 had DNA fragments up to −209 and −39, respectively, plus the *cat* reporter gene and the rest of *ahpC*. These mini-Tn*5* constructs were mobilized and transposed into the *X. campestris* pv. *phaseoli* chromosome. The *ahpC* promoter activity in *X. campestris* pv. *phaseoli* TnCP1 and TnCP2 was determined by Western analysis of Cat (Fig. 6). Densitometer analysis of the data showed that MD treatment induced more than twofold increased Cat levels in the strain containing TnCP1. Similar treatment did not result in increased Cat levels in the strain containing TnCP2. Oxidant-inducible *cat* expression in the strain containing TnCP1 and lack of induction in the strain containing TnCP2 confirmed the proposed binding site of the oxidized OxyR required for *in vivo* activation of the promoter. Unexpectedly, basal Cat levels in the strain containing TnCP2 were threefold less than those in the strain containing TnCP1. The *Cla*I deletion of the *ahpC* promoter removed most of the reduced OxyR binding sites, but left the promoter intact. This should relieve reduced OxyR repression of the promoter, leading to higher levels of gene expression from the *ahpC* promoter similar to the higher levels of *ahpC* expression in the *oxyR* mutant (Fig. 1). The contradictory results shown in Fig. 6 suggested that the *ahpC* promoter might require regions upstream of −35 for full uninduced activity. Interestingly, the *Cla*I deletion of the *ahpC* promoter (TnCP2) had half

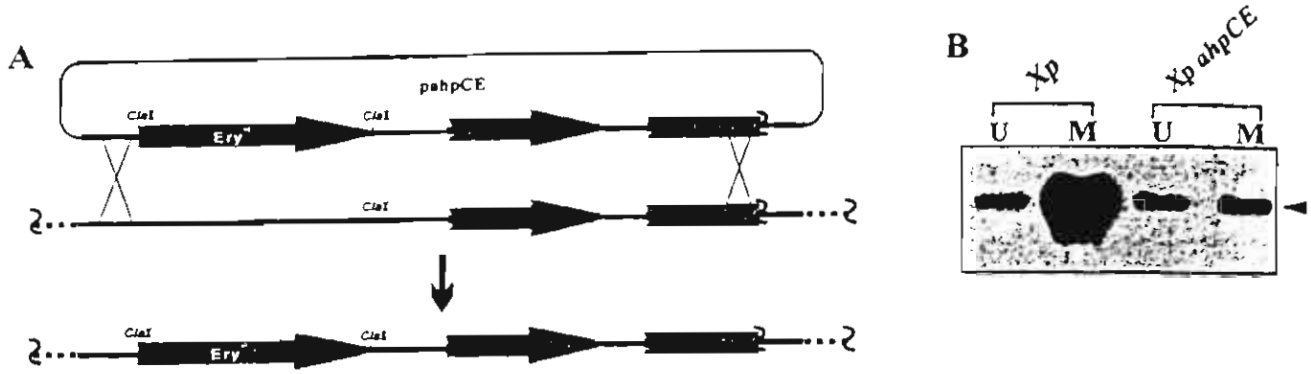


Fig. 7. Construction and analysis of the *ahpCE* mutant that uncoupled OxyR regulation from basal expression of *ahpC*.
A. Construction of the *ahpCE* mutant and its marker exchange into the chromosome of *X. campestris* pv. *phaseoli* is shown.
B. Northern analysis of *ahpC* expression in the mutant (*Xp ahpCE*) and a parental strain (*Xp*). Total RNA (5 μg) was loaded into each lane after gel electrophoresis; fractionated RNA samples were transferred to a nylon membrane and probed with radioactively labelled *ahpC*. The arrow indicates the position of positively hybridized *ahpC* mRNA.

the extended reduced OxyR binding site that contained a CTAT palindrome sequence and a conserved LysR-binding motif (T-N_{11}-A) (Schell, 1993) intact (Fig. 5). In *Mycobacterium*, a shorter region containing one palindrome sequence and a conserved LysR binding site is thought to be sufficient for OxyR binding (Dhandayuthapani *et al.*, 1997; Pagan-Ramos *et al.*, 1998). This shorter OxyR binding site has not been demonstrated to be functional in Gram-negative bacteria. In order to account for the Cat levels in the strain containing TnCP2 (Fig. 6), we proposed that, in the absence of normal OxyR binding sites, it is possible that OxyR could bind to the half-palindrome CTAT site, as exposure to an oxidant did not effect this repression, suggesting that both reduced and oxidized OxyR could bind to the half-site. The half OxyR binding site and the conserved LysR-binding motif are located between the −35 and −10 regions of the *ahpC* promoter. Binding of either reduced or oxidized OxyR to these sites prevents RNA polymerase binding to the promoter by blocking the −35 region. The results in Fig. 6 show that basal levels of Cat specified by TnCP2 in the *oxyR* mutant were higher than the Cat level attained in a parental strain. These results suggested that, in the absence of OxyR, no repression occurred at the *Cla*I-deleted *ahpC* promoter.

Characterization of a regulatory mutant of ahpC

Alterations in *ahpC* expression in various bacteria have important physiological consequences (Antelmann *et al.*, 1996; Dhandayuthapani *et al.*, 1996; Sherman *et al.*, 1996; Fuangthong and Mongkolsuk, 1997; Rocha and Smith, 1999). The majority of known mutations are located in promoter or structural regions. No mutants have been constructed in genes for oxidative stress protection that separated oxidant regulation from basal expression levels without inactivation of either structural or regulatory genes. Such mutants would permit analysis of the role of oxidant-induced alteration in gene expression on bacterial physiological responses. We were interested in making an *ahpC* mutant that uncoupled gene regulation from the basal level of gene expression without knocking out either *ahpC* or *oxyR*. This would provide a unique opportunity to examine the physiological

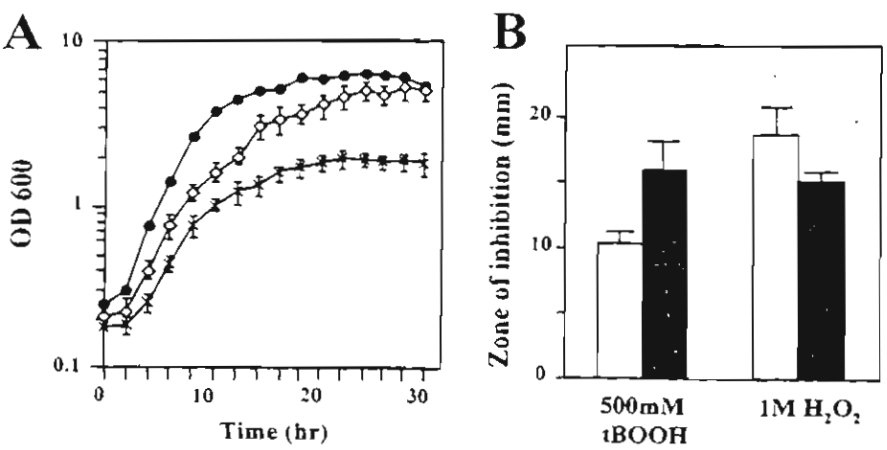


Fig. 8. Physiological analysis of a *X. campestris* pv. *phaseoli ahpCE* mutant.
A. Aerobic growth of *X. campestris* pv. *phaseoli* (●), the *ahpCE* mutant without (○) and with 150 μM tBOOH (x) in SB at 28°C was monitored spectrophotometrically at A_{600}.
B. The diameter of the zone of growth inhibition; 7 μl of the indicated concentrations of tBOOH (500 mM) and H_2O_2 (1 M) were spotted onto 6 mm paper discs before being placed on the *ahpCE* mutant (shaded box) and a parental strain (unshaded box) cell lawn. All experiments were repeated four times. Error bars represent the standard error of the mean.

roles of oxidant induction of *ahpC* expression during normal growth and peroxide stress. An *ahpC* mutant was constructed by the insertion of an ery^R at the *ClaI* site (Fig. 7A). This separated OxyR binding sites from the *ahpC* promoter. The mutated gene was transferred into *X. campestris* pv. *phaseoli* and marker exchanged into the chromosome, as described in *Experimental procedures* and Fig. 7A. This resulted in a *X. campestris* pv. *phaseoli ahpCE* mutant (Fig. 7). The integrity of the mutant was confirmed by Southern analysis (data not shown). Northern analysis revealed that *ahpC* was no longer inducible by menadione in the mutant (Fig. 7B). The data confirmed that insertion of an ery^R gene between the OxyR binding site and the promoter separated OxyR activation of gene expression from normal promoter functions.

We have observed that mutations in genes involved in oxidative stress response often resulted in altered aerobic growth and sensitivity to oxidants (Mongkolsuk *et al.*, 1996; 1998a). Mutations in *ohr*, a gene involved in organic peroxide protection in *Xanthomonas*, show increased sensitivity to organic peroxide killing (Mongkolsuk *et al.*, 1998b). Next, we determined the physiological consequences of separation of OxyR regulation from basal *ahpC* promoter functions. First, the mutant aerobic growth rate was determined in a complex SB medium (Fig. 8A). Under these conditions, the mutant had a slower doubling time of 140 min, compared with 110 min for the parental strain. Nevertheless, the mutant reached a similar density to the parental strain by stationary phase. Next, we examined growth of the mutant in the presence of a low concentration (150 μM) of tBOOH. In the presence of tBOOH, the mutant had a doubling time of 170 min, compared with 110 min for the parental strain (Fig. 8A). We extended these observations by determining the mutant resistance levels to killing concentrations of tBOOH and H_2O_2. With killing concentrations of tBOOH and H_2O_2, respectively, the mutant showed a growth inhibition zone of 16 mm and 15 mm, compared with 10.5 mm and 19 mm in the parental strain (Fig. 8B). Normal aerobic metabolism generated H_2O_2 and organic peroxide, which could induce *ahpC* expression via OxyR (Gonzalez-Flecha and Demple, 1997). In the mutant, the inability to respond to increased levels of oxidant probably led to the accumulation of toxic organic peroxides that resulted in the observed slower growth rate (Fig. 8A). This deficiency was accentuated when the mutant was exposed to exogenous organic peroxide, as shown by severely reduced growth rate and resistance levels. Unexpectedly, the mutant had small increases in resistance to H_2O_2 killing, which correlated with a small increase in total catalase levels. The mutant had 3.5 U mg^{-1} compared with 5.6 U mg^{-1} protein in the parental strain. The small increase in catalase levels could result from a compensatory response in the mutant for its inability to increase *ahpC* expression in response to changes in the environment. A similar compensatory response has been observed in *Bacillus subtilis* (Antelmann *et al.*, 1996; Bsat *et al.*, 1996).

In the absence of exogenous oxidants or under growth conditions in which fewer oxidants were being generated intracellularly, *ahpC* expression would be repressed by reduced OxyR. However, when cells are exposed to oxidants up-expression of the gene can be achieved rapidly by activation of the promoter by oxidized OxyR. The dual regulation of the *ahpC* promoter by both reduced and oxidized forms of OxyR allows fine tuning of AhpC levels. Analysis of the mutant that lacks OxyR regulation of *ahpC* expression physiological responses suggested that fine tuning of gene regulation is important to the cells. In the mutant, the gene basal level of expression was not sufficient to protect cells from oxidants generated intracellularly, as reflected by a slower aerobic growth rate. This deficiency was more pronounced when the mutant was challenged with organic peroxide.

Experimental procedures

Growth, culture and oxidant killing conditions

All *Xanthomonas* strains were grown aerobically in SB medium at 28°C. *E. coli* strains were grown in LB at 37°C. For *Xanthomonas*, the following concentrations of antibiotics were used; kanamycin 30 μg ml^{-1}; erythromycin 100 μg ml^{-1}. To test the effects of oxidants on the growth rate of *Xanthomonas*, overnight cultures were diluted into fresh SB medium to give A_{600} of 0.1. The cultures were allowed to grow for 1 h before oxidants were added to give desired final concentrations. Growth was monitored spectrophotometrically at A_{600}. The killing zone method for determining the sensitivity of a strain to oxidant killing was performed by adding 10^8 log phase cells to 3 ml of warm top SB agar. The mixture was poured onto an SB agar plate. Then, 6 mm paper discs containing 7 μl of desired concentrations of oxidants were placed on the cell lawn. It was essential to use cells from similar stages of growth, as levels of resistance to oxidant killing in *Xanthomonas* varies with stage of growth (Vattanaviboon *et al.*, 1995). Zone of growth inhibition was measured after 24 h incubation.

Nucleic acids isolation and analysis

Genomic DNA extraction from *Xanthomonas* strains and Southern blot experiments were performed as described previously (Mongkolsuk *et al.*, 1996). Total RNA was isolated using the modified hot acid phenol method (Mongkolsuk *et al.*, 1996). For Northern blot experiments, RNA samples were separated on formaldehyde agarose gels and subsequently capillary transferred to nylon membranes. Radioactive labelled *ahpC* probes were prepared from polymerase chain reaction (PCR) fragments containing the *ahpC* coding region (Mongkolsuk *et al.*, 1997). PCR fragments were purified from agarose gels and radioactively labelled using

a commercial random prime kit with [α-^{32}P]-dCTP. The probes were boiled and added to hybridization bags. Prehybridization, hybridization and washing were performed under high-stringency conditions as described previously (Mongkolsuk *et al.*, 1996).

Primer extension experiments were carried out to determine *ahpC* transcription start sites (Storz and Altuvia, 1994). PE1 primer (5′-TTGCCGTTGTGGTACGCATT-3′) located at nucleotide position 80–99 of *ahpC* (Fig. 5) was labelled with T4 polynucleotide kinase and [γ-^{32}P]-ATP. The labelled primer was annealed with 5 μg of total RNA and incubated further at 50°C for 30 min. Then, 200 units of Superscript II MMLV reverse transcriptase was added to the reaction, and incubation was continued at 42°C for 60 min. The extension products were analysed on sequencing gels next to sequence ladders.

Purification of OxyR.

Plasmid pOXX (Mongkolsuk *et al.*, 1998a) containing *oxyR* was used as a template in PCR reactions with primer A (5′-CGTCTAGAAGGCTGCTGCATAT-3′) and primer B (5′-TTGTCGACAGCCGCAACCGCCTT-3′), which covered the 5′ and 3′ regions of the gene respectively. The 940 bp PCR products were digested with *Sal*I and *Xba*I and cloned into pCYB4 (Biolabs) digested with *Xho*I and *Xba*I. The recombinant plasmid pINT-oxyR was transformed into an *E. coli* GSO8 (*oxyR* ::*kan*^{R}>). The OxyR fusion to intein was verified using both anti-intein and anti-OxyR antibodies. For purification of OxyR, GSO8 harbouring pINT-oxyR were grown in LB to log phase and induced with 2 mM IPTG for 6 h at 28°C. We found that induced expression of *oxyR* at 28°C gave better product yields. Cells were pelleted and washed once with Tris buffer, pH 7.8. The pellet was resuspended in binding buffer (20 mM Tris, pH 8.0, 500 mM NaCl and 0.1 mM EDTA) plus 0.1% Triton X-100 before being sonicated on ice. A clear lysate was obtained after centrifugation at 10 000 *g* for 10 min, and it was used to bind to chitin beads for 30 min before the mixture was loaded into a column and washed extensively. Bound fusion OxyR protein was cleaved by incubating the column content in a cleavage buffer (binding buffer plus 300 mM DTT) overnight at 4°C. Fusion protein was eluted by washing the column with cleavage buffer, and 0.5 ml fractions were collected. These fractions were analysed by SDS–PAGE gels, and fractions that contained a high concentration of purified OxyR protein were pooled and dialysed against the binding buffer at 4°C overnight. OxyR protein purity was greater than 90% judged by SDS–PAGE.

Gel shift and DNase I footprinting

Both gel shift and DNase I protection experiments were performed as described previously (Storz and Altuvia, 1994). End-labelled DNA fragments (205 bp) were used. Radioactively labelled DNA fragments were prepared by labelling primer PE1 with [γ-^{32}P]-ATP and T4 kinase. The labelled primer was mixed with plasmid pKSahpC and a second unlabelled primer 127 (5′-TAGGATCCACTGCGACTG-3′) in PCR reactions performed for 25 cycles. The 205 bp labelled products were purified from agarose gel and used in gel shift and DNase I footprinting experiments. For DNase I footprinting of the top strand, primer 127 was end labelled with [γ-^{32}P]-ATP and T4 kinase. The labelled primer was mixed with pahpC and primer 213 (5′-CCGACCTTGCGACGAA-3′) in PCR reactions. The 746 bp PCR products were then cleaved with *Nae*I, end labelled with a 270 bp fragment purified from agarose gels and used in DNase I footprinting experiments.

The gel shift reactions were performed by adding 3 fmol of labelled probe to TM buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM $MgCl_2$, 20% glycerol, 1 mM EDTA, pH 8.0, 0.1% Nonidet P-40 and 100 mM KCl). Purified OxyR was added to give a final concentration of 0.5 × TM in 25 μl. To assay OxyR binding under reducing conditions, 200 mM DTT was added to binding reactions (Storz and Altuvia, 1994). For DNase I protection assays, the same labelled fragment and OxyR binding conditions were used as described in the gel shift assay. After 10 min incubation, 25 μl of Mg^{2+} and Ca^{2+} and 0.5 U of DNase I were added. The reaction was continued for 1 min before 200 μl of a stop solution (20 mM EDTA, pH 8.0, 1.0% SDS, 0.2 M NaCl and 250 μg ml^{-1} tRNA) was added. The mixture was extracted with phenol–chloroform and ethanol precipitated. Dry pellets were mixed with a sequencing loading buffer and loaded onto a sequencing gel. The DNA sequence ladder was performed using fmol^{R} sequencing kits (Promega) and radioactively labelled primer 192 on pKSahpC template.

Molecular cloning of ahpC *and promoter analysis*

Study of regulated promoters on a multiple-copy promoter probe vector often leads to deregulation of the promoter caused by titrating out a limited amount of regulatory factors. To avoid this problem, *ahpC* promoter activity was monitored in cells with transposons containing *ahpC* promoter fused to a reported gene integrated into the chromosome. PCR fragments (895 bp) containing *Xp ahpC* from −208 to +687 bp were cloned into pBluescript KS. The PCR primers corresponding to 5′ (5′-TAGGATCCGGCGAAGCAACTG-3′) and 3′ (5′-GTAAGCTTCCGGCACCGGCTC-3′) of *ahpC* were added to 0.5 μg of *Xp* genomic DNA, dNTP and buffer and amplified using the following conditions: 96°C denaturing, 60°C annealing of primers and 72°C extension of polymerase reaction. *Taq* polymerase (2 U) was added at the start of the reaction. PCR products were purified and cloned into pBluescript KS. The recombinant plasmid pKSahpC was transformed into *dam*$^{-}$ *E. coli*. The promoterless chloramphenicol acetyltransferase gene (*cat*) from pSM-cat1 (Mongkolsuk *et al.*, 1993) digested with *Bam*HI and *Bgl*II was gel purified and cloned into pKSahpC digested with *Bcl*I. This resulted in a new recombinant plasmid pKSahpCcat containing an *ahpC–cat* transcriptional fusion. The plasmid was then digested with *Xho*I–*Hin*dIII, and a 1.7 kb fragment containing the *ahpC–cat* fusion was cloned into *Sal*I–*Hin*dIII-digested pUC18Sfi, resulting in pUC18Sfi-ahpCcat1 (De Lorenzo and Timmis, 1994). A second construct containing the *Cla*I deletion was made by digesting pUC18Sfi-ahpCcat1 with *Cla*I. It was then gap filled with DNA polymerase and *Sma*I, followed by blunt-ended DNA ligation. This resulted in pUC18Sfi-ahpCcat2. The *ahpC–cat* fusions in pUC18Sfi were cloned

into a mini-Tn*5* vector pUTTn5lacZ1 (De Lorenzo and Timmis, 1994). pUTTn5lacZ1 was then digested with *Sfi*I. The vector portion containing a mini-Tn*5* and a selectable marker was gel purified and ligated with the *Sfi*I fragments containing *ahpC–cat* from *Sfi*I-digested pUC18Sfi-ahpCcat1 or pUC18Sfi-ahpCcat2. This resulted in two new recombinant plasmids pUTahpCcat1 and pUTahpCcat2. These plasmids were transformed into *E. coli* S-17λ*pir* (Tp^R Sm^R *recA, thi, pro, hsdR⁻M⁺* RP4:2-Tc:Mu:Km^R Tn*7*, λ*pir*) and subsequently conjugated into *X. campestris* pv. *phaseoli*. Conjugants were selected for Rif^R and Kan^R and scored for carbeniciline sensitivity. To determine the *in vivo ahpC* promoter activity, cells containing TnCP1 and TnCP2 were grown under uninduced and menadione-induced (100 μM) conditions. Cat levels were monitored using Western blot analysis with an anti-Cat polyclonal antibody. Experiments were repeated four times.

Construction of an ahpCE *mutant*

ery^R derived from pE194 was digested with *Bam*HI and *Eco*RV followed by gap filling with DNA polymerase I. The blunt-ended 0.9 kb fragment was gel purified and cloned into the *Cla*I site (Fig. 5) of pahpC4.1 (Loprasert *et al.*, 1997), resulting in pahpCE, which conferred Ap^R and Ery^R. pahpCE was electroporated into *X. campestris* pv. *phaseoli* using previously described conditions (Mongkolsuk *et al.*, 1997). Ery^R could arise from a single recombination of the plasmid with the chromosome. Transformants were selected for Ery^R and scored for Ap^S. Several putative *ahpCE* mutants were characterized at both Southern (data not shown) and Western levels. One of the mutants that showed correct restriction enzyme patterns was selected for physiological analysis.

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Catalase has a novel protective role against electrophile killing of *Xanthomonas*

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The ability of *Xanthomonas campestris* pv. *phaseoli* to protect itself against lethal concentrations of man-made (*N*-ethylmaleimide, NEM) and endogenously produced (methylglyoxal, MG) electrophiles was investigated. Pretreatment of *X. c.* pv. *phaseoli* with a low concentration of NEM induced protection against lethal concentrations of NEM and MG. MG pretreatment weakly induced protection against NEM but not against MG itself. NEM-induced protection against electrophile killing required new protein synthesis and was abolished by the addition of a protein synthesis inhibitor. By contrast, MG-induced protection against NEM killing was independent of *de novo* protein synthesis. *X. c.* pv. *phaseoli* harbouring an expression vector carrying a catalase gene was over 100-fold more resistant to MG and NEM killing. High expression levels of genes for other peroxide-protective enzymes, such as those for alkyl hydroperoxide reductase (*ahpC* and *ahpF*) and *ohr*, failed to protect against electrophile killing. Thus, catalase appears to have a novel protective role(s) against electrophile toxicity. This finding suggests that in *X. c.* pv. *phaseoli* NEM and MG toxicity might involve accumulation and/or increased production of H_2O_2. This idea was supported by the observation that addition of 10 mM sodium pyruvate, a compound that can react chemically with peroxide or hydroxyl radical scavengers (DMSO and glycerol), was found to protect *Xanthomonas* from electrophile killing. The protective role of catalase and the role of H_2O_2 in electrophile toxicity are novel observations and could be generally important in other bacteria. In addition, unlike other bacteria, *Xanthomonas* in stationary phase was more susceptible to electrophile killing compared to cells in exponential phase.

Keywords: methylglyoxal, *N*-ethylmaleimide, resistance, catalase

INTRODUCTION

Xanthomonas spp. are important bacterial plant pathogens. In the environment and on plants, bacteria are exposed to a variety of chemicals, some of which could modulate bacterial physiological responses. Increased production and accumulation of reactive oxygen species, including H_2O_2, organic peroxide and superoxide anions, are important components of plant active defence responses to microbial invasion (Levine *et al.*, 1994; Sutherland, 1991). Exposure to low concentrations of peroxide and superoxide anions affects the stress responses of *Xanthomonas* spp. in complex ways (Loprasert *et al.*, 1996; Mongkolsuk *et al.*, 1997b). These alterations in physiological responses could affect disease progression and outcome. Understanding complex bacterial physiological processes not only contributes to our understanding of pathological processes but also might reveal new targets for drug development to control bacterial proliferation.

In nature, bacteria are exposed to toxic electrophiles both from within the cell and from their environment. Methylglyoxal (MG) is the major electrophile produced intracellularly by bacterial cells. Millimolar concentrations of MG are produced during growth under certain conditions (Ferguson *et al.*, 1998b, 1999). In addition, electrophiles are released into the environment

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Abbreviations: MG, methylglyoxal; NEM, *N*-ethylmaleimide; SB, Silva-Buddenhagen.

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in the form of herbicides and chemicals used in the poultry industry (Stevens *et al.*, 1995; Zablotowicz *et al.*, 1995). The toxicities of these compounds are believed to stem from their interactions with nucleophilic centres of macromolecules. Electrophiles react with amino acid residues such as arginine, lysine and cysteine (Lo *et al.*, 1994). These compounds are known also to react with DNA and are highly mutagenic (Ferguson *et al.*, 1998a; Ferguson *et al.*, 2000). Electrophiles must be rapidly detoxified to prevent cellular and genetic damage. In bacteria, MG and NEM detoxification involves glutathione. MG is detoxified by glutathione-dependent glyoxalase systems (Ferguson *et al.*, 1995; MacLean *et al.*, 1998). In addition, both MG and NEM glutathione adducts activate the potassium transport system, KefB/KefC, resulting in acidification of the cytoplasm and protection against electrophile killing (Ferguson *et al.*, 1995).

Recently, we have shown that electrophiles such as NEM can modulate the oxidative stress responses in *Xanthomonas* spp. (Mongkolsuk *et al.*, 1997b; Vattanaviboon *et al.*, 1999). Exposure to low concentrations of NEM was shown to induce high levels of the peroxide-scavenging enzymes alkyl hydroperoxide reductase subunit C and catalase in an OxyR-dependent manner. This gives high-level protection against peroxide toxicity. Exposure of bacteria to low concentrations of some compounds can induce protection to subsequent exposure to lethal concentrations of the same compound (adaptive) or of other non-related compounds (cross-protection). These responses are important strategies for bacterial survival under stressful conditions. Thus, exposure to electrophiles will affect various physiological processes. Here, we determined the effects of pre-exposure to low concentrations of electrophiles on the response to subsequent exposure to lethal concentrations of electrophiles. A novel protective mechanism against electrophile toxicity was also investigated.

METHODS

Bacterial growth conditions. All *Xanthomonas campestris* pv. *phaseoli* strains were grown aerobically in SB (Silva-Buddenhagen) medium (Mongkolsuk *et al.*, 1996) at 28 °C. To ensure synchronous exponential growth, overnight cultures were subcultured into fresh SB medium to give OD_{600} of 0·1. Bacterial growth was monitored spectrophotometrically at OD_{600} using a MAKE AND MODEL TO BE ADDED spectrophotometer. Exponential-phase cells (OD_{600} of 0·5, after 4 h of growth) and stationary-phase cells (OD_{600} of 5·5 after 24 h of growth) were used in experiments, as indicated.

Quantitative determination of resistance levels to oxidants. The induced adaptive and cross-protection response experiments were performed by adding either 0·01 % (w/v) MG, 100 μM NEM or 100 μM iodoacetamide to exponential-phase *X. c.* pv. *phaseoli* cultures. The cultures were allowed to grow for 1 h before aliquots of cells were treated with lethal concentrations of MG (0·1 % w/v) or NEM (1·0 mM). At the indicated times, samples were removed and washed twice with fresh SB medium before cells were plated on SB agar. Colonies were counted after 36 h incubation at 28 °C. Surviving fractions are defined as the number of c.f.u. recovered after the treatment divided by the number of c.f.u. prior to the treatment. To test the effect of sodium pyruvate on electrophile killing, experiments were performed as described, except that cells were plated on SB agar with and without 10 mM sodium pyruvate. The effects of hydroxyl radical scavengers were determined as previously described (Vattanaviboon & Mongkolsuk, 1998) by exposing *X. c.* pv. *phaseoli* cultures to protecting concentrations of DMSO (0·4 M) and glycerol (1·0 M) for 30 min prior to exposure to lethal concentrations of NEM (1·0 mM). All experiments were repeated at least four times and means and standard deviations are shown.

Qualitative determination of levels of resistance to lethal concentration of NEM. The levels of resistance against a lethal concentration of NEM was qualitatively determined by using the inhibition zone method (Mongkolsuk *et al.*, 2000). Exponential-phase cells (10^8 c.f.u. ml^{-1}) were mixed with SB top agar and overlaid onto SB agar plates. Then 6 mm diameter filter paper discs soaked with 5 μl of 10 mM NEM were placed on top of the *Xanthomonas* cell lawn. The zone of growth inhibition was measured after 24 h incubation.

Catalase activity gel and assays. Total catalase activity was assayed as previously described (Mongkolsuk *et al.*, 1996). Preparation of cell lysates for enzyme assays and catalase activity gels were prepared according to Mongkolsuk *et al.* (1996). Essentially, cells were pelleted and washed once with 50 mM sodium phosphate buffer pH 7·0 and lysed by brief sonication followed by centrifugation at 10000 *g* for 10 min. Cleared lysates were used for enzyme assay and catalase activity gels. Catalase isozymes were visualized on native PAGE gels as described by Vattanaviboon & Mongkolsuk (2000). After electrophoresis gels were immersed at room temperature for 45 min in 50 mM sodium phosphate buffer pH 7·0 containing 50 μg ml^{-1} horseradish peroxidase (Sigma) and then in 50 mM sodium phosphate buffer pH 7·0 containing 5 mM H_2O_2 for 10 min. Subsequently, each gel was washed twice and finally treated with 0·5 mg ml^{-1} diamino benzidine. Catalase activity appeared as colourless bands against a dark brown background.

RESULTS AND DISCUSSION

Adaptive and cross-protection responses to electrophile killing

The effect of pretreatment of *X. c.* pv. *phaseoli* with low concentrations of electrophiles on survival to subsequent challenge with lethal concentrations of the chemicals was investigated. Two model electrophiles were used in the study, NEM, a man-made agent, and MG, an endogenously generated compound. Pretreatment of *X. c.* pv. *phaseoli* with 100 μM NEM induced a more than 100-fold increase in resistance when cells were subsequently exposed to a lethal concentration (1·0 mM) of the same compound (Fig. 1a). By contrast, pretreatment of the bacteria with 0·01 % (w/v) MG did not induce protection against a lethal concentration of MG (Fig. 1c). Several other pretreatment concentrations of MG were also tested, none of which induced protection against MG killing (data not shown). The ability of the electrophiles to induce cross-protection against exposure to their lethal concentrations was also investigated. The results in Fig. 1(b) show that NEM pretreatment gave some protection to *X. c.* pv. *phaseoli*

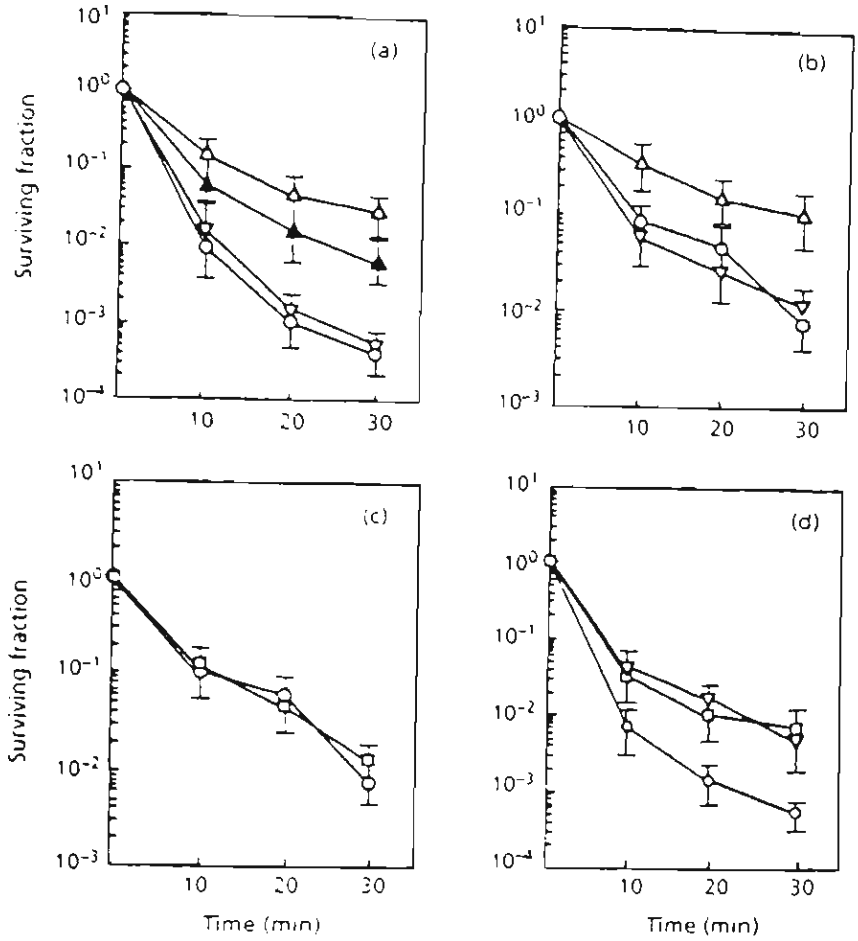

Fig. 1. Electrophile-induced adaptive and cross-protection responses in *X. c.* pv. *phaseoli*. Effects of pretreatment with 100 μM NEM alone [in (a) and (b); △] and in the presence of 150 μg ml^{-1} chloramphenicol [in (a) and (b); ▽], 100 μM DTT [in (a), ▲] or 0·01% w/v MG alone [in (c) and (d); □] and in the presence of 150 μg ml^{-1} chloramphenicol [in (d); ▽] on subsequent exposure to 1·0 mM NEM [in (a) and (d)] and 0·1% w/v MG [in (b) and (c)] compared to uninduced cells [in (a), (b), (c) and (d); ○]. Experiments were performed as described in Methods. Values are means of four replicates and error bars indicate SD.

against MG killing. Conversely, pretreatment of *X. c.* pv. *phaseoli* cultures with 0·01% w/v MG induced a small (approx. 10-fold) increase in resistance to NEM killing (Fig. 1d).

In *Escherichia coli*, cytoplasm acidification confers protection against electrophile killing and is independent of *de novo* protein synthesis (Ferguson *et al.*, 1995). Accordingly, we tested whether the electrophile-induced protection responses to MG and NEM killing required new protein synthesis. Adaptive and cross-protection experiments were repeated with addition of chloramphenicol at 150 μg ml^{-1} [a concentration of drug which has been shown to inhibit protein synthesis in *Xanthomonas* spp. (Mongkolsuk *et al.*, 1997b)]. NEM did not induce resistance to MG and NEM killing in chloramphenicol-treated cultures (Fig. 1a, b). This suggests that the NEM-induced adaptive and cross-protection responses require *de novo* protein synthesis and probably result from NEM-induced expression of stress-protective genes. By contrast, MG-induced protection against NEM killing was not affected by addition of chloramphenicol (Fig. 1d), implying that new gene expression was not required. The data clearly show that MG and NEM induce different protective responses to electrophile killing.

The different electrophile-induced adaptive responses were unexpected. They suggested that *X. c.* pv. *phaseoli* has evolved the ability to mount an adaptive response against a man-made electrophile NEM but not against an endogenously produced one, MG. The ability of NEM to induce high-level protection against both MG and itself implied that it is a potent inducer of a pathway which can detoxify both NEM and MG. In contrast, MG is not an inducer of this detoxification pathway(s) and is unable to confer protection against itself. Since MG-induced protection against NEM was found to be independent of new protein synthesis, MG probably effects some modification of existing cellular components that results in an increase in resistance to NEM killing. A likely mechanism for MG-induced resistance is via the KefB/KefC system induced by MG/glutathione conjugates, resulting in cytoplasmic acidification and protection against NEM killing (Ferguson *et al.*, 1995; Ferguson, 1999). This process is independent of new protein synthesis (Ferguson *et al.*, 1995). In *Xanthomonas*, unlike *E. coli*, cytoplasm acidification is not sufficient to confer protection against MG toxicity, as shown by lack of MG-induced adaptation. In addition, the inability of MG to induce high-level protection against electrophile killing could be due to the ability of the bacteria to metabolize MG effectively. This could prevent the intracellular concentration of MG from reaching the critical level required to activate a protective pathway(s), while NEM is more slowly metabolized; so its concentration could increase rapidly

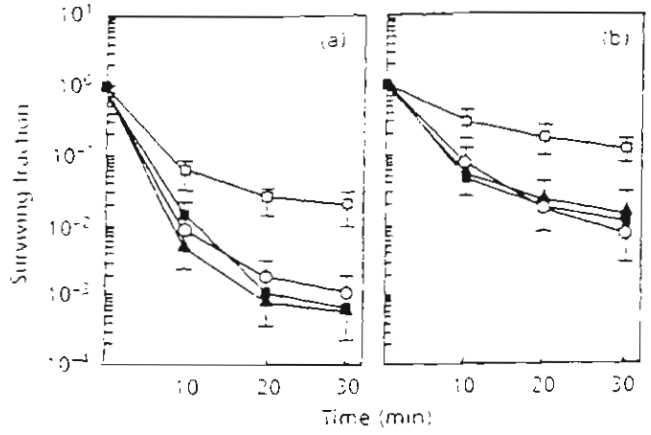

Fig. 2. Effects of high-level expression of various peroxide stress protective genes on NEM and MG killing. *X. c.* pv. *phaseoli* strains harbouring the expression vector pUFR047 alone (○) or containing genes involved in peroxide protection, catalase (pkat, □), alkyl hydroperoxide reductase C and F subunits (pahpCF, ■) and Ohr (pohr, ▲) were treated with lethal concentrations of either NEM (a) or MG (b).

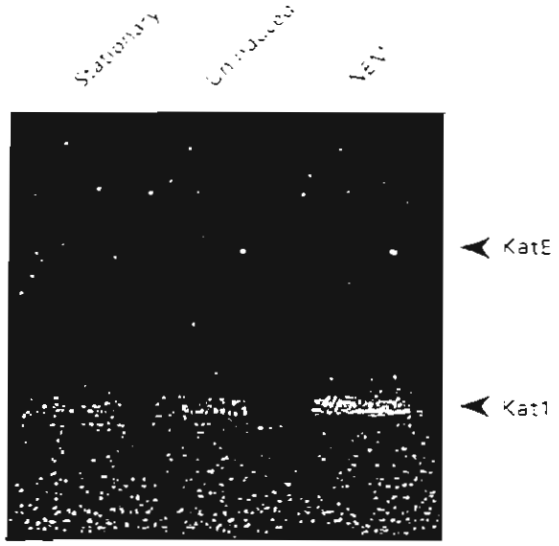

Fig. 3. NEM-induced KatT Catalase activity gel displaying the form of catalase induced by 100 μM NEM (NEM) for 1 h in exponential-phase cells. Also shown are catalase profiles of extracts of uninduced cells from exponential phase (Uninduced) and from stationary phase (Stationary). Protein (80 μg) was loaded into each lane and catalase activity was detected as described in Methods.

to the level needed to induce the protective pathways. The data show that in *Xanthomonas*, NEM and MG toxicity arise from several routes and there are protective pathways against these compounds. Variation in the ability of each electrophile to induce a protective response(s) is likely to have significant physiological effects.

Catalase has important protective roles against electrophile killing

We were interested to determine the mechanisms responsible for induced adaptive and cross-protection responses to MG and NEM killing. We have shown that exposure of *Xanthomonas* spp. to low concentrations of NEM induces cross-protection to H_2O_2 killing (Vattanaviboon *et al.*, 1999). NEM induces a more than 10-fold increase in each of the peroxide-scavenging enzymes, catalase and alkyl hydroperoxide reductase subunit C (AhpC) in an OxyR (a peroxide sensor and transcription regulator)-dependent manner (Vattanaviboon *et al.*, 1999). This mechanism is likely to be responsible for NEM-induced cross-resistance to peroxide killing. We have observed that a *X. c.* pv. *phaseoli* H_2O_2-resistant mutant has a more than 200-fold increase in both catalase and alkyl hydroperoxide reductase activities and is more resistant to NEM killing (Fuangthong & Mongkolsuk, 1997). *E. coli* strains with suppressors of *oxyR* mutants which have high levels of catalase and AhpC show reduced susceptibility to NEM killing (Greenberg & Demple, 1988). We suspect that peroxide-scavenging enzymes might help protect against electrophile killing. We tested this idea by determining the resistance levels to MG and NEM killing in *X. c.* pv. *phaseoli* harbouring recombinant plasmids containing genes for peroxide-scavenging enzymes. *X. c.* pv. *phaseoli* harbouring pUFR047 vector alone), pahpCF (containing genes for alkyl hydroperoxide reductase C and F subunits, *ahpC* and *ahpF*; Loprasert *et al.*, 1997; Mongkolsuk *et al.*, 1998a), pkat (containing the monofunctional catalase gene, *katX*; Mongkolsuk *et al.*, 1996), pohr (containing a gene conferring organic peroxide resistance; Mongkolsuk *et al.*, 1998a)] were treated with lethal concentrations of MG and NEM. The results (Fig. 2a, b) clearly showed that only cells expressing high levels of catalase were protected from MG and NEM killing. High-level expression of genes for all other peroxide-scavenging enzymes tested had no protective effect. The ability of catalase to protect bacterial cells against MG and NEM killing is a novel finding and could be generally important in other bacteria.

Our results show that low concentrations of NEM are potent inducers of protective pathways against electrophile killing and also of catalase enzyme (Figs 1a, b and 3). There appears to be a correlation between the abilities of electrophiles to induce catalase and to induce protection against electrophile killing. The idea was further tested by determining the effects of pretreatment of *X. c.* pv. *phaseoli* with MG on catalase activity. Several inducing concentrations of MG were tested. MG did not significantly induce catalase at any of the concentrations tested (data not shown). Thus, the ability of NEM to induce catalase correlates with its ability to induce resistance to MG and NEM. In contrast, MG is unable to induce catalase and does not induce protection against itself. MG-induced resistance against NEM

killing was independent of new protein synthesis and appeared to involve some other protective pathway(s).

There are at least two monofunctional catalases and a bifunctional catalase/peroxidase in *X. c.* pv. *phaseoli* (Vattanaviboon & Mongkolsuk, 2000). We have determined that NEM did not induce bifunctional catalase/peroxidase (data not shown). Thus, it was considered important to determine the form of monofunctional catalase induced by NEM. Analysis of catalase activity gels showed that Kat1, the major form of catalase in *X. c.* pv. *phaseoli*, was induced by NEM (Fig. 3). This induction was abolished in an *oxyR* mutant (Mongkolsuk *et al.*, 1998b). MG did not induce Kat1 (data not shown). Additional evidence for the correlation of catalase induction and induced resistance to electrophile killing came from the observation that a *Xanthomonas oxyR* mutant (Mongkolsuk *et al.*, 1998b) that could not induce catalase was more sensitive to NEM killing than was the parent strain. The parental strain had a zone of growth inhibition caused by NEM of 27·0 mm, compared to 30·5 mm for the *oxyR* mutant (zone of inhibition experiments were performed as described in Methods). These data strongly support the conclusion that Kat1 is responsible for resistance to electrophile killing. Nonetheless, we have not been able conclusively to establish the role of Kat1 in the mechanism of protection against electrophile killing due to the lack of a knock-out mutant in the gene. Experiments are in progress to isolate the gene and construct a *kat1* knock-out mutant.

These observations raised the question of how catalase protects cells from electrophile toxicity. H_2O_2 is the only substrate for monofunctional catalase. It is unlikely that the enzyme can react directly with either MG or NEM, suggesting that MG and NEM toxicity might reflect accumulation and/or increased production of H_2O_2. Studies on MG and NEM toxicity in other bacteria have not conclusively shown that H_2O_2 is involved in electrophile-induced toxicity (Ferguson *et al.*, 1995, 1998a; Ferguson, 1999). Nevertheless, there is evidence that oxidative stress might be involved in electrophile toxicity. For example, expression of an oxidative stress protective gene (*dps*) has been shown to increase resistance to electrophile killing (Ferguson *et al.*, 1998a). Support for the role of H_2O_2 in electrophile toxicity came from two observations. First, addition of 10 mM sodium pyruvate [a compound that chemically inactivates peroxide (Nath *et al.*, 1995)] to the growth medium increased *X. c.* pv. *phaseoli* resistance levels more than 100-fold to MG and NEM killing (Fig. 4). Secondly, H_2O_2 killing of bacteria involves production of highly reactive hydroxyl radicals. In *Xanthomonas*, compounds which absorb hydroxyl radicals protect cells from H_2O_2 killing (Vattanaviboon & Mongkolsuk, 1998). The effects of hydroxyl radical scavengers (DMSO and glycerol) on NEM killing was investigated. The results in Fig. 5(a) show that both DMSO and glycerol produced around 10-fold protection against NEM killing. The question as to how exposure to NEM or MG leads to an increase in the level of H_2O_2 remains obscure. Electrophiles are known to react with amino acids in proteins, especially with cysteine residues (Lo *et al.*, 1994). Many peroxide-scavenging enzymes, such as alkyl hydroperoxide reductase or the regulator of the peroxide stress response OxyR, have cysteine residues at their active sites (Loprasert *et al.*, 1997; Mongkolsuk *et al.*, 1997a). Thus, electrophiles could react directly with cysteine residues at active sites of these proteins and inactivate their biological functions. This, in turn, would reduce the cell's ability to detoxify toxic peroxides, leading to intracellular accumulation of these compounds. Glutathione is an important component of electrophile-detoxification systems. High levels of glutathione have protective effects against electrophile toxicity (Ferguson *et al.*, 1995). Electrophiles could lower the ratio of reduced versus oxidized glutathione. Depletion of glutathione would directly affect the rate of electrophile metabolism, electrophile resistance levels and the redox balance of the cell. The condition not only reduces the rate of electrophile metabolism and the associated resistance level but also the inducing oxidative stress condition(s). These factors might be expected to combine to increase intracellular concentrations of H_2O_2. Thus, a thiol reagent such as iodoacetamide that causes depletion of glutathione (Kondo *et al.*, 1987) is expected to affect electrophile killing of the bacteria. *Xanthomonas* cultures were pretreated with 100 μM iodoacetamide prior to exposure to lethal concentrations of NEM. The results clearly showed that iodoacetamide induced high-level (1000-fold) protection against NEM killing (Fig. 5b). Iodoacetamide caused over 10-fold induction of total catalase activity (data not shown). In addition, we observed that a reducing agent such as DTT partially reversed the effects of NEM-induced protection against itself (Fig. 1a). Thus, in cells that produce high levels of catalase, or when grown in media with high levels of pyruvate or hydroxyl radical scavengers (DMSO or glycerol), H_2O_2 accumulation is prevented. This results in the observed increase in resistance to electrophile killing.

Growth-phase-dependent resistance to electrophile killing

In the environment, bacteria have a short period of rapid exponential growth when nutrients are plentiful followed by a long stationary phase during nutrient limitation. The ability to survive stresses during the stationary phase is important for bacteria in the environment. Bacterial stress resistance varies greatly with growth phase (Vattanaviboon *et al.*, 1995; Ferguson *et al.*, 1998a). In general, stationary-phase cells are more resistant than exponential-phase cells to a variety of stresses. Moreover, the level of stationary-phase stress resistance often does not correlate with the levels of known stress-protective enzymes, suggesting that other factors such as alterations in membrane structure, altered metabolic activity and non-specific DNA-binding proteins are more important (Ferguson *et al.*, 1998a; Martinez & Kolter, 1997). Here, we investigated MG and NEM killing during exponential and stationary

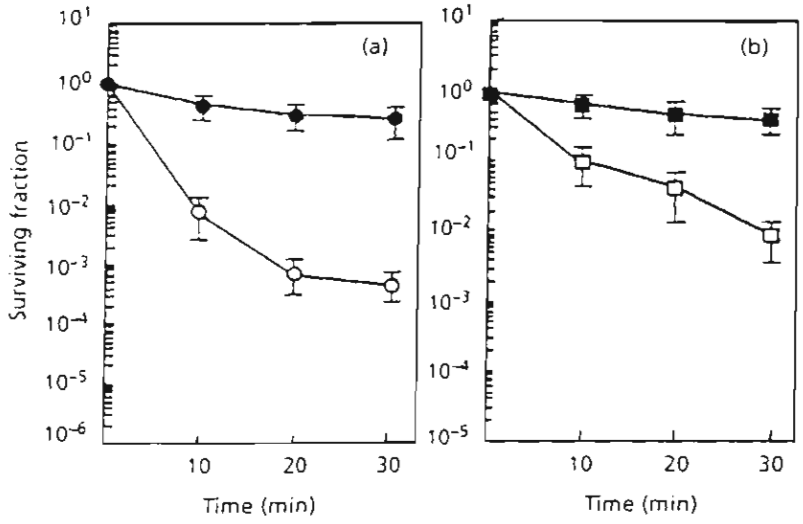

Fig. 4. Effects of addition of 10 mM pyruvate on NEM and MG killing. *X. c.* pv. *phaseoli* were treated with 1·0 mM NEM (a) or 0·1% w/v MG (b). At the indicated times, samples were removed, washed twice and plated on SB agar with 10 mM sodium pyruvate (●, ■) or without addition (○, □). Values are means of four replicates and error bars indicate SD.

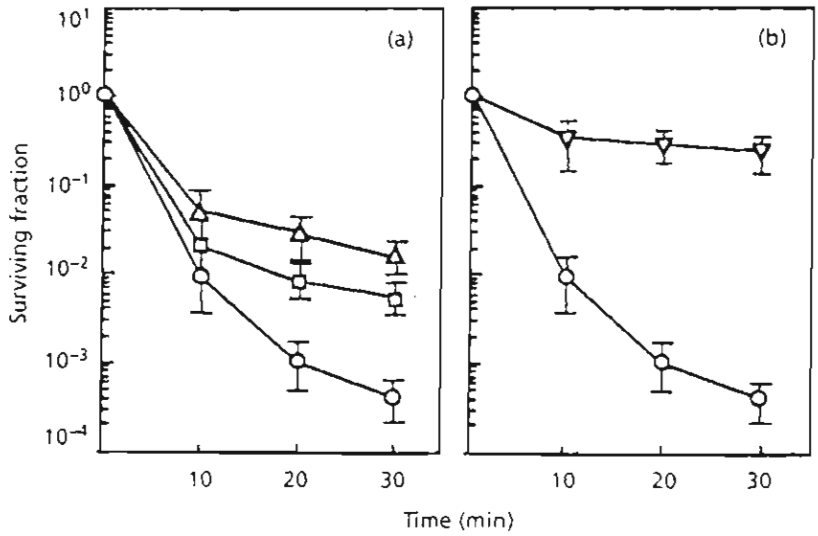

Fig. 5. Effects of hydroxyl radical abosorbers and a thiol reagent on NEM killing. In (a), exponential-phase uninduced cells of *X. c.* pv. *phaseoli* were treated with 1·0 mM NEM in the absence (○), or in the presence of hydroxyl radical scavenger, 1·0 M glycerol (△) or 0·4 M DMSO (□). In (b), *X. c.* pv. *phaseoli* cultures were induced with 100 µM iodoacetamide for 1 h (▽) or grown uninduced (○) prior to exposure to 1·0 mM NEM. Growth and electrophile killing conditions were as described in Methods. Values are means of four replicates and error bars indicate SD.

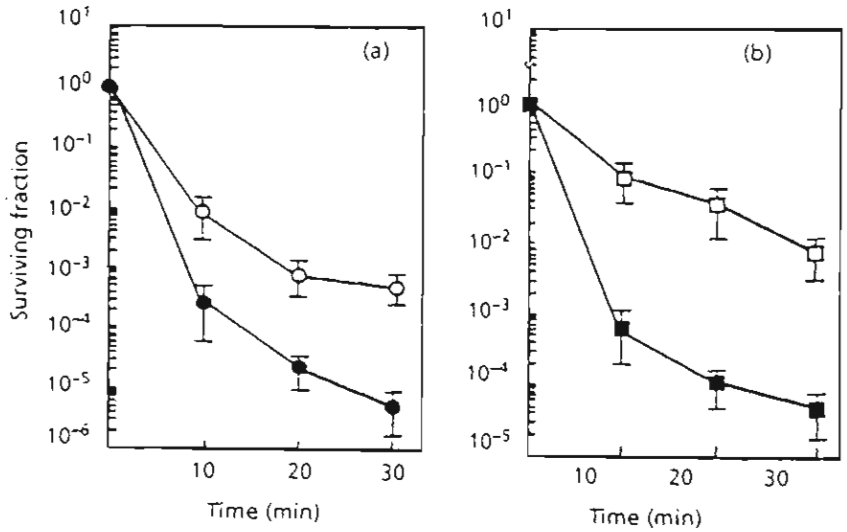

Fig. 6. Growth phase variation in resistance levels to NEM and MG killing. Exponential-phase (○, □) or stationary phase (●, ■) cells were treated with 1·0 mM NEM (a) or 0·1% w/v MG (b). Growth and electrophile killing conditions were as described in Methods. Values are means of four replicates and error bars indicate SD.

phases. The results presented in Fig. 6 show that stationary-phase cells were over 100-fold more sensitive than exponential-phase cells to both NEM and MG killing, a finding in contradiction to previous reports (Ferguson *et al.*, 1998a). In *E. coli*, resistance to electrophile killing was found to increase during station-

ary phase (Ferguson *et al.*, 1998a). The products of genes in the *rpoS* regulon and a non-specific DNA binding protein (Dps) have been shown to contribute to the stationary-phase electrophile resistance phenotype (Ferguson *et al.*, 1998a). In *Xanthomonas*, increased sensitivity to electrophile killing during stationary phase could be due to a decrease in the total catalase level. Unlike other bacteria, in *Xanthomonas* total catalase activity decreases in the stationary phase (Loprasert *et al.*, 1996). Analysis of catalase activity gels loaded with lysates prepared from a *X. c.* pv. *phaseoli* culture during exponential and stationary growth phases shows that the activity of the major form of monofunctional catalase (Katl) decreased as cells entered stationary phase. Even though a minor form of growth-phase-regulated catalase KatE increases during stationary phase (Vattanaviboon & Mongkolsuk, 2000), the increase in KatE activity did not compensate for the decrease in Katl activity, resulting in a lower total catalase activity during the stationary phase (Vattanaviboon & Mongkolsuk, 2000). In addition, while in stationary phase, cells are starved of nutrients, resulting in alterations in flux through various biochemical pathways. This, in turn, could affect the ratio of glutathione to glutathione disulphide. Lowering the availability of glutathione not only reduces the capacity to detoxify electrophiles but also reduces the resistance to electrophiles.

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A *Xanthomonas* Alkyl Hydroperoxide Reductase Subunit C (*ahpC*) Mutant Showed an Altered Peroxide Stress Response and Complex Regulation of the Compensatory Response of Peroxide Detoxification Enzymes

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Alkyl hydroperoxide reductase subunit C (AhpC) is the catalytic subunit responsible for alkyl peroxide metabolism. A *Xanthomonas ahpC* mutant was constructed. The mutant had increased sensitivity to organic peroxide killing, but was unexpectedly hyperresistant to H_2O_2 killing. Analysis of peroxide detoxification enzymes in this mutant revealed differential alteration in catalase activities in that its bifunctional catalase-peroxidase enzyme and major monofunctional catalase (Kat1) increased severalfold, while levels of its third growth-phase-regulated catalase (KatE) did not change. The increase in catalase activities was a compensatory response to lack of AhpC, and the phenotype was complemented by expression of a functional *ahpC* gene. Regulation of the catalase compensatory response was complex. The Kat1 compensatory response increase in activity was mediated by OxyR, since it was abolished in an *oxyR* mutant. In contrast, the compensatory response increase in activity for the bifunctional catalase-peroxidase enzyme was mediated by an unknown regulator, independent of OxyR. Moreover, the mutation in *ahpC* appeared to convert OxyR from a reduced form to an oxidized form that activated genes in the OxyR regulon in uninduced cells. This complex regulation of the peroxide stress response in *Xanthomonas* differed from that in other bacteria.

Increased rates of production and accumulation of reactive oxygen species (ROS), including H_2O_2, organic peroxide, and superoxide, are important components of active plant defense responses to microbial invasion (11). In addition, normal aerobic metabolism also generates large quantities of ROS (6, 7). For successful plant invasion, these ROS must be rapidly detoxified. Monofunctional catalases are major H_2O_2 scavenging enzymes in *Xanthomonas* (26), while detoxification of organic peroxides is more complex. We have identified in *Xanthomonas* alkyl hydroperoxide reductase genes (*ahpC* and *ahpF* [12, 16]) and a novel family of organic peroxide resistance genes (*ohr* [17]) which are involved in organic peroxide protection. Alkyl hydroperoxide reductase (AhpCF) is the best characterized microbial enzyme involved in organic peroxide metabolism. AhpCF consists of a catalytic 22-kDa C subunit (AhpC) and a reductase 52-kDa F subunit (AhpF) (19, 24). The *ahpC* gene has been highly conserved in evolution and is found in organisms ranging from bacteria to humans (5). Inactivation of *ahpC* in various bacterial mutants results in increased sensitivity to organic peroxide killing and to spontaneous mutagenesis (1, 3, 8, 20, 29). In addition, since mutants show additional alterations in oxidative stress response that range from increased sensitivity to hyperresistance to oxidative stress (1, 3, 21, 29), we have isolated and characterized *Xanthomonas* genes for both the catalytic (*ahpC*) and the reductase (*ahpF*) subunits (12, 16). *ahpC* has a unique form of regulation in which reduced OxyR represses *ahpC* expression while oxidized OxyR activates its expression (18).

Recently, we have shown that an *ahpCE* mutant with OxyR regulation separated from basal *ahpC* promoter activity has a lower aerobic growth rate and increased sensitivity to organic peroxide resistance (13). However, the lack of an *ahpC* knockout mutant in *Xanthomonas* has hampered analysis of the gene's physiological functions and its role in protection against peroxide stress. In this communication, we describe the construction and physiological characterization of an *ahpC* knockout mutant. The mutant showed atypical alterations in resistance to peroxide killing and deregulation of genes for peroxide scavenging enzymes.

Construction of an *ahpC* knockout mutant. An *ahpC* mutant was constructed by integration into *Xanthomonas campestris* pv. phaseoli chromosome of a recombinant plasmid, pKSahpC1. Essentially, primers corresponding to amino acid residue numbers 63 to 70 and 126 to 133 of *ahpC* were used to amplify a 157-bp DNA fragment containing an internal coding region of *ahpC* that was subsequently cloned into pKSKm (16). The resultant plasmid, pKSahpC1, was electroporated into *X. campestris* pv. phaseoli, and transformants were selected for Km^r. This yielded the *ahpC1* mutant. Southern analysis of genomic DNA digested with *Sac*II from the mutant and probed with the *ahpC* probe showed a positive hybridization band with an increase of 3.5 kb compared to similarly digested DNA from the parental strain (data not shown). This pattern was consistent with the idea that pKSahpC1 had correctly integrated and disrupted the gene. Results of Western immunoblot analysis of lysates confirmed the lack of AhpC in the mutant (data not shown).

Altered sensitivity to peroxide killing in the mutant. The levels of peroxide resistance in various *ahpC*-minus mutants

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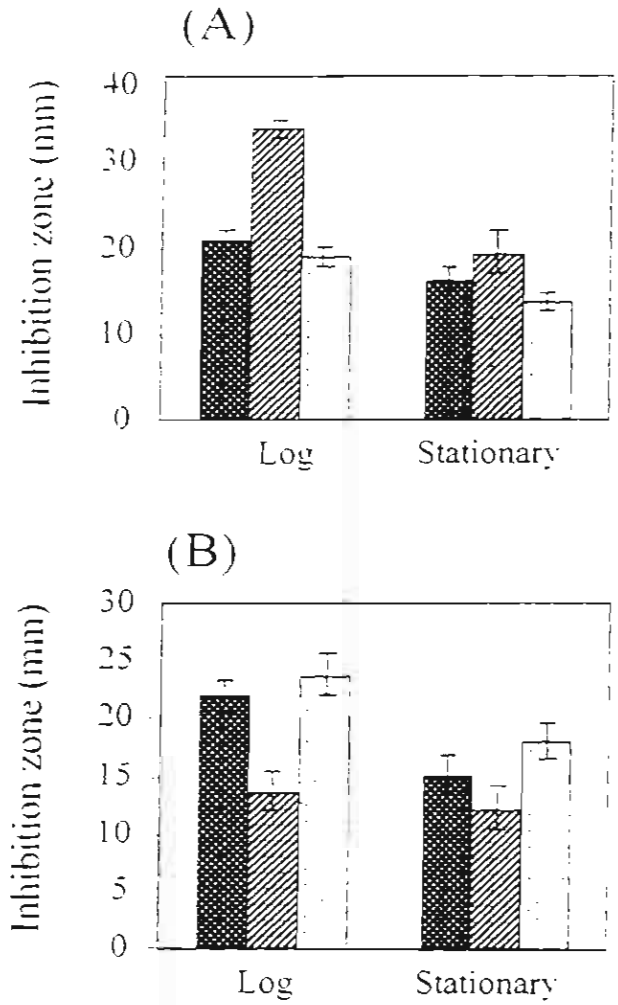

FIG. 1. Determination of levels of resistance to peroxide killing in the *ahpC* mutant and the parental strain. All *Xanthomonas* strains were grown aerobically in SB medium at 28°C. The exponential and stationary phases of growth were defined as 4 h (4.0 × 10^8 CFU/ml) and 30 h (4.0 × 10^9 CFU/ml) after inoculation, respectively. For determination of levels of resistance to peroxide killing, bacterial cells from the exponential or stationary phase were mixed with SB top agar and poured onto SB plates. Thus, 5 μl of 0.5 M tBOOH (A) or H_2O_2 (B) was spotted onto paper discs and placed on the cell lawn. The zone of growth inhibition around the disc was measured after 24 h of incubation. The experiments were repeated four times, and error bars indicate the standard error of the mean. ■, parental strain; ▨, *ahpC* mutant; ▩, mutant harboring pahpC.

differ widely (1, 3, 20, 21). Hence, levels of resistance to killing concentrations of organic peroxide and H_2O_2 in the *ahpC* mutant were determined during the exponential and stationary phases of growth. The mutant was more sensitive to *tert*-butyl hydroperoxide (tBOOH) killing than the parental strain during the exponential phase of growth (Fig. 1). This observation confirmed the important role of *ahpC* in protection against organic peroxide toxicity. A similar phenotype has been observed in other *ahpC* mutants (1, 20, 21, 24, 29). In contrast, *Xanthomonas ahpC1* was more resistant to H_2O_2 killing (Fig. 1). Purified AhpCF is capable of using both H_2O_2 and organic peroxide as substrates (19). Thus, it was unexpected that inactivation of the gene for the catalytic subunit of the AhpCF would result in increased H_2O_2 resistance. Nonetheless, a similar phenotype has been observed in *Bacillus subtilis ahpC* (1, 3). The mutant was transformed with pahpC (an expression vector containing a functional *ahpC* gene [12, 16]). pahpC complemented alterations in peroxide resistance levels in the mutant. The mutant harboring pahpC1 had levels of resistance to tBOOH and H_2O_2 similar to those of the parental strain (Fig. 1).

TABLE 1. Determination of enzymes involved in peroxide resistance in various *Xanthomonas* strains

Xanthomonas pv. phaseoli type	Enzyme activity (U/mg of protein)[a]				
	Catalase	Peroxidase (10^-3)	SOD	GR	G6PDH (10^-1)
Wild type	4.9	1.6	5.3	1.9	27.5
ahpC1	38.4	18.4	5.1	2.1	29.4
ahpC1 pahpC1	2.6	1.1	5.5	1.8	28.4
ahpC1 ohrR	1.8	12.8	4.9	1.8	26.2
ohrR	2.1	1.3	ND[b]	ND	ND

[a] Crude lysates from exponential-phase cultures of *Xanthomonas* strains were prepared as previously described (26). Assays to determine the activities of catalase (26), peroxidase (14), superoxide dismutase (SOD) (15), glutathione reductase (GR) (22), and glucose-6-phosphate dehydrogenase (G6PDH) (25) were performed as previously described. Values are means of four replicates.

[b] ND, not done.

In all bacteria thus far studied, levels of peroxide resistance significantly increase in the stationary phase (10, 27). Thus, we determined the levels of resistance to peroxide killing during the stationary phase. In the stationary phase, both the mutant and parental strains were more resistant to peroxide killing than in the exponential phase (Fig. 1). During the stationary phase, the mutant was also more resistant to H_2O_2 killing than the parental strain. However, differences in stationary-phase organic peroxide resistance levels between the mutant and the parental strain were less pronounced than differences during the exponential phase. The results suggested that AhpC was not an important factor in determining organic peroxide resistance levels during the stationary phase.

Altered levels of peroxide detoxification enzymes in the *ahpC* mutant. The unusual phenotype of the mutant prompted us to determine the activities of various enzymes involved in oxidative stress protection and peroxide detoxification. The results in Table 1 show intricate changes in the activities of these enzymes. The activities of oxidative stress protection enzymes such as superoxide dismutase, glucose-6-phosphate dehydrogenase, glutathione reductase, and Ohr were similar in the mutant and the parental strains. In contrast, increased activities of the peroxide detoxification enzymes catalase (8-fold) and peroxidase (11-fold) were observed in the mutant (Table 1). These increases were due to inactivation of *ahpC* and could be complemented by pahpC (Table 1). Thus, lack of AhpC led to compensatory increase in the activities of these enzymes.

We have observed several forms of monofunctional catalases in *Xanthomonas* by using catalase activity gels (26), and analysis of cloned *kat* genes indicates that various forms of catalase are products of different genes (26; S. Mongkolsuk, unpublished data). Using catalase and peroxidase activity gels, we have shown that the levels of a major monofunctional catalase, Kat1 (26), increased severalfold in the *ahpC* strain compared to those in the parental strain (Fig. 2). Kat1 accounts for over 80% of total catalase activity, as judged by analysis of catalase activity gels (26). Increased Kat1 activity was responsible for the observed increase in total catalase activity (Table 1). Analysis of peroxidase activity gels showed only one positive activity band (Fig. 2). The intensity of this band was severalfold higher in the mutant. When a similar gel was stained for catalase activity, a catalase activity band was observed at the same position as the peroxidase activity band

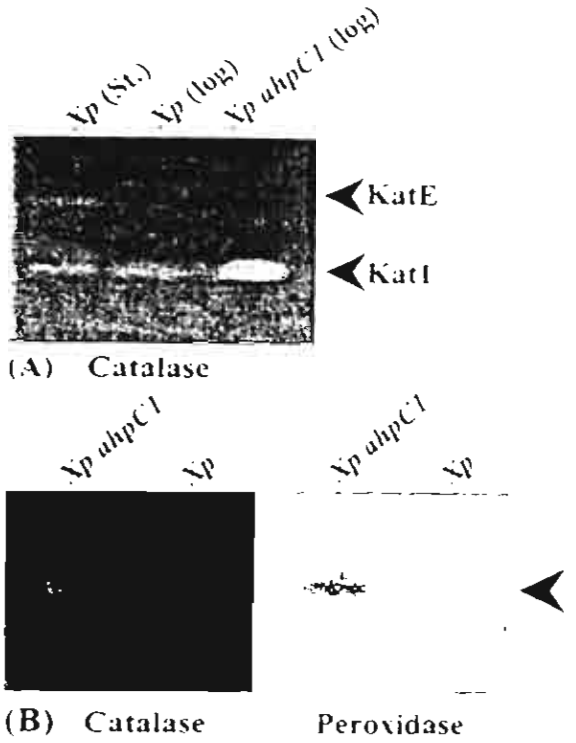

FIG. 2. Analysis of various forms of catalase and peroxidase in the *ahpC* mutant. (A) Forty micrograms of cell lysates prepared from exponential-phase (log) or stationary-phase (St.) cultures of parental *X. campestris* pv. phaseoli (Xp) and exponential-phase *ahpC* mutant (Xp *ahpC1* [log]) cultures was loaded into each lane. Lysate preparation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and renaturing of gels were performed as previously described (29). Catalase activity staining with 3-amino-9-ethylcarbazole was performed as described by Vattanaviboon and Mongkolsuk (29). The positions of KatI and KatE are shown. (B) Eighty micrograms of lysate from the exponential-phase *ahpC* mutant (Xp *ahpC1*) and the parental strain (Xp) was loaded into each lane. Cell electrophoresis was performed with a 7.5% polyacrylamide native gel (14). Subsequently, the gel was split into two halves, and each half was stained for peroxidase activity with diaminobenzidine (28) or for catalase activity (29). The arrowhead indicates the position of the catalase-peroxidase bifunctional enzyme.

(Fig. 2). This suggested that the enzyme was a bifunctional catalase and peroxidase. Indeed, most of the bacterial peroxidases are bifunctional catalase-peroxidase enzymes (9). Analysis of catalase and peroxidase activity gels suggested that the bifunctional enzyme contributed less than 10% to total catalase activity (data not shown). Thus, the 11-fold increase in peroxidase activity (Table 1) could be assigned to increases in activity of the catalase-peroxidase bifunctional enzyme. The compensatory increases in activities of both enzymes were abolished in the mutant harboring pahpC. The levels of a third form of growth-phase-regulated monofunctional catalase (KatE [26]) were similar in the two strains (data not shown). Increased catalase activity in the mutant could account for the increased H_2O_2 resistance during the exponential and stationary phases.

The compensatory increases in monofunctional and bifunctional catalases were mediated by different regulators. Compensatory alterations in gene expression resulting from either gene inactivation or altered gene expression are important reactions for bacterial survival under stressful conditions. In almost all cases, the regulation of these processes is unknown. OxyR is a peroxide sensor and a transcription regulator (23, 25). Thus, the role of OxyR in regulation of the catalase compensatory response in the *ahpC1* mutant was investigated. An *ahpC1 oxyR* double mutant was constructed by transformation with chromosomal DNA from an *X. campestris* pv. phaseoli *oxyR*::Gm' mutant (18) into the *ahpC1* mutant. Southern and Western analyses were used to confirm the integrity of the double mutant (data not shown). The levels and forms of catalases in the *ahpC1* and the *ahpC1 oxyR* mutants were determined and are shown in Fig. 2 and Table 1. The *ahpC1 oxyR* double mutant showed levels of catalase similar to those of the *oxyR* mutant (Table 1), and these were 10-fold less than those in the *ahpC1* mutant. Thus, *oxyR* mutation completely eliminated the compensatory increase in total catalase activity in the *ahpC1* mutant. Surprisingly, the *ahpC1 oxyR* and *ahpC1* mutants had comparable peroxidase levels. Both were 10-fold higher than those of the parental strain and the *oxyR* mutant (Table 1). Since *oxyR* mutation had no effect on the compensatory increase in the levels of bifunctional peroxidase and catalase in the *ahpC1* mutant, the process had to be regulated by another unknown regulator. Thus, the data suggested that *Xanthomonas* has at least two regulators which responded to changes in levels of peroxide. Dual regulation of the catalase compensatory response could be a means of ensuring sustained activity even if one of the regulators was incapacitated, and the response could be vital to bacterial survival in the absence of a functional *ahpC* gene.

Mutation in *ahpC* altered expression of OxyR-regulated genes. We wished to elucidate how OxyR could activate catalase expression responsible for the compensatory response in the *ahpC1* mutant. OxyR can exist in either a reduced or oxidized form (23). In uninduced cells, OxyR exists in the reduced form (2, 30). Upon exposure to H_2O_2, highly conserved cysteine residues of OxyR are oxidized to form a disulfide bond, converting it to the oxidized form (30). In *Xanthomonas*, OxyR is required for oxidant-induced expression of catalase and *ahpC* genes (18). Oxidized OxyR probably activates *katI* expression. Since we could not directly determine the in vivo redox status of OxyR, an alternative approach was used. This was based on the fact that the *ahpC* promoter in *Xanthomonas* is transcriptionally activated by oxidized OxyR and repressed by reduced OxyR (13, 18). Thus, *ahpC* promoter activity can be used to reflect the redox status of OxyR. An experiment was designed to test whether the mutation in *ahpC* altered the redox status of OxyR. This was done by monitoring levels of a chloramphenicol acetyltransferase gene (*cat*) used as a reporter transcriptionally fused to the *ahpC* promoter in the *ahpC1* mutant and the parental strain. The *ahpC* promoter fused to the *cat* gene was inserted into a mini-Tn*10* transposon, resulting in TnCP1 (13). The construct was subsequently transposed into the parental strain. Chromosomal DNA of *X. campestris* pv. phaseoli TnCP1 was extracted and electroporated into the *ahpC1* mutant. Integration of TnCP1 in the mutant and the parental strain was confirmed by PCR and Southern analysis (data not shown). Results of *cat* Northern analysis in strains containing TnCP1 are shown in Fig. 3. The parental strain containing TnCP1 showed low levels of uninduced *cat* expression. We have observed that menadione consistently induced *ahpC* expression in an *oxyR*-dependent manner (16, 18). Exposure to 50 μM menadione induced high levels of *cat* expression (Fig. 3). This result was consistent with the observations that reduced OxyR repressed the *ahpC* promoter and oxidized OxyR activated it (13). However, even in the absence of oxidant induction, high levels of *cat* mRNA were detected in the *ahpC1* mutant containing TnCP1 (Fig. 3). The high *cat* mRNA level in the uninduced mutant was similar to the *cat* mRNA level in the oxidant-induced parental strain containing TnCP1 (Fig. 3). Complementation in the *ahpC1* TnCP1 strain with pahpC resulted in uninduced *cat* mRNA levels similar to those of the uninduced parental strain con-

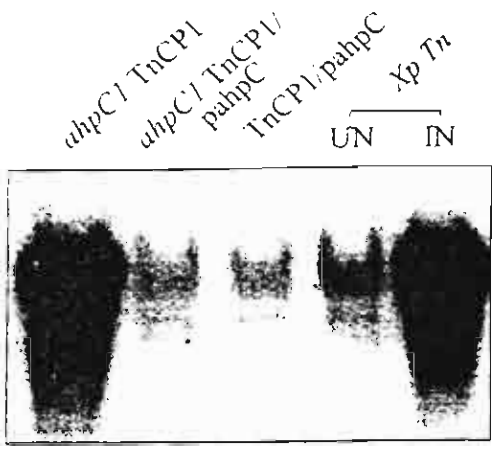

FIG. 3. Northern analysis of the *ahpC* promoter fused to *cat* in both the *ahpC1* mutant and the parental strain. All *Xanthomonas* strains used in this study contained TnCP1 (13). Total RNA was extracted by a hot phenol method from the mutant (*ahpC1* TnCP1), the mutant harboring pahpC (*ahpC1* TnCP1 pahpC), the parental strain (*Xp Tn*, uninduced [UN] or induced with 50 μM menadione [IN]) and the parental strain harboring pahpC (TnCP1 pahpC). RNA (20 μg) was then loaded into each lane and separated on a formaldehyde agarose gel. Gel electrophoresis, blotting hybridization, washing, and preparation of the *cat* probe were performed as previously described (13).

taining TnCP1 (Fig. 3). Furthermore, activation of the *ahpC* promoter in the uninduced mutant required functional OxyR, since it was eliminated in *oxyR*-minus derivatives of the mutant (data not shown). These data and data from Table 1 support the idea that OxyR existed in an oxidized form in the uninduced *ahpC1* mutant and that this oxidized OxyR was responsible for activation of genes in the OxyR regulon, including *katI*. The question of how OxyR was converted to the oxidized form in uninduced cells remains unanswered. The physiological substrates of AhpC are not known. AhpC can metabolize a wide range of organic peroxides, such as nucleotide peroxides and lipid peroxides (19, 24). Our observations implied that the *ahpC1* mutant probably accumulated various organic peroxides which converted OxyR from the reduced form to the oxidized form. This suggested, in turn, that various organic peroxides could act as intracellular signals to activate a global peroxide defense response via OxyR. We could not rule out that increased organic peroxide levels could lead to a transient increase in H_2O_2 levels sufficient to activate OxyR. Additional support for the role of organic peroxides and not H_2O_2 as the signal for activation of the OxyR-dependent compensatory response in the *ahpC1* mutant came from the observation that the mutant had eightfold higher catalase levels than the parental strain. High catalase activity should efficiently prevent accumulation of H_2O_2 in the mutant. Moreover, addition of sodium pyruvate to SB medium did not affect the *cat* levels in the *ahpC1* TnCP1 strain. At present, we cannot conclusively prove this hypothesis, because in our hands, the levels of organic peroxide could not be accurately determined. Definitive support for the theory must wait for accurate measurement of all organic peroxides in mutant and parental strains. In *Xanthomonas*, OxyR can function as a sensor for both H_2O_2 and organic peroxide. The proposed role of organic peroxides as signal molecules is novel but not unique to *Xanthomonas*. An analogous observation has been reported in *B. subtilis ahpC* mutants. Several groups have suggested that *ahpC* mutation can lead to accumulation of organic peroxides and result in inactivation of PerR, a peroxide-sensitive transcription repressor (1, 3, 4). This can lead to increased expression of genes in the PerR regulon (4). The role of organic peroxides as signal molecules is likely to be generally important in a wide range of bacteria.

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