BRG/10/2543 รศ.ดร. ศกรณ์ มงคลสุข

รายงานวิจัยฉบับสมบูรณ์

โครงการ
การศึกษาระดับโมเลกุล และสรีรวิทยาของยีนซึ่งมีความสัมพันธ์กับการตอบสนองต่อ Peroxide stress ในเชื้อ *Xanthomonas*

โดย รศ.ดร. ศกรณ์ มงคลสุข และคณะ

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สัญญาเลขที่ BRG/10/2543

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
ชุดโครงการทุนองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา

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CONTENT

Executive summary

1. ชื่อโครงการ (ภาษาไทย) การศึกษาระดับโมเลกุล และสรีรวิทยาของยีนซึ่งมีความสัมพันธ์กับการตอบสนองต่อ Peroxide stress ในเชื้อ *Xanthomonas.*

(ภาษาอังกฤษ) Molecular and Physiology Analysis of Genes Involved in Peroxide Stress Response in *Xanthomonas.*

2. ชื่อหัวหน้าโครงการ หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail

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3. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

In response to microbial infection, plant synthesize large quantity of reactive oxygen species including H_2O_2, organic peroxide and superoxide anions. These ROS function directly in inhibiting bacteria proliferation and act as signals for further activation of plant defense response. *Xanthomonas* belong to important groups of bacterial phytopathogen known to effect every major economically important crops. For bacteria to grow and establish themselves, they must over come host generated oxidative stress. Current evidence suggest that many aspects of *Xanthomonas* oxidative stress response are differed from other bacteria. Although, much work still need to be done in order to achieve a better understanding of the process. Understanding of bacteria response to the stress could lead to new strategies for controlling bacterial proliferation and eventual outcome of the disease.

4. วัตถุประสงค์

The goal of the research is to gain a better understanding of the complex regulation of oxidative stress response in a bacterial phytopathogen, *Xanthomonas.*

8.1) Isolation and characterization of catalase (*katA*). The gene transcription organization, transcription start sites and promoter sequences will be done. The role of *oxyR* in regulation of the gene will be investigated using Northern analysis.

8.2) Construction and physiological analysis of *katA* mutant to investigate the role of the gene in oxidative stress response.

8.3) Isolation and characterization of methionine sulfoxide reductase (*msrA*). Gene and transcription organization will be determined as well as regulation of the gene. Promoter and other transcription regulatory elements will be investigated.

8.4) Construction of *msrA* mutant and examine its physiological roles in oxidative stress response.

8.5) Transcription analysis of an important regulator *oxyR* will be performed by gene fusion technology. This will allow monitoring of transcription regulation of *oxyR* which is differed in *Xanthomonas* from all other bacteria thus far studied.

Understanding of these genes will give better insight into pathogenesis processes and might lead to novel strategies for controlling bacteria proliferation.

5. ระเบียบวิธีวิจัย

1) Isolation and characterization of katA.

1.1 Making a katA specific probe.

Several *katA* homologues have been isolated and comparison of their amino acid sequences showed highly conserved regions suitable for reverse genetics/PCR approach of gene isolation. The degenerate primers will be used to maximize possibility of gene isolation. PCR will be performed at several annealing temperatures to established the most suitable conditions for these primers. The putative *katA* will be sequenced and the resultant sequence used to search Genbank. The probe will be used to screen previously constructed genomic library in the lambda cre-lox phage vector. The phages containing putative *katA* clones will be plaque purified three times and plasmids excised from phages using automatic excision system of cre-lox positive cells.

1.2 Characterization of putative katA clones.

The putative *katA* will be sequenced in both directions using the primer walking technique and Prism DNA sequencing kits on an automated DNA sequencer. The sequence data will be analyzed using DNA analysis programs. Transcription organization of the gene will be determined by Northern analysis. Expression analysis of the gene will be performed by Northern experiments. Oxidants induction of *katA* will be investigated.

1.3 Construction of katA mutant and physiological analysis.

To specifically construct a knock out mutation in *katA*, part of the coding region of *katA* will be cloned into a vector that cannot replicate in *Xanthomonas* (i.e. pUC18). The constructed plasmid will be transformed into *Xanthomonas* by electroporation as previously described (17). Transformants will be selected for ampicillin resistance and checked by PCR to determine the plasmid integration into *Xp* chromosome. A single recombination between plasmid and chromosome should result in inactivation of *katA*. Physiological characterization will be performed on the mutant.

2) Regulation of oxyR.

Xanthomonas regulation of *oxyR* is unique. In response to oxidants, not only OxyR changes form from reduced to oxidized forms but also increased its concentration. The *cat-kan*^{R} genes cassette will be purified by *Hind*III digestion, gap filled with DNA polymerase I and blunt end cloning into gap filled *Kpn*I site of pOXX containing *Xp oxyR*. The *oxyR::cat-kan*^{R} will be eletroporated into *Xp* selecting for Kan^R. The transformant will be designated *Xp oxyR::cat*. Construction of the mutant will be confirmed by Southern analysis. The strain will allow monitoring of *oxyR* transcription activity by assaying Cat.

The effect of high level expression of *oxyR* and autoregulation will be investigated in *Xp oxyR::cat*.

3) Isolation and characterization of msrA.

One of the better characterized system for repairing of oxidative damage proteins is the methionine sulfoxide reductase (MsrA). The gene is highly conserved and has been found from bacteria to man. Little is know how the gene regulated. In this proposal, the gene will be isolates and characterized.

3.1 Isolation and Characterization of msrA.

The initial sequencing of the cloned fragment from *Xp* pOXX (13,18) that contained *oxyR* showed that down stream of the *oxyR* there are several genes of interests. Downstream of this *orf* located a truncated *msrA*. The truncated gene will be used as a probe to screen a *Xp* genomic library constructed in a lambda phagemid vector. The *Xp* DNA in the plasmids will be sequenced using primers walking technique and an automated DNA sequencer.

The genome organization of *msrA* will be performed by first determine its copy number. Then, transcription organization will be performed by hybridizing *msrA* probe against total RNA isolated by a hot phenol method and separated on formaldehyde agarose gels. This will give information whether *msrA* is transcribed as a monocistronic or polycistronic.

3.2 Expression analysis of msrA.

msrA expression will be performed using Northern hybridization. Total RNA will be isolated using hot phenol method and separated on formaldehyde agarose gels.

To aid understanding of transcription regulation of the gene, the transcription start site will be determined by primer extension.

3.3 Construction and physiological analysis of *msrA* mutant.

A knockout mutant in *msrA* will be constructed via a single recombination and gene inactivation. The putative mutant will be characterized using PCR and confirmed by Southern hybridization.

Physiological analysis of the mutant will lead to better understanding of the function of the gene. Sensitivity of the mutant to various oxidants, and stresses will be determined using both low and high concentrations.

6. แผนการดำเนินการวิจัยตลอดโครงการ

1) Making *Xanthomonas katA* specific probe

2) *katA* isolation and sequencing

3) Construction of *katA* mutant

4) Physiological analyzed of *katA* mutant

5) Expression analysis of *katA*

6) Isolation and characterization of *msrA*

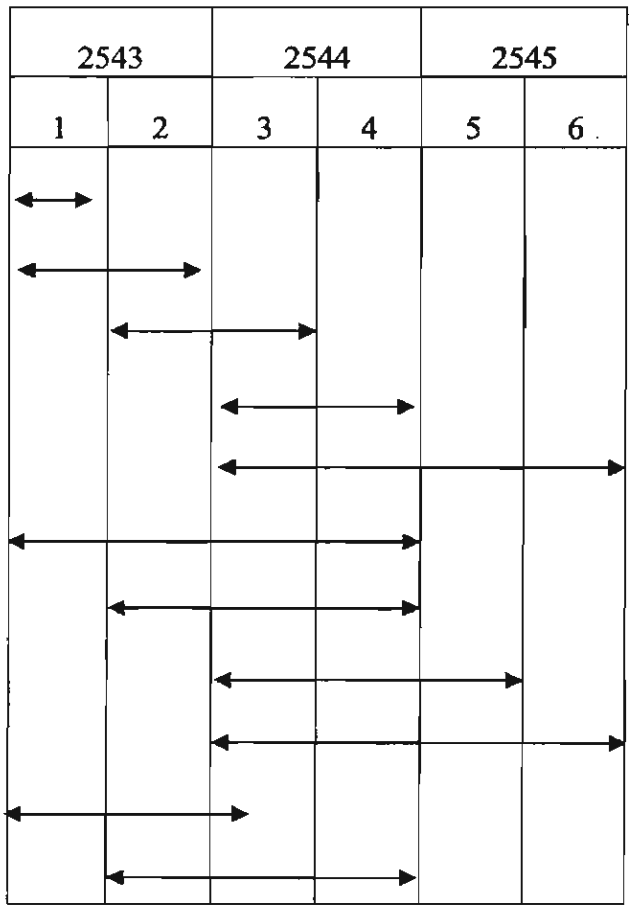
7) Construction of *msrA* mutant

8) Physiological analysis of *msrA* mutant

9) Regulation of *msrA* expression

10) Construction of *oxyR* gene fusion

11) Analysis of *oxyR* regulation



7. ประโยชน์ที่คาดว่าจะได้รับ

Understanding of how bacterial phytopathogen response to plant defense mechanisms could potentially lead to new disease management strategies. Generation of

oxidative stress is an important components of plant defense response. Currently not much is know how bacteria coped with increased oxidative stress generated by plants. Analysis of the role catalase, methionine sulfoxide reductase play in oxidative stress response could lead to development of inhibitors that could disrupt the bacterial ability to defense themselves from oxidative stress. This would make bacteria more susceptible to plant defense response and potentially could effect disease outcome. This could have big impact on agricultural practises and disease control of economically important crops.

8. หัวขอเรื่องและวารสารที่คาดว่าจะตีพิมพ์

1. Isolation and characterization of *katA*. **Journal of Bacteriology**
2. OxyR regulation of *katA*. **Journal of Bacteriology**
3. Isolation and characterization of *msrA*. **Journal of Bacteriology**
4. Unusual regulation of *oxyR* in *Xanthomonas*. **Journal of Bacteriology**

CHAPTER I

KatA: Monofunctional catalase in *Xanthomonas campestris* pv. *phaseoli*

ABSTRACT

Xanthomonas campestris pv. *phaseoli katA*, the gene for major monofunctional catalase was cloned using the reverse genetic technique. Two degenerated primers were designed from a partial amino acid sequences of KatA in *Xp* and from the conserved region of monofunctional catalases. The PCR using degenerate primers and *Xp* genomic DNA was done and giving 344 bp-PCR product that was used as a DNA probe to screen a full length *katA* gene from *Xp* genomic library λ-ZipLox phages by plaque hybridization. A complete positive clone was named pKat29 containing complete sequence of *katA*. An open reading frame of 1,521 bp, encoding a protein of 507 amino acids with theoretical molecular weight about 56 kDa. The apparent Km and Vmax values are about 39 mM of H_2O_2 and 2.3 x 10^5 μmol/min/mg protein, respectively. The k_{cat} at Vmax point is 2.132 x 10^5 sec^{-1}. Both KatA from *Xp*w/t and *Xp*HR were sensitive to 3 Amino-1,2,4 Triazole. They are unusual catalase in several respects, including the apparent of narrower pH optimal and less heat stable than normal tetrameric catalase. The *Xp* KatA is classified in the bacteria group I from the results of amino acid sequences similarity to all principal monofunctional catalase. To examine the *Xp katA*' s role against oxidative strass, *katA* mutant was constructed by insertional inactivation using a piece of *katA* inserted into non-replicated plasmid. *Xp katA* mutant was ascertained by Southern analysis and catalase activity gel staining. The mutant produced significantly decreased level of total catalase activity at log phase of growth, and became extremely sensitive to H_2O_2 killing comparing with the wild type strain. Thus, all results suggest the *katA* played an important role in *Xp* against oxidative stress by detoxification of exogenous H_2O_2. Expression analyses by Northern blot hybridization revealed that katA was transcribed as a polycistronic RNA with the adjacent protein called ankyrin like protein. The expression of katA could be induced by exposure to oxidants including H_2O_2, organic hydroperoxide and particularly menadione and was mediated by *oxyR*, a global peroxide sensor and transcription regulator.

บทคัดย่อ

คณะผู้วิจัยได้ทำการแยกยีน katA ของแบคทีเรีย *Xanthomonas campestris* pv. *phaseoli* ซึ่งเป็นยีนที่ code เอ็นไซม์ monofunctional catalase โดยใช้วิธี reverse genetic ด้วยการออกแบบ degenerate primers จากลำดับกรดอะมิโนที่ conserved และได้ใช้ primers ดังกล่าวทำ PCR โดยใช้ genomic DNA ของแบคทีเรีย *X. campestris* pv. *phaseoli* เป็นต้นแบบ จากการPCR ทำให้ได้ DNA fragment ขนาด 344 bp ซึ่งต่อมาได้นำเอา DNA fragment นี้มาเป็น probe โดยการติดฉลากด้วยสารรังสี ^{32}P เพื่อนำไปใช้ในการหายีน katA จากห้องสมุด DNA ของ *X. campestris* pv. *phaseoli* ที่สร้างขึ้นใน λ-ZipLox phages จากการ screen โดยใช้ plague hybridization ทำให้ได้พลาสมิด pKat29 ที่มี *katA* เต็มยีน เมื่อวิเคราะห์จากลำดับเบสของยีน *katA* พบว่ายีนนี้มีขนาด 1521 bp สำหรับสร้างโปรตีนขนาด 56 kDa เมื่อนำโปรตีน KatA ที่ purified จากแบคทีเรียกลายพันธ์ที่สร้าง KatA ในปริมาณสูงมาทำการวิเคราะห์ทางชีวเคมีพบว่ามีค่า apparent Km and Vmax values เท่ากับ 39 mM of H_2O_2 and 2.3 x 10^5 μmol/min/mg protein ตามลำดับ มีค่า k_{cat} at Vmax point is 2.132 x 10^5 sec^{-1} และมีความไวต่อสาร 3 Amino-1,2,4 Triazole เหมือนกับ typical catalase ทั่วไป จากการสร้าง phylogenetic tree โดยใช้ลำดับกรดอะมิโนของ catalase จากสิ่งมีชีวิตต่างๆ ใน database พบว่า KatA จัดอยู่ในประเภท bacteria group I เพื่อศึกษาถึงบทบาทของ KatA ต่อการอยู่รอดของแบคทีเรียภายใต้สภาวะที่มีอนุมูลอิสระออกซิเจนสูง คณะผู้วิจัยจึงได้สร้างแบคทีเรียกลายพันธ์ที่ไม่สามารถสร้าง KatA ได้โดยใช้วิธี insertional inactivation จากชิ้นส่วนของยีน*katA* ที่อยู่ในพลาสมิดที่ไม่สามารถแบ่งตัวเพิ่มจำนวนได้ใน *X. campestris* pv. *phaseoli* เมื่อนำ *katA* mutant ไปศึกษาพบว่าระดับของ total catalase activity ลดลงอย่างมากในช่วง log phase เมื่อนำไปทดสอบการต้านทานต่อสาร peroxide ต่างๆพบว่า *katA* mutant ไวต่อสาร H_2O_2 อย่างมากแสดงให้เห็นว่า KatA มีความสำคัญต่อการอยู่รอดของแบคทีเรียในสภาวะที่มี H_2O_2 ในการวิเคราะห์การแสดงออกของยีน *katA* โดยใช้วิธี Northern blot hybridization พบว่าการแสดงออกของยีนนี้สามารถถูกเหนี่ยวนำได้เมื่อแบคทีเรียสัมผัสต่อสารประเภท oxidants เช่น H_2O_2, organic hydroperoxide และ menadione โดยการแสดงออกนี้อยู่ภายใต้การควบคุมของ *oxyR* ซึ่งเป็น a global peroxide sensor and transcription regulator นอกจากนี้ยังพบว่า katA ถูก transcribed เป็น polycistronic RNA ร่วมกับโปรตีนอีกชนิดหนึ่งที่อยู่ใกล้เคียงที่มีชื่อว่า ankyrin like protein และ

INTRODUCTION

Xanthomonas spp. is an important group of bacterial phytopathogen. During plant-microbe interactions, the initial plant defense response involves increased production of reactive oxygen species [1]. They function as bacteriocidal agents and as secondary signal molecules to further activate plant defense responses [2]. To survive and proliferate, bacterial pathogens must overcome reactive oxygen species. Microbial defense against oxidative stress required well-orchestrated expression of enzymes involving both detoxification of the stress and repair processes. Catalases (E.C. 1.11.1.6) have important protective roles against H_2O_2 , the first ROS released, from plants [3,4]. There are two types of catalase enzymes, a bifunctional catalase-peroxidase and a monofunctional catalase [5]. In this study we are interesting in *Xanthomonas campestris* pv. *phaseoli* (*Xp*). It is a causative agent of common blight disease in dry beans (*Phaseolus valgaris* and *Phaseolus lunatus*) [6]. The oxidative stress responses of *Xp* is quite complex [7]. The previous study suggested that *Xp* possesses at least two distinct monofunctional catalase isozymes on the basis of the observation of two main activity bands on non denaturating polyacrylamide gel electrophoresis [8]. The stationary-phase dependent catalase denoted KatE has been cloned and characterized [8]. This is an essential step in order to understand this bacteria. In this study, the major form of monofunctional catalase denoted KatA (formerly name Kat1) was purified, cloned and characterized.

METHODS

Bacterial cultures and media.

All *Xanthomonas* strains were grown aerobically at 28°C in Silva-Buddenhagen (SB) media containing appropriate antibiotic, respectively [9]. To ensure synchronous exponential growth, overnight cultures were subcultured into fresh medium to give OD_{600} of 0.1.The culture was allowed to grow until an exponential phase culture represented density had reached 0.5 OD_{600} or a stationary phase culture presented 24 h up to the experiments. All *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) media.

Cell lysate preparation and catalase activity gel staining

Preparation of cell lysates for activity gel analysis was according to Vattanaviboon and Mongkolsuk [8]. Essentially, 20 ml log-phase cultures were pelleted and washed once with 50 mM sodium phosphate buffer pH 7.0. Bacterial cells were lysed by brief sonication followed by centrifugation at 10,000 g for 10 min. Cleared lysates were used for catalase activity gels. Catalase isozymes were visualized on native PAGE gels performed by the method of Kim *et al.* with some modifications [8,10].

Purification of the *Xp* catalase KatA.

All manipulations were carried out at 4°C. The crude extract was prepared by using the Aminco French press at 20,000 psi (1 psi = 6.894757 kPa) and a 2.5 % streptomycin sulfate precipitation was carried out as described [11], except that 1mM EDTA was present in the buffer. The ammonium sulfate precipitation was performed to separate the catalase A (KatA) and also to achieve a greater increase in the specific activity of enzyme. The first fraction was carried out at 30, 40, 50 and 60% saturation with solid ammonium sulfate. At each step, the solution was stirred gently to ensure complete dissolution and precipitation. For the *Xanthomonas* catalase, the 60% fraction had the highest catalase specific activity. This fraction was dialyzed overnight at 4°C against 4 L of 50 mM potassium phosphate buffer (pH 7.0) and loaded on a 2 x 10 cm hydroxylapatite column equilibrated in the same buffer. The column was washed until the absorbance at 280 nm of the elution had dropped below 0.05. The enzyme was eluted with a linear gradient of NaCl (0.05-0.5 M). The fraction showing catalase activity were pooled, concentrated by ultrafiltration (Amicon), dialyzed overnight in the same buffer, and stored at –70°C.

Partial amino acids determination

KatA was purified as previously decribed. Purified KatA was run on the gel eletrophoresis after digested by BrCN to eliminate blocking and bloted onto immobilon filter. The catalase band was cut from the filter to determined partial amino acid sequence by the Tripartite Microanalytical Center at the University of Victoria, Victoria, Canada.

DNA isolation, cloning, PCR and nucleotide sequencing.

Xanthomonas genomic DNA was purified as previously described [12]. Primers for amplification of *Xp katA* were designed base on partial amino acid sequence of KatA protein (NGVHAYKLVNAQG) and the conserved region of monofunctional catalases bias toward the high G+C codon usage and modified to be the complementary strand (DNFFQETEQ). Both of them were BT50 [5′ AAC GG(G/C) GT(G/C) CA(C/T) GC(G/C) TA(C/T) AA(A/G) CT(G/C) GT(G/C) AA(C/T) GC(A/G) CA(A/G)GG 3′], BT54 [5′ TG(C/T) TC(A/G/C) GT(C/T) TC(C/T) TG(A/G) AA(A/G) AA(A/G) TT(A/G) TC 3′] respectively. Genomic DNA (0.5μg) from *Xp* was added to PCR reaction containing 25 pmol of primers, a commercial PCR reaction buffer and nucleotide mix (Promega), 2 units of Taq polymerase. The PCR reactions were performed using the following cycle conditions: denatured at 94°C 1 min, annealed at 55 °C 1 min and reaction at 72 °C 1 min for 30 cycles. The PCR products were cloned into pGEM-T easy vector (Promega) and use as a probe for screening *Xp* genomic library [13] if the results from nucleotide sequencing by fluorescence dye terminator ABI prim kits and analyzed on an automated DNA sequencer ABI 377 showed a high degree of identity with monofunctional catalase of related species.

Analysis of peptide KatA and deduced amino acid sequence.

The nucleotide sequence was analyzed for possible ORF by DNASIS program. The protein related to monofunctional catalase were detrived from databases using the BLAST program. These amino acid sequences were aligned using the CLUSTAL W (version 1.7) [14]. The results were drawn by PHYLODENDRON (version 0.8d) of D.G. Gilbert, Department of Biology, University of Indiana, USA (http://iubio.bio.indiana.edu).

Enzyme assay and protein estimation

Absorbance at 280 nm was used for monitoring proteins in column elutes. Catalase activity was estimated by the method of Rorth and Jensen [15] in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase in defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min at 37°C

Enzyme kinetics.

Michaelis-Menten constant (K_m), maximum velocity (V_{max}) and the turn over rate of enzyme (k_{cat}) were determined as a function of the H_2O_2 concentration at 37°C at pH 7.0

Temperature sensitivity.

To estimate thermal stability, the purified enzyme was incubated at for 10 min at different temperatures, and the residual activity was measured at 37°C

Effect of pH.

The purified enzyme was incubated in 50 mM potassium phosphate buffer at the various pH values for 1 min prior to commencing the assay by adding H_2O_2 [16]. For values beyond the buffering capacity, the pH was adjusted by the addition of HCl or NaOH directly before the assay as previously described [17].

Inhibitory effect of 3-amino-1,2,4-triazole on the purified catalase A .

The catalase enzyme was incubated in 50 mM potassium phosphate buffer at various concentration of inhibitor for 1 min. prior to commencing the assay by adding H_2O_2 [18].

Construction of *Xp katA* insertional inactivation mutant.

The fragment *katA* coding region in plasmid vector will transferred in to *Xp* wild type by electroporation. The transformants will be selected on the appropriate antibiotic SB plate. All antibiotic resistance colonies were characterized for inactivation of *katA* by Southern blot analysis.

Purification of Kat A from *Xp*HR

*Xp*HR is a peroxide resistant mutant strain over producing KatA enzyme [19] as result from the activity gel of lysate when compare with lysate from normal cell (Fig 2A). KatA was purified by $(NH_4)_2SO_4$ precipitation followed by hydroxylapatite. The specific activity of the KatA was about 150,000 U/mg protein. It is quite high specific activity when compare with other monofunctional catalases. The final precipitation migrated product showed a single band of protein on denaturing SDS acrylamide gels, with the corresponding to a molecular weight of 54 kDa (Fig 2B)

Cloning of *Xp katA* and analysis of catalase nucleotide sequence and the deduced amino acid sequence.

To determine the role of catalase in the defense of *Xp* against H_2O_2 toxicity, we employed a genetic approach to clone and characterize a *katA* from this organism. We took advantage of the high homology between region of monofunctional catalases from various bacteria. Degenerate oligonucleotides derived from the conserved region of monofunctional catalases bias toward the high G+C codon usage (DNFFQETEQ) and designed base on partial amino acid sequence of KatA protein (NGVHAYKLVNAQG). A 344 bp PCR product was amplified from *Xp* genomic. To determine whether the PCR product was derived from a *Xp* catalase gene, we cloned this fragment into pGem T-easy plasmid vector yielding pKat111. The DNA inserted in this recombinant plasmid was sequenced by both orientations.Analysis of its nucleotide sequence showed a high degree of identity to various monofunctional catalases. This 344 bp DNA fragment was used as a probe to screen a full length *katA* gene from *Xp* genomic library constructed in λ ZipLox phages by plaque hybridization. A positive clone named pKat29 containing a fulll length of *katA* was isolated. The open reading frame of 1,521 bp capable encoding a protein of 507 amino acids with a predicted molecular mass 56 kDa was identified. This molecular weight was consistent with that of the purified KatA determined SDS polyacrylamide gel electrophoresis. The codon usuage of this ORF is typical of G+C rich which is the character of this family. Homology search against GenBank databases showed high degree of amino acid sequence identity to all principal monofunctional catalase, especially to *P. syringae* and *P. aeruginosa* catalases.

Phylogenetic relationships of monofunctional catalases.

The alignments of amino acid sequences were created the multiple-alignment among the monofunctinal catalase using CLUSTAL W (version 1.7) [14]. Estimates of the evolutionary relationships of sequences and drawn unrooted tree by PHYLODENDRON (version 0.8d) of D.G. Gilbert, Department of Biology, University of Indiana, USA (http://iubio.bio.indiana.edu). The results from Fig.3 showed that KatA of *Xp* is closely related to the bacteria group I. This group was derived from opportunistic or nonpathogenic bacteria that are widespread in nature and include representatives from such diverse taxa as ?-proteobacterial florescent pseudomonads [20].

RESULTS AND DISCUSSION

1. Multiple catalase isozymes in *X. campestris* pv. *phaseoli*

Multiple catalase isozymes have been reported in many bacteria especially in *Pseudomonas*, a closely related genus to *Xanthomonas*. *P. putida* exhibits three catalase isozymes encoded from three distinct genes. In order to differentiate catalase isozymes in *Xp*, a catalase activity staining SDS-PAGE gel was performed. Equal amount of protein in crude extracts prepared from *X. campestris* pv. *phaseoli* cultures at all stage of growth were separated on 7.5% SDS-PAGE. After electrophoresis, polyacrylamide gel was renatured and stained for catalase activity as describe in the Materials and Methods. The protein bands having catalase activity would appear as colorless bands in dark brown background. The results from activity gel showed that *Xp* produced two detectable catalase isozymes (Fig. 1). The low apparent mass isozyme, called KatA, was expressed at all growth-phases. KatA was produced in higher quantity at log-phase of growth and declined slightly when cells entering stationary phase. The other isozyme of catalase, named KatE, had relatively higher apparent mass than KatA. The expression of Kat2 was turned on when cells entering stationary phase. Both KatE and KatA activity bands did not have peroxidase activity when a corresponding gel was stained for peroxidase activity (data not shown). These results suggested that both KatA and KatE were monofunctional catalases.

2. Induction of KatA isozyme by oxidants

In many microorganisms, catalase activity increases in response to oxidative stress. Either bifunctional catalase i.e. *E.coli* KatG or monofunctional catalase i.e. *Haemophilus influenzae* HktE, and *Vibrio fischeri* KatA whose expressions could be inducible by exposure to low concentrations of H_2O_2. In *Xanthomonas*, the studies in *X. oryzae* reveal that *X. oryzae* produces only one detectable monofunctional catalase isozyme, KatX. The expression of KatX is inducible in response to various oxidants including MD, tBOOH and H_2O_2. Unlike *X. oryzae*, *Xp* possesses two monofunctional catalase isozymes expressed in different fashion (Fig.33). Induction of catalase expression by oxidants was investigated in *Xp* at both log- and stationary-phase of growth. Log phase or stationary

phase cells were pretreated with MD, tBOOH, H_2O_2 or NEM for 1 h. The lysates prepared from induced or uninduced culture were assayed for total catalase activity and protein content. The results (Table 1) showed that treatment of log-phase cultures with H_2O_2, tBOOH, MD or NEM could induce accumulation of catalase by 2.7, 4.5, 8.4 and 2.4 fold, respectively, when compared to uninduced cells. The catalase activity staining of SDS-PAGE of those extracts (Fig. 2) revealed that Kat1 isozyme was increased in response to the treatment of oxidants whilst the activity of Kat isozyme was still non-detectable. The intensity of KatA bands increased concomitantly with the fold of induction in total catalase activity.

Induction of stationary phase cells with oxidants showed different profile although the total catalase activity appeared to be inducible (Table 1). The folds of induction by H_2O_2, tBOOH, MD or NEM were 1.7, 1.9, 2.1 and 1.7, respectively, which dramatically lower than those of induction in log-phase cells. The catalase activity staining of SDS-PAGE (Fig. 3) showed that the intensity of KatA isozyme was increased and likely to be responsible for an enhancement of total catalase activity. On the other hand, the expression of KatE isozyme was not obviously inducible by these tested oxidants. These results clearly showed that the expression of KatA isozyme could be induced by H_2O_2, tBOOH, MD or NEM at both log- and stationary-phase of growth.

3. The induction of KatA was mediated by OxyR

Since H_2O_2, tBOOH, MD and NEM have been reported to be potent inducers of *Xanthomonas oxyR*, a global regulator and peroxide sensor. Thus, KatA expression and induction might be regulated via OxyR,. To test this hypothesis, induction experiments were repeated in *Xp oxyR*⁻ mutant (*oxyR*::Gm). As expected, *Xp oxyR*⁻ mutant produced non-inducible level of catalase activity corresponding to the low intensity of KatA band in activity gel (Table 2, Fig. 4). These results indicated that induction of KatA expression in *Xp* requires functional *oxyR*.

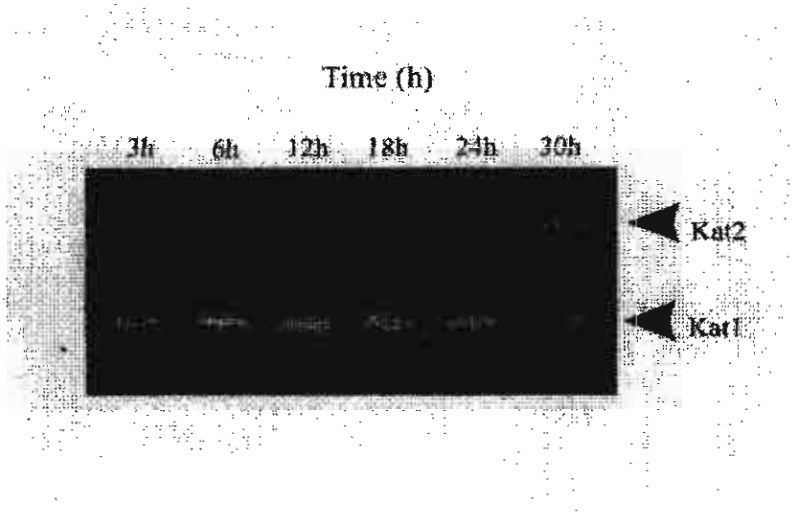


Fig. 1. Multiple catalase isozymes in *Xp*.

Catalase ctivity staining of proteins separated on 7.5% SDS-PAGE to determine various catalase isozymes in crude extracts at different growth phases. Lane: 1, early log (3h); 2, mid-log (6h); 3, early stationary (12h); 4, stationary (18h); 5, late stationary (24h); and 6, late stationary (30h).

Table. 1 Total catalase activity of *Xp* at log-phase and stationary-phase in response to different inducers.

Inducers (concentration)	Total catalase activity [a] (fold of induction)	
	log-phase cells	stationary-phase cells
Uninduced	5.0±1.1 (1.0)	3.9±1.0 (1.0)
H_2O_2 (100μM)	13.9±1.4 (2.7)	6.6±1.2 (1.7)
tBOOH (100μM)	22.8±2.4 (4.5)	7.2±1.1 (1.9)
MD (100μM)	42.2±3.6 (8.4)	8.3±1.0 (2.1)
NEM (100μM)	12.3±2.0 (2.4)	6.7±1.3 (1.7)

[a]All experiments were independently repeated 3 times and mean ± S.D. values are shown.

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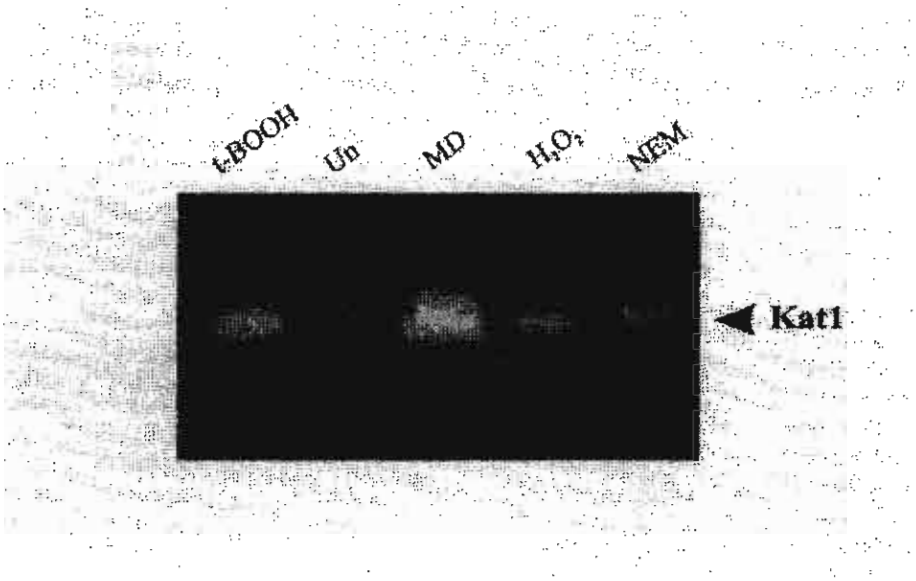

Fig. 2. Induction of catalase isozymes in *Xp*.

Activity staining of catalases separated on 7.5% SDS-PAGE. Log-phase cells induced with different oxidants, Lane 1, tBOOH-induced; 2, uninduced; 3, MD-induced; 4, H_2O_2-induced; and 5 , NEM-induced.

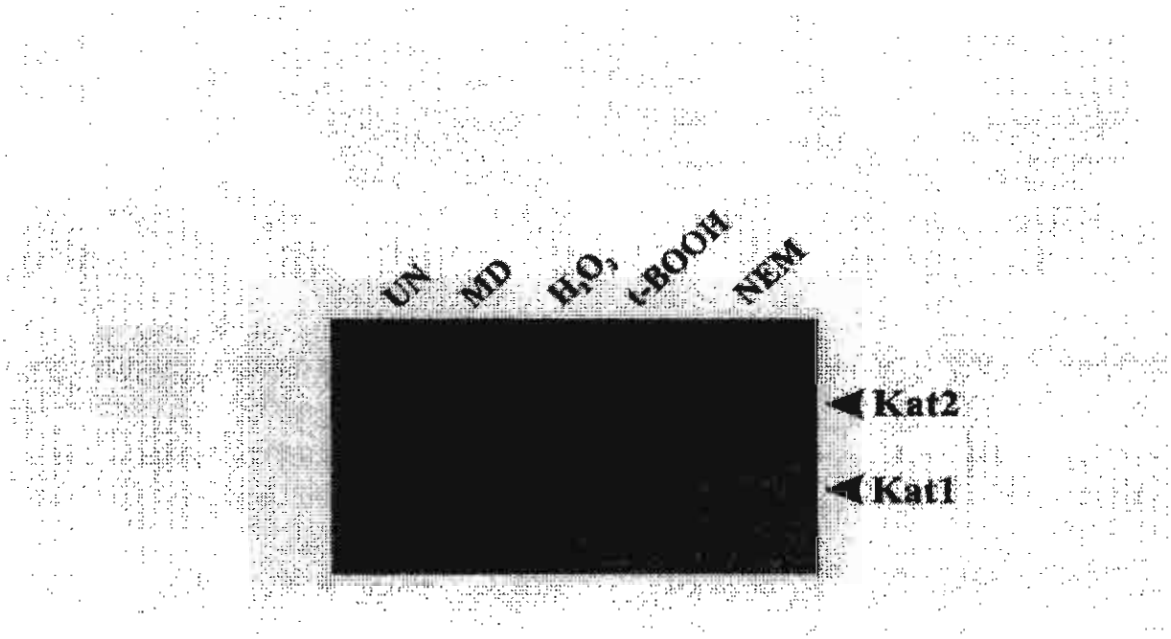

Fig. 3. Induction of catalase isozymes in *Xp*

Catalase activity staining of proteins separated on 7.5% SDS-PAGE to visualize catalase isozymes in samples prepared from stationary phase cells induced with different oxidants, Lane 1, uninduced; 2, MD-induced; 3, H_2O_2-induced; 4, tBOOH-induced; and 5, NEM-induced.

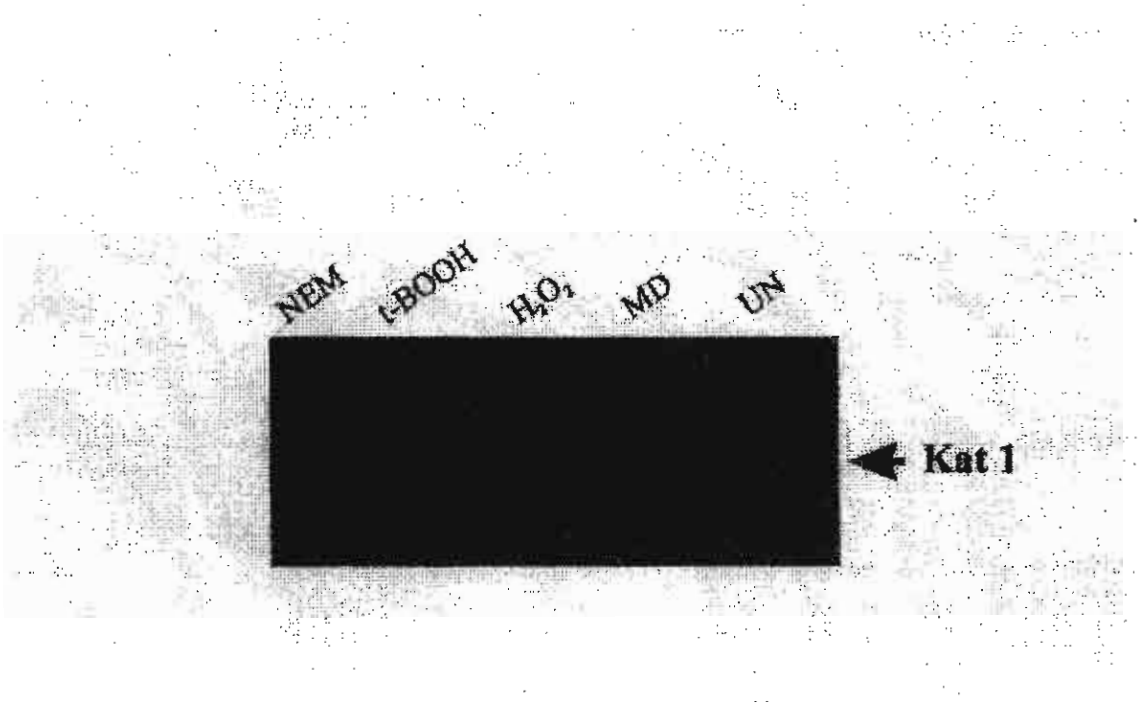

Fig. 4. Induction of catalase isozymes in an *Xp oxyR*⁻ mutant.

Catalase activity staining of proteins separated on 7.5% SDS-PAGE to visualize catalase isozymes in samples prepared from log-phase culture induced with different oxidants. Lane 1, uninduced; 2, tBOOH-induced; 3, H_2O_2-induced; 4, NEM-induced; and 5, MD-induced.

Table 2. Total catalase activity of *Xp oxyR*⁻ mutant in response to oxidants.

Inducers (concentration)	Catalase (U/mg protein)
Uninduced	1.7 ± 0.4
H_2O_2 (100μM)	1.5 ± 0.5
tBOOH (100μM)	1.6 ± 0.4
MD (100μM)	1.6 ± 0.3
NEM (100μM)	1.9 ± 0.4

[a]All experiments were independently repeated 3 times and mean ± S.D. values are shown.

4. KatE was inducible by starvation.

The expression of *E.coli* monofunctional catalase (KatE) is regulated by σ^S sigma factor whose expression is turn on when cell entering stationary phase or in starvation condition. Similar to *E.coli* KatE, *Xp* Kat2 was produced at stationary phase of growth. The effect of starvation on the expression of Kat2 was observed in *Xp*. An overnight culture was subcultured into 20-ml SB broth. The log-phase (3h) growing cells were collected by centrifugation at 28 °C. Cell pellet was resuspended in 20 ml M9 minimal salt (without glucose) and grown for 3h in aerobic condition. For non-starved control, the 3h-culture in SB was continued growing for 3h in the same condition. The lysates prepared from control and starved cells were loaded into SDS-PAGE for catalase activity staining. As shown in Fig. 5, starvation could induce the expression of KatE catalase isozyme.

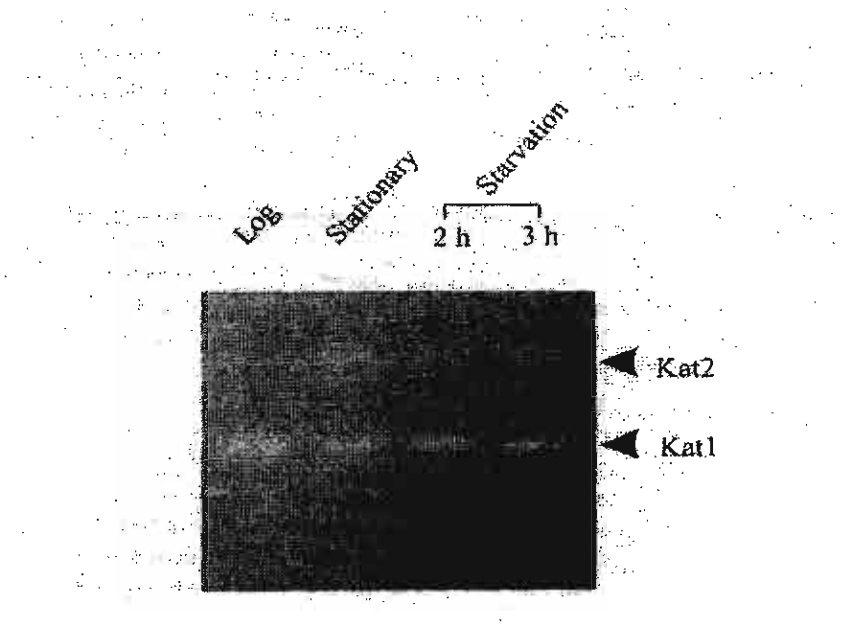

Fig. 5. Induction of catalase isozymes by starvation.

Catalase activity staining of proteins separated on 7.5% SDS-PAGE to visualize catalase isozymes in samples prepared from log phase cultures of *Xp* in complex medium (non-starved, lane 1), under carbon starvation for 2 h (lane 3), 3h (lane 4) and a stationary phase culture (lane 2).

5. Cloning and characterization of *Xp katA*

The *katA* gene from *Xp* was cloned using a reverse genetic approach. The partial amino acid sequence of KatA, NGVHAYKL, was determined from trypsin digest fragments. The KatA amino acid sequence and another sequence derived from a highly conserved region of bacterial monofunctional catalases were used to design two degenerate oligonucleotide PCR primers which yielded a 344 bp PCR product that was cloned into pGEM-T-easy vector giving pKat111. The nucleotide sequence of the fragment showed significant similarity to other monofunctional catalases. The 344 bp DNA fragment was then used as a probe to screen for the full-length *katA* gene from an *Xp* genomic library. A positive clone named pKat29 containing *katA* was isolated and sequenced. An open reading frame of 1,521 bp encoding a 507 amino acid protein with a predicted molecular mass of 56 kDa was identified. The encoded protein showed a high degree of amino acid sequence identity with other monofunctional catalases, including 84% and 78% identity with CatF of *P. syringae* and KatB of *P. aeruginosa*, respectively. The gene was therefore designated *katA*. A phylogenetic analysis of a limited group of catalase sequences was carried out revealing that *katA* is a clade I catalase more closely related to plant than to mammalian catalases (Fig. 6).

Xp katA was sub-cloned from pKat29 into a pGEM-5Zf vector giving pGemkatA for transformation into the catalase deficient *E. coli* strain UM255 (*katG2, katE12::Tn10,recA*) [9]. Cell lysates prepared from UM255 harboring pGemkatA and from *Xp* were compared on non-denaturing gels stained for catalase activity revealing a similar band of catalase in both extracts. Expression of KatA in UM255 harboring pGemkatA was more efficient at 28°C (955 units mg of protein^{-1}) as compared to 37°C (119 units mg of protein^{-1}). This effect may be the result of poorer folding of KatA in the heterologous *E. coli* host at 37°C and to the enhanced temperature sensitivity noted above.

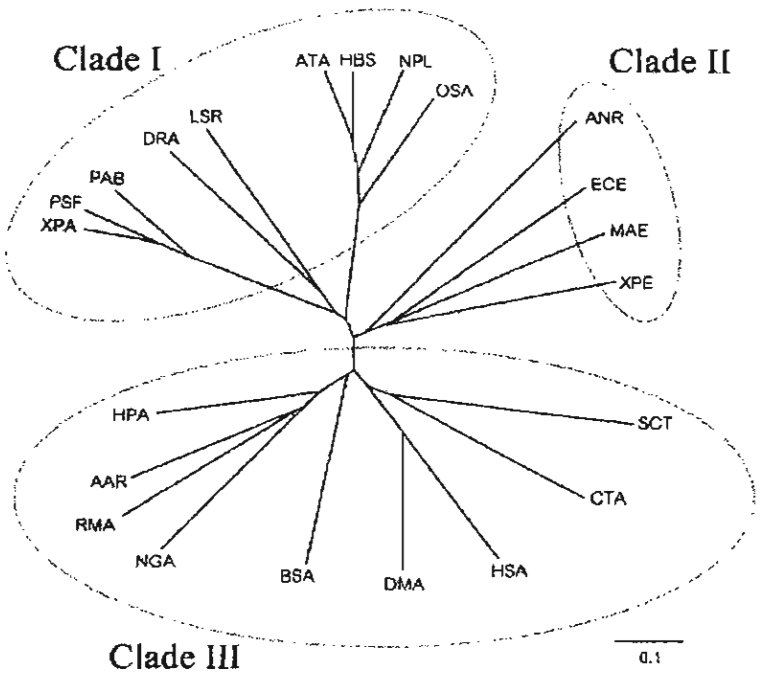

Fig. 6. Fig. 3. A phylogenetic tree of monofunctional catalases.

A phylogenetic tree was constructed by the neighbor joining method using the Tree program for the phylogenetic analysis of Clustal W [12]. The results were drawn using the program PHYLODENDRON (Version 0.8 d 1994; Department of Biology, University of Indiana [http://iubio.bio.indiana.edu]). XPA, *X. campestris* pv. phaseoli KatA (AF461425); PSF , *P. syringae* CatF (U03465); PAB, *P. aeruginosa* KatB (U34896); DRA, *Deinococus radiodurans* KatA (D63898); LSR, *Listeria seeligeri* Kat (M75944); ATA, *Arabidropsis thaliana* CatA (X64271); HBS, *Hevea brasiliensis* (AF151368); NPL, *Nicotiana plumbaginifolia* Cat2 (Z36976); OSA, *Oryza sativa* CatA (X61626); ANR, *Aspergillus niger* CatR (Z23138); ECE, *E.coli* KatE (M55161); MAE, *Mycobacterium avium* KatE (L41246); XPE, *X. campestris* pv. *phaseoli* KatE (AF170449); SCT, *Saccharomyces cerevisiae* CatT (X04625); CTA, *Candida tropicalis* CatA (M18832); HAS, *Homo sapiens* Catalase (P04040); DMA, *Drosophila melanogaster* CatA (X52286); BSA, *B. subtilis* KatA (M80796); NGA, *Neisseria gonorrhoeae* KatA (U35457); RMA, *Rhizobium meliloti* KatA (U59271); AAR, *Actinobacillus actinomyceterium* (AF162654); HPA, *Helicobacter pylori* KatA (U67458). Bar, 0.1 change per site.

6. Purification of KatA from XpHR

Catalase activity gels have shown that *Xp* produces two isozymes of monofunctional catalases coded by distinct genes. Because many catalase genes have been isolated from bacteria, but very few enzymes have been purified and characterized, the purification and characterization of KatA, the major catalase in *Xp*, was initiated. *Xp* has a low total catalase activity that made purification of the enzyme difficult, but a multiple peroxide resistant mutant designated *Xp*HR that produces 200-300 fold higher levels of catalase has been isolated (Fig. 7A). This mutant was used as the source for KatA, the major catalase of *Xp* (Fig. 7). The purified catalase has a specific activity of 150,000 U mg protein^{-1} (at 60 mM H_2O_2), somewhat higher than some other bacterial catalases such as *E. coli* KatE and *P. aeruginosa* catalases. SDS-PAGE analysis of the purified enzyme showed that the preparation was greater than 90% pure and the major protein band had a molecular weight of 56 kDa (Fig. 7A) similar to other small subunit monofunctional catalases.

7. Biochemical characterization of KatA

The apparent K_m and V_{max} values of the KatA for H_2O_2 at pH 7 and 37°C were determined to be 75 mM of H_2O_2 and 2.74 × 10^5 μmol H_2O_2 μmol heme^{-1}sec^{-1}, respectively. The k_{cat} is 2.55 × 10^5 sec^{-1}. Catalases generally have low substrate affinities with apparent K_m values for H_2O_2 of 10.6 and 44.7 mM for KatB and KatA from *P. aeruginosa* and 60 mM for CatF from *P. syringae* [2,4] and *Xp* KatA is similar to these.

KatA is very sensitive to the catalase inhibitors, 3-amino-1,2,4-triazole (K_i = 2.0 mM) and NaN_3 (K_i = 1.0 μM) in sharp contrast to *E.coli* KatE which is highly resistant to both inhibitors but similar to the catalases from *Pseudomonas*. KatA has a broad pH range for optimal activity from 6.0-9.0, outside of which activity dropped off rapidly (Fig. 8B). This differs from monofunctional catalases from *E.coli* and *Bacillus subtilis*, which have even wider ranges of optimum pH. Incubation of KatA for 10 minutes at 30 °C resulted in a loss of 20% of activity while incubation for 10 minutes at 55 °C resulted in a loss of 50 % of activity (Fig. 8C) making KatA was much more sensitive to heat inactivation than many other catalases. In conclusion, KatA from *Xp*HR differs in some properties and is similar in others compared to characterized bacterial catalases.

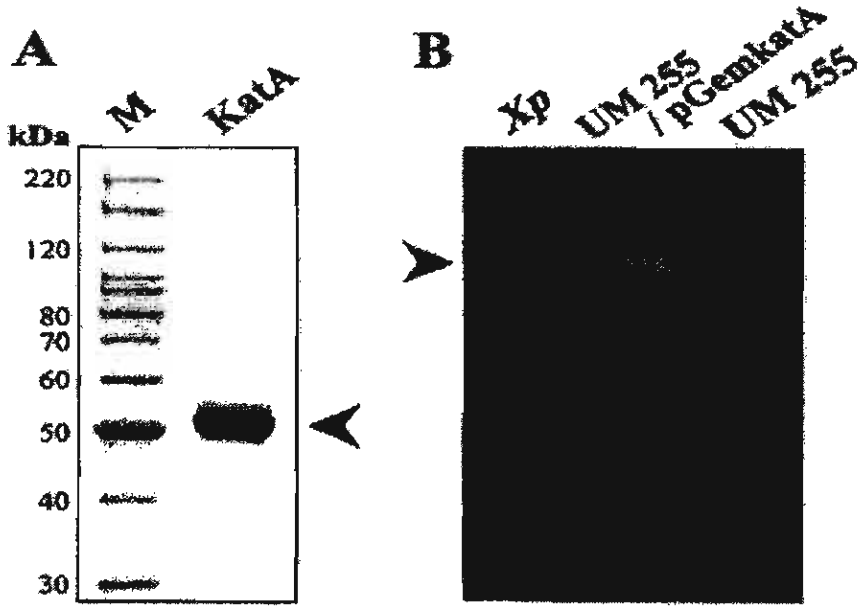

Fig. 7. Analysis of purifed KatA and catalase activity gel.

In A, 5 μg of purifed KatA was analyzed on a 8% SDS-PAGE gel stained with Coomassie Blue (KatA). M represented protein molecular weight markers. In B, catalase activity staining of the protein lysates separated on 7.5 % SDS-PAGE to visualize KatA in UM255 (100 μg total protein), UM255 harboring pGemkatA (5 μg total protein) and exponential phase *Xp* (100 μg total protein), respectively. Arrow indicated the position of KatA.

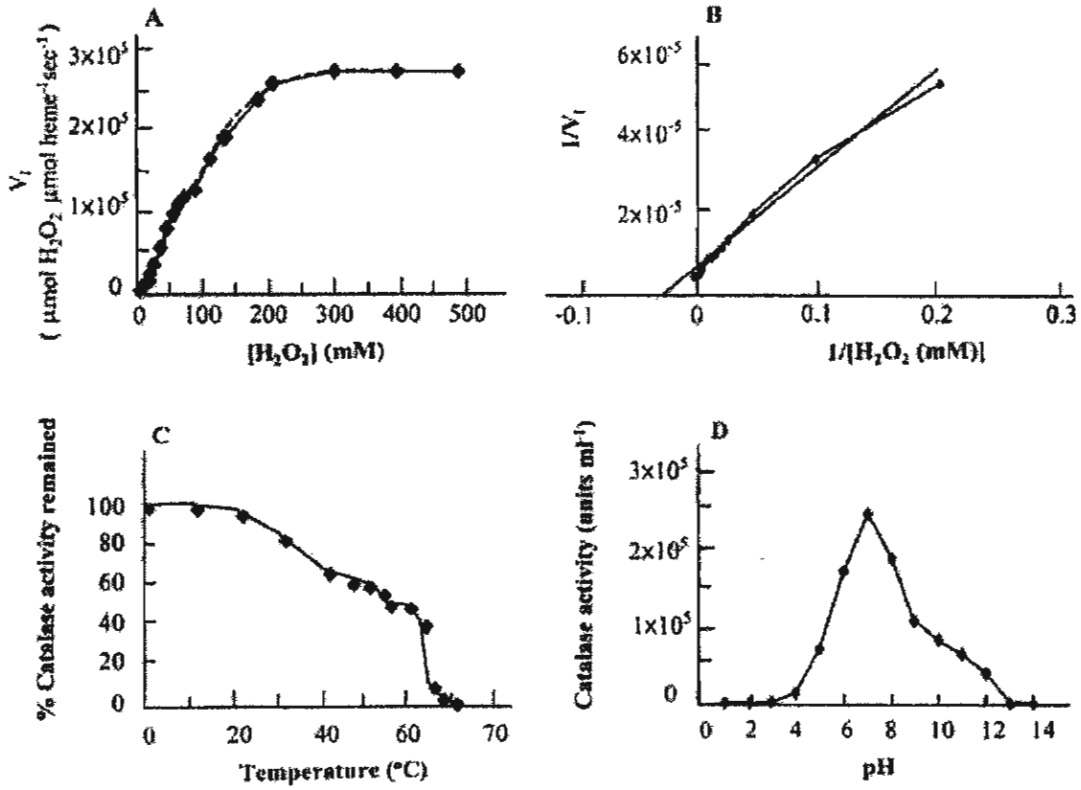

Fig. 8. Biochemical characterization of KatA.

The apparent V_{max} (A) and K_m (Lineweaver-Burk plot, B) determinations with different concentrations of H_2O_2 as substrate. The effect of temperature (C) and pH (D) on catalase activity. All experiments were performed as described in the materials and methods.

8. Construction of *katA* mutant.

Part of the coding region of *katA* cloned into a vector that cannot replicate in *Xp* (the pKat111) was introduced into *Xp* by electroporation. A single recombination between a katA fragment in non-replicated plasmid and a counter part on the chromosome resulted inactivation of *katA*. The Southern analysis showed the result to confirm the knock out of *katA* gene in *Xp katA* mutant (Fig.9). The mutant produced significantly decreased level of total catalase activity at log phase of growth, and very sensitive to H_2O_2 killing when comparing the parental strain (Table 3). Thus, all results suggest the *katA* played an important role in *Xp* against oxidative stress by detoxification of exogenous H_2O_2.

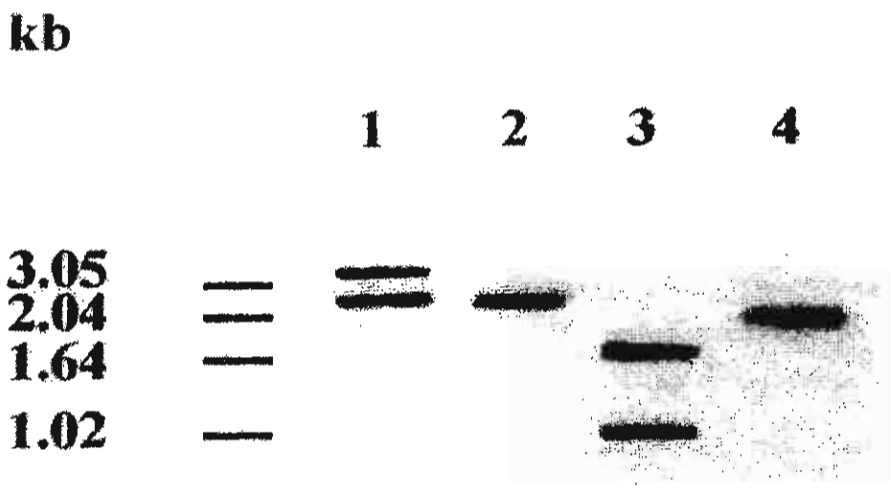

Fig. 9. Southern blot analysis of *Xp katA* and its parental strain (*Xp*).
Genomic DNA of *Xp katA* (lane 1 and 3) and *Xp* (lane 2 and 4) were digested with *EcoR*I and *Sal*I respectively. The blot was hybridized with radioactively labeled *katA* probe.

Table 3. The resistant level against oxidants in *Xp katA* mutant and its parental strain.

Strains	**Inhibition Zone (mm)**		
	H_2O_2	Menadione	tBOOH
Xp	14.0	24.5	29.0
Xp katA	24.0	24.5	28.0
Xp katA/pKatA	10.0	22.5	31.0

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CHAPTER II

ohrR: organic peroxide sensor and transcriptional repressor in *X. campestris* pv. *phaseoli*

ABSTRACT

We report the physiological role of OhrR as an organic peroxide sensor and transcription repressor in *Xanthomonas campestris* pv. *phaseoli*. *In vivo* exposure of *X. campestris* pv. *phaseoli* to either *tert*-butyl or cumene hydroperoxides efficiently neutralized OhrR repression of expression from the OhrR regulated P1 promoter. H_2O_2 was a weak and non-physiological inducer of the system while other oxidants and metabolites of organic peroxide metabolism did not induce the expression from the P1. Northern blotting results indicated a correlation between concentrations of *tert*-butyl hydroperoxide used in the treatment and the induction of *ohr* (an OhrR-regulated gene) expression. In addition, the levels of *ohr* mRNA in cultures induced by various concentrations of *tert*-butyl hydroperoxide were reduced in cells with high levels of an organic peroxide metabolising enzyme (AhpC-AhpF) but not in cells with high catalase levels suggesting that organic peroxide interacts with OhrR. DNA band shift experiments using purified OhrR and the P1 promoter fragment showed that organic peroxide treatment prevented binding of the protein to the P1 promoter by oxidation of OhrR, since the inhibition of binding to the P1 promoter was reversed by addition of a reducing agent, DTT. The highly conserved cysteine residue C22 of OhrR is required for organic peroxide inducible gene expression. A mutant protein, OhrRC22S can repress the P1 promoter activity but insensitive to organic peroxide treatment. Thus, OhrR is the first transcription repressor characterized that appeared to evolve to physiologically sense organic peroxides.

INTRODUCTION

Organic peroxides are highly toxic compounds to biological systems due to their abilities to react with intracellular macromolecules and to generate reactive organic radicals (Halliwell and Gutteridge, 1984). Bacteria have evolved several pathways to ensure efficient removal of organic peroxides. Alkyl hydroperoxide reductase (AhpC) is widely distributed in diverse organisms ranging from bacteria to humans and is the best characterized organic peroxide detoxification system that metabolizes organic peroxides to their corresponding alcohols (Chae *et al.*, 1994; Poole and Ellis, 1996). A second organic peroxide protection system, organic hydroperoxide resistance protein (Ohr), was first identified in *X. campestris* pv. *phaseoli* (Mongkolsuk *et al.*, 1998) and subsequently shown to be present in many gram-positive and gram-negative bacteria (Atichartpongkul *et al.*, 2001; Fuangthong *et al.*, 2001; Ochsner *et al.*, 2001; Shea and Mulks, 2002). Inactivation of *ohr* results in increased sensitivity towards organic peroxides (Atichartpongkul *et al.*, 2001; Mongkolsuk *et al.*, 1998). How the Ohr system works is not known.

The abilities to sense changes and respond to oxidative stress are crucial for aerobic organisms. Bacteria have complex sensing mechanisms and response regulators which alter patterns of gene expression as to prevent oxidative damages to cells. Two of the most well characterized bacterial sensors of oxidative stress and transcription regulators are OxyR, a global peroxide sensor (Toledano *et al.*, 1994; Zheng and Storz, 2000) and SoxR, a global superoxide sensor (Demple *et al.*, 2002). AhpC and Ohr appear to have overlapping physiological functions, but their patterns of expression and regulation differ. *ahpC* is regulated by OxyR (Loprasert *et al.*, 2000), while *ohr* is regulated by a novel, organic hydroperoxide inducible, transcription repressor, OhrR, that belongs to the MarR family. *ohrR* is found in both gram-negative and gram-positive bacteria (Fuangthong *et al.*, 2001). Interestingly, genes in the OxyR regulon are highly induced by treatment of cells with H_2O_2 and organic peroxide whereas genes in the OhrR regulon are highly induced only by organic peroxide (Loprasert *et al.*, 2000; Sukchawalit *et al.*, 2001). In *X. campestris* pv. *phaseoli*, *ohrR* is uniquely regulated at both the transcriptional and post-transcriptional levels. OhrR autoregulates expression from its own promoter (P1), and RNA processing and production of a highly labile *ohrR* mRNA, coupled with inefficient translation of the mRNA limits the intracellular level of OhrR (Mongkolsuk *et al.*, 2002). Here, we report that OhrR is evolved to sense and respond to changes in organic peroxide levels. The sensing mechanism involved oxidation of the highly conserved C residue that prevents the protein from binding to its target site in the P1 promoter region.

METHODS

Bacterial culture conditions

All *Xanthomonas* strains were grown aerobically in SB (Silva-Buddenhagen medium, 0.5% peptone, 0.5% yeast extract, 0.5% sucrose and 0.1% glutamic acid, pH 7.0, Mongkolsuk *et al.*, 1997) at 28°C. The oxidant induction experiments were performed on exponential phase cells by addition of stated concentrations of oxidants to cultures followed by incubation for additional 15 min for Western analysis and 10 min for Northern analysis before cells were harvested for lysate preparation and RNA extraction. Antibiotics were used at the following concentrations: for selection of chromosomal integrated mutants 15 μg ml^{-1} kanamycin; and for selection of plasmids, 15 μg ml^{-1} gentamicin and 30 μg ml^{-1} kanamycin. All plasmids were transferred into *X. campestris* pv. *phaseoli* by electroporation using previously described conditions (Mongkolsuk *et al.*, 1998).

Western immuno detection of Cat

Cell lysates were prepared by resuspended cell pellets from 10-ml log-phase cultures in 0.5 ml of 50 mM sodium phosphate buffer pH 7.0. The cell suspension was sonicated intermittently for 2 min and spun at 10 000×g for 20 min. Protein concentration in clear lysate was measured using the Bradford assay (Bradford, 1976). Subsequently, 20 μg total protein was loaded into each lane of an 10% SDS-PAGE gel. The separated proteins were electrophoretically transferred to a sheet of PVDF membrane. The blocking of membrane, primary antibody reaction, washing and subsequent detection of immune reaction by alkaline phosphatase conjugated second antibody were done as previously described (Loprasert *et al.*, 2000). Densitometer analysis was performed as previously described (Sukchawalit *et al.*, 2001).

Northern analysis of *ohr* expression

X. campestris pv. *phaseoli* strains were treated with different concentrations of tBOOH or H_2O_2 for 10 min. Cells were pelleted and total RNA was extracted using the modified hot phenol method (Mongkolsuk *et al.*, 1997). In the induction kinetic experiment, aliquots of cells were removed at the indicated time and cells were pelleted by centrifugation before total RNA was extracted. RNA samples were loaded into 1.5% formaldehyde agarose gel and after electrophoresis the separated RNA samples were transferred to a nylon membrane. Pre-hybridization, hybridization, stringent washing conditions and *ohr* probe preparation were done as previously described (Mongkolsuk *et al.*, 1998).

Purification of OhrR

The poly His-OhrR fusion protein was made using BT377 (5'ATTCTCGAGTCCCGCGCCAA GGCT 3') and BT 378 (5'CGAATTCGCCGATGGTCCC 3') primers and pBBRohrR as DNA

template in a PCR reaction using previously described conditions except 1 U of *pfu* polymerase was used instead of *Taq* polymerase. The 560 bp PCR products were digested with *Nco*I and *Xho*I and cloned into similarly digested pET Blue-2 (Invitrogen). This gave pET-OhrR3His that has OhrR fused to poly-His at the carboxyl terminus. The gene sequence was confirmed by DNA sequencing. The fusion protein was purified from *Escherichia coli* BL21(DE3)/pLysS harbouring pETohrR3His. Essentially, cell pellet was resuspend in resuspension buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, 1 mM PMSF and 10% glycerol) and lysed by intermittent sonication. The cell debris was removed by centrifugation at 10 000×g at 4°C for 30 min before loading into a heparin agarose column equilibrated with binding buffer without 100 mM NaCl. Bound protein was eluted with NaCl gradient (0.05-1.0 M). Fractions containing OhrR was pooled and concentrated before loading onto Superdex 75 column and protein eluted at the rate of 0.5 ml min^{-1} in binding buffer with 100 mM NaCl. Fractions containing OhrR was pooled and protein concentration was determined by a dye-binding method (Bradford, 1976). The purity of OhrR is greater than 90%.

DNA band shift assay

The DNA band shift reactions were performed by adding 3 fmol of labelled 170 bp P1 promoter fragment probe to the binding buffer (20 mM Tris pH 7.0, 50 mM KCl, 1mM EDTA, 5% glycerol, 50 μg ml^{-1} BSA, 5 μg ml^{-1} calf thymus DNA, 0.8 μg ml^{-1} polydI/dC). Then 15 ng of purified OhrR was added and the reaction incubated at 37°C for 30 min (Loprasert *et al.*, 2000).

Site-directed mutagensis of *ohrR*

Polymerase chain reaction (PCR) based site-directed mutagenesis was used to change the OhrR C22S. Essentially, mutagenic primers BT17 (5'-GAGCTGTCCTTTGCGTTGT-3') and BT18 (5'ACAACGCAAAGGACAGCTC-3') were used and pBBRohrR as DNA template were mixed with PCR reaction and performed using previously described conditions (Mongkolsuk *et al.*, 2000). Two PCR products were re-annealed and re-PCR with M13-Forward and M13-Reverse primers giving 710 bp PCR products which were digested with *EcoR*I and *Sac*I and cloned into pBBR1 MCS-5 (Kovach *et al.*, 1994) giving pBBRohrRC22S. The sequence of the mutated DNA was verified using an automated DNA sequencer.

Construction of *Xp* P1lacZ

A 170 bp fragment containing P1 was cloned into a mini-Tn5 vector pUTPlacZ1 (de Lorenzo and Timmis, 1994) in front of a promoterless *lacZ* giving pP1lacZ. The promoter probe vector was transferred into *X. campestris* pv. *phaseoli* and selected for Km^R and Ap^S phenotypes for transposition of the mini-Tn. The intergration of the mini-Tn containing P1lacZ was confirmed

by PCR using primers located in P1 and *lacZ*. β-galactosidase assays were done according to Ochsner *et al.*, (2001).

RESULTS AND DISCUSSION

1. OhrR binding to P1

We assessed the binding of purified OhrR to 293 bp fragment containing the P1 in the DNA band shift assay. The results of the DNA band shift assay show that OhrR binds specifically to P1 (Fig. 1). The OhrR binding to P1 was abolished by unlabelled competing P1 fragment but not by unrelated DNA sequence (Fig. 1). Moreover, substitution of a protein unrelated to OhrR did not produce mobility shift of P1 (Fig. 1). Addition of increasing concentrations of OhrR to the P1 fragment did not produce additional species of slower migrating bands suggesting that there was no co-operative binding of OhrR to the operator and probably only one binding site for the protein was present within the P1 fragment (Fig. 1).

The location of the OhrR binding site within the P1 promoter fragment was determined by DNaseI protection assay (Fig. 2). Analysis of the footprint patterns shows that OhrR binding to P1 produced a DNaseI protected region of 44 bp extending from -1 to -45 on the coding strand and from -7 bp to -51 bp on the non-coding strand (Fig. 2). The DNaseI protected regions completely overlap the -35 and -10 regions of P1. Thus, the binding of OhrR prevents RNA polymerase from binding to the promoter resulting in repression of gene expression.

Analysis of the DNA sequence within the OhrR protected region reveals several features which could constitute the OhrR operator site. There are several of atypical AT rich regions of two direct repeats ATAAATCG (D1) separated by 22 bp, TTGCAA (D2) separated by 21 bp and an inverted repeats TTGCAATT-AATTGCAA (I1) separated by 17 bp surrounded by normal GC rich *Xanthomonas* DNA. These elements are all located in the close proximity of the -35 and -10 regions of P1 and binding of OhrR to these sites would prevent RNA polymerase from binding to the promoter. The analysis of P1 deletion showed that half of the ATAAATCG (D1) direct repeat could be removed (Deletion #87) without altering the ability of OhrR to repress *cat* expression indicating that this motif was not crucial for the binding of OhrR to P1. In the *B. subtilis* system, the proposed sequence for the putative binding site for OhrR located in front of the *ohrA* also has AT rich regions of overlapping inverted and direct repeats (Fuangthong *et al.*, 2001). The importance of these elements as putative OhrR binding sites is suggested by analysis of a non-inducible *ohrA* mutant that has a deletion which removes half of the inverted and direct repeats (Fuangthong *et al.*, 2001). OhrR from *Xanthomonas* and *B.*

subtilis also share high levels of homology at the amino acid sequence level suggesting that they might recognize similar DNA motifs as the binding site. Comparison of the AT rich regions of P1 and the *ohrA* promoter revealed a region with a high degree of similarity. Base on the analysis of the sequence alignment of these regions (data not shown), we proposed the putative OhrR operator site to be an inverted repeat TTnCAATT-(16-17)-AATTGnAA. The site consists of AT rich inverted repeats separated by a relatively long space of 16-17 bp. In *X. campestris* pv. *phaseoli*, the putative OhrR operator consisted of a perfect inverted repeat separated by 17 bp (I1) located between the -35 and 3' of -10 regions of the P1 promoter. Results of both the DNA footprinting of OhrR binding to P1 and the *in vivo* deletion analysis suggests that the binding site of OhrR is located within the -35 and -10 regions of P1. In *B. subtilis*, the OhrR operator has an inverted repeat containing three mismatches and separated by 16 bp and the operator overlaps the -35 and -10 regions of the *ohrA* promoter (Fuangthong *et al.*, 2001). OhrR appears to recognize an extended operator site of about 32-33 bp. This probably accounts for the observed long (44 bp) protected region resulting from OhrR binding to P1 in the DNaseI footprinting experiment (Fig. 5). At present we do not know whether the D2 direct repeats contributes to OhrR binding to P1. The importance of the inverted and direct repeats is being investigated.

Overlapping binding sites for OhrR and RNA polymerase suggest that the repressor binding to the operator prevents RNA polymerase from initiating transcription at P1. Generally, a repressor has a higher binding affinity for an operator site than does RNA polymerase for a promoter. In uninduced cells, most of the OhrR binds to the operator resulting in repression of P1. Exposure to organic peroxide likely inactivates OhrR and prevents it from binding to the operator which allows RNA polymerase to bind and initiate transcription. This assumption is supported by the primer extension data showing the tBOOH treatment increased the transcription initiation at P1. Moreover, preliminary investigations suggested that, *in vitro*, organic peroxide might directly modify OhrR and prevents it from binding to P1 (data not shown).

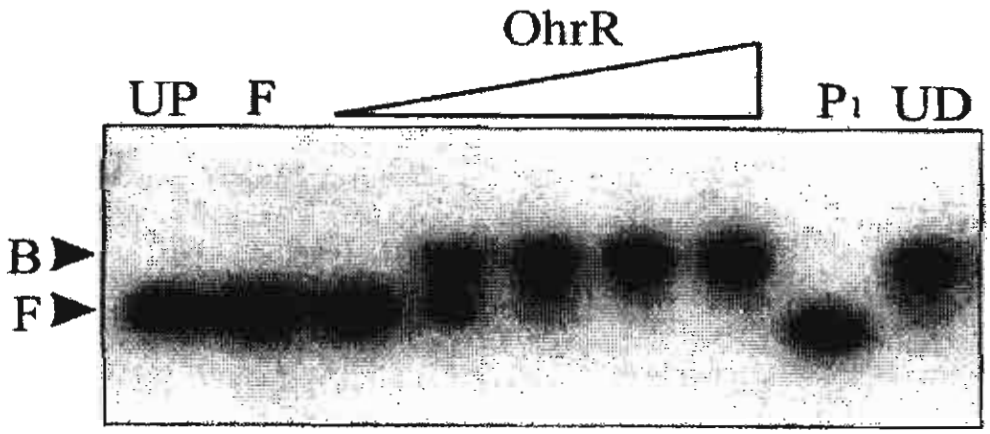

Fig. 1. DNA band shift assay for binding of OhrR to P1.

Purified OhrR was added to 293 bp of radioactively labelled P1 fragment in the binding buffer and separated in a polyacrylamide gel done as described in the materials and methods. The binding reaction consisted of labelled P1 fragment and 400 ng OhrR. UP is adding of 2 μg of unrelated protein (BSA) to the binding reaction ; FP is free P1 probe; addition of increasing concentrations of OhrR 100, 200, 400, 800 and 1200 ng to labeled P1 probe; P1 is adding of 2 μg of unlabelled P1 DNA to the binding reaction; UD addition of 3 μg of unrelated DNA (pUC18 plasmid) to the binding reaction. The positions of free (F) and bound (B) P1 probe are shown to the left.

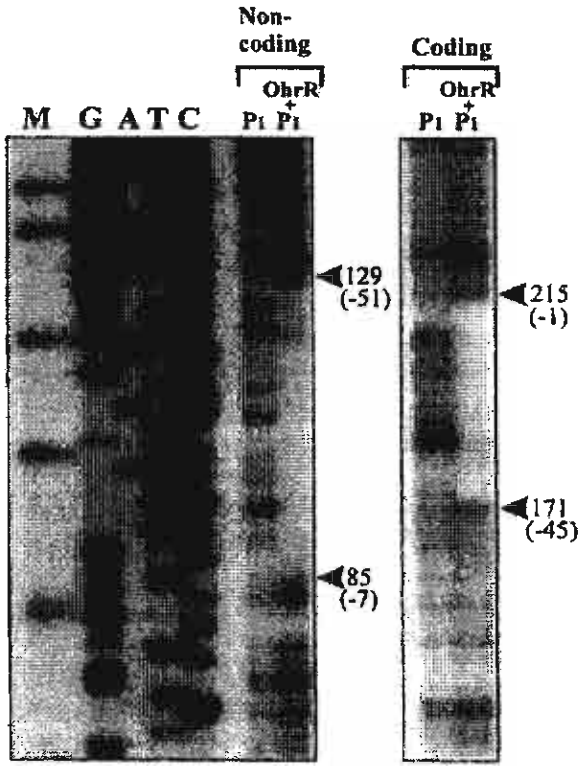

Fig. 2. DNaseI protection assay to locate OhrR binding site to P1.

The DNaseI protection assay for the binding of OhrR to P1. P1 represents the DNA fragments treated with DNaseI; P1+OhrR represents the binding of OhrR to the DNA fragments prior to DNaseI treatment. The labelling of non-coding and coding strands are done as described in the materials and methods. The arrows indicate the size of the fragments in bp and numbers in the brackets indicate the position of the protected regions with respect to the +1 transcription initiation site. G, A, T, C are the sequence ladder. M is radioactively labelled ϕX174 *Hin*fI molecular weight markers.

2. The stability of *ohrR-ohr* and *ohr* mRNA

We have shown that the bicistronic *ohrR-ohr* mRNA is processed by cutting in the loop section of the stem and loop structure 3' of *ohrR* possibly by a RNase III-like enzyme to give monocistronic *ohr* mRNA and rapidly degraded monocistronic *ohrR* mRNA (Sukchawalit *et al.*, 2001). This reduces the functional level of *ohrR* mRNA and ultimately effect the level of OhrR. Since, transcription of *ohrR* and *ohr* are driven from the P1, the stability of these mRNA would have important effects on their expression. Here, we determined the stability of *ohrR-ohr* and *ohr* mRNA by measuring the half-life of these mRNA in tBOOH induced cells. The data show that *ohr* mRNA is highly stable and has a half-life of greater than 15 minutes (Fig. 6). By contrast, the bicistronic *ohrR-ohr* mRNA was highly labile and the unprocessed bicistronic could not be detected 6 minutes after addition of rifampicin (Fig. 3). Consistent with previous observations, no monocistronic *ohrR* mRNA could be detected (Fig. 3 and Sukchawalit *et al.*, 2001). This differential stability of *ohrR* and *ohr* mRNA would result in high concentrations of functional *ohr* mRNA giving correspondingly high Ohr levels and would reduce the level of *ohrR* mRNA. Since, Ohr is responsible for detoxification of organic peroxide, high level of the protein would be beneficial to the bacterial during an exposure to organic peroxide stress. While low levels of OhrR also prevents excessive repression of the operon by the autoregulatory process. Thus, the differential stability and rapid processing of the mRNA exerts an additional post transcription step to regulate *in vivo* concentration of OhrR. This mechanism is not unique to the regulation of *ohrR*. Post transcription regulation at the level of mRNA stability had been observed in diverse bacteria (Takata *et al.*, 1989; Nilsson *et al.*, 1996; Herbermehl and Klug, 1998; Homuth *et al.*, 1999).

3. *ohrR* is inefficiently translated

We have observed in both uninduced and tBOOH induced cells that OhrR is barely detectable by Western analysis despite the gene having a highly efficient promoter (data not shown). Although, we identified a post-transcription regulatory step which involves the differential stability of the mRNA, lack of correlation between the promoter strength and the concentration of OhrR suggested further regulation perhaps at the translational level. *ohrR* produced a leaderless mRNA. An alternative interpretation that *ohrR* mRNA could be translated from other translation initiation codons located further downstream of the ATG is unlikely since there is no other commonly used gram negative translation initiation codons (ATG to GTG) nearby. Furthermore, studies on the translation of leaderless mRNA indicate that the ATG codon is required (Wu and Janssen, 1997; Winzeler and Shapiro, 1997). Here,

we investigated the role of the ATG codon in the transcription of the gene and the translation of *ohrR* mRNA. A site directed mutagenesis of the gene was done to change the "ATG" codon to a rarely used translation initiation codon, "CTG" (Fig. 4). Subsequently, the levels of *ohrR* transcription and translation *in vivo* was determined by making transcription (*cat2*, Mongkolsuk *et al.*, 1993) and translation (*cat3*, Mongkolsuk *et al.*, 1993) fusions of *cat* reporter genes to the mutated CTG-*ohrR* at the *Pst*I site. Cat levels specified by *Xanthomonas* harbouring pOPCcat2 and pOPCcat3 were determined and compared to the levels attained by the strains harbouring non-mutated *ohrR-cat* fusion plasmids (Fig. 4). The results show that changing of the translation initiation codon from ATG to CTG did not affect the level of transcription (pOPCcat2). By contrast, the translation fusion of CTG-*ohrR* to *cat3* (pOPCcat3) was abolished and no Cat fusion protein was detected (Fig. 4). The evidence confirmed that the translation of *ohrR* mRNA occurred at the proposed ATG and the codon was required. However, the ATG codon was not important to the transcription of the gene.

We also further investigated the level of transcription and translation of *ohrR* using the *cat* reporter gene fusions. Initially, the transcription and translation fusions were made at the *Kpn*I site located in the middle of *ohrR* (Fig. 4). The results of densitometer analysis show that the Cat levels specified by transcriptional fused pOKcat2 was at least 5 fold higher than the levels specified by the translational fused pOKcat3 (Fig. 4). To confirm these observations, additional transcription and translation *cat* fusions were made at the *Pst*I site located closer to the translation initiation of *ohrR* (Fig. 4). Cat levels specified by pOPcat2 was at least 5 fold higher than the level attained by pOPcat3. These fusions gave similar patterns regardless of the locations of the gene fusions indicated that *ohrR* mRNA is inefficiently translated and the translation level of the *ohrR* mRNA occurs in only about 20% of the total *ohrR* mRNA.

4. High levels of OhrR reduce organic peroxide resistance

The existence of multiple regulatory mechanisms to ensure that OhrR is not produced at high levels implies that the tight regulation of the gene must have important physiological consequences on the cells ability to response to oxidative stress. The assumption was tested by measuring the effects of killing concentrations of H_2O_2, a superoxide generator (menadione) and organic peroxides on *X. campestris* pv. *phaseoli* harbouring pBBRohrR. In the bacteria harbouring pBBRohrR, tBOOH and cumene hydroperoxide gave zones of growth inhibition of 32 mm and 31 mm, respectively compared to 26 mm for the bacteria harbouring pBBRMCS-1. H_2O_2 and menadione produced similar sizes of zone of growth inhibition in

both strains. The data indicate that high level of OhrR has detrimental effects on oxidative stress response by decreasing the organic peroxide resistance level, most likely by repression of the *ohr* expression. Thus, it is not surprising that *Xanthomonas* has evolved multiple mechanisms to ensure proper regulation of *ohrR*. At present, we don't know other genes in the OhrR regulon thus it is possible that other stress responses could be affected by high levels of OhrR.

5. A model for transcription and post transcription of regulation of *ohrR*

The transcription regulation of *ohrR* involves the autoregulation of the gene at the P1 promoter by OhrR. The binding target for OhrR overlaps with the -35 and -10 sites enabling repressor to block RNA polymerase from binding to the promoter. In uninduced cells, *OhrR* represses expression of its own operon. The post transcriptional regulation of *ohrR* occurs at two steps: First, the bicistronic *ohrR-ohr* mRNA is rapidly processed giving high levels of *ohr* mRNA and rapid degradation of *ohrR* mRNA. *ohr* mRNA is highly stable in contrast to highly labile bicistronic *ohrR-ohr*. This greatly reduces the functional concentration *ohrR* mRNA. Second, *ohrR* mRNA is inefficiently translated and translation of the mRNA occurred at only 20% of the transcription level. Multiple regulatory mechanisms at transcriptional and post transcriptional levels ensure that the intracellular level of OhrR remains at a low level. When cells are exposed to organic peroxides, presumably they inactivate OhrR and prevent the repressor from binding to the operator resulting in high levels expression of *ohrR* and *ohr*. Once Ohr removes organic peroxide, OhrR would then autoregulate itself and represses expression of the operon (Fig. 5).

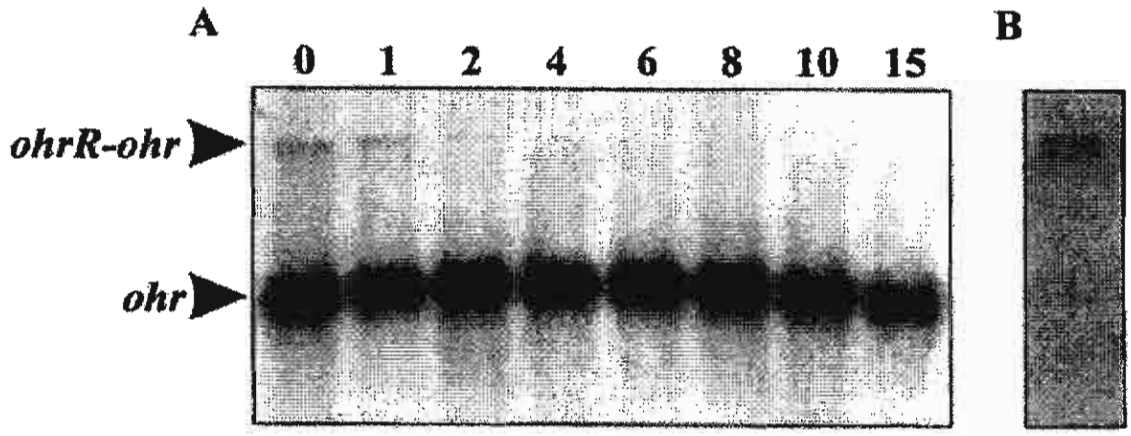

Fig. 3. Analysis of bicistronic *ohrR-ohr* and *ohr* mRNA stability.

Total RNA was isolated culture treated with 100 μM tBOOH for 10 min before addition of Rifampicin. Time zero represents the steady state level of mRNA before addition of 150 μg ml^{-1} Rifampicin was added. At stated time after rifampicin addition samples were withdrawn and total RNA extracted. 15 μg of RNA from each time point was loaded into each well and separated in a formaldehyde gel. The samples were blotted to a nylon membrane and probed with radioactively labelled *ohr* in A. The steady state (0) RNA sample was probed with *ohrR* in B.

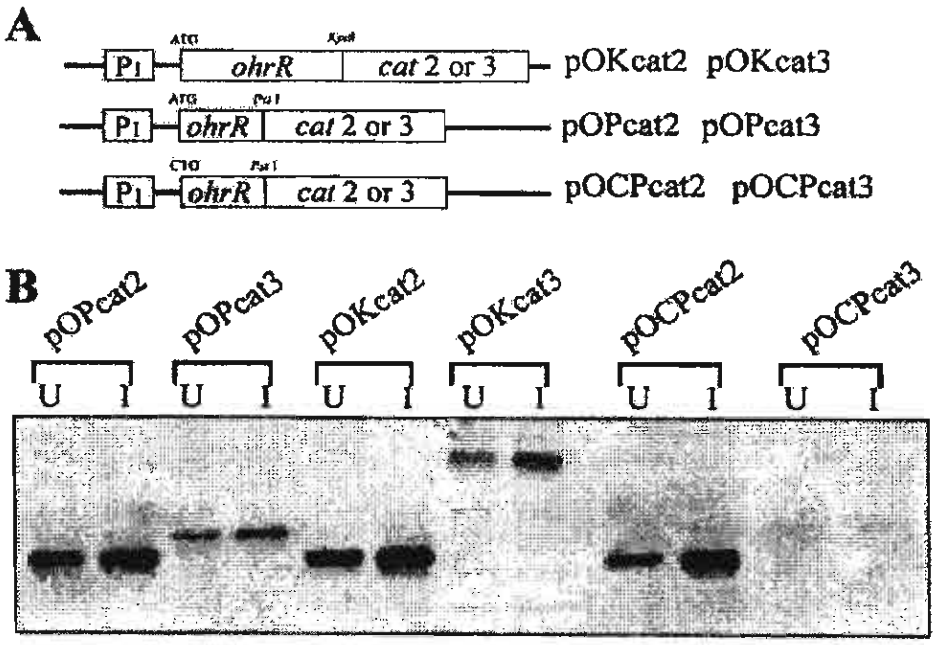

Fig. 4. *ohrR* mRNA is inefficiently translated.

In A, Diagrammatic representation of various plasmids showing the site of either transcriptional or translational fusions between *ohrR,* mutated CTG-*ohr* and *cat*. *cat2* and *cat3* fusions represent transcriptional and translational fusions, respectively. In B, 10 μg of total protein prepared from cultures of *Xanthomonas* harbouring various plasmids was loaded into each lane. Separated protein samples were transferred to membranes after gel electrophoresis. Cat was detected by Western blot. U and I are uninduced and 100 μM tBOOH induced cultures, respectively.

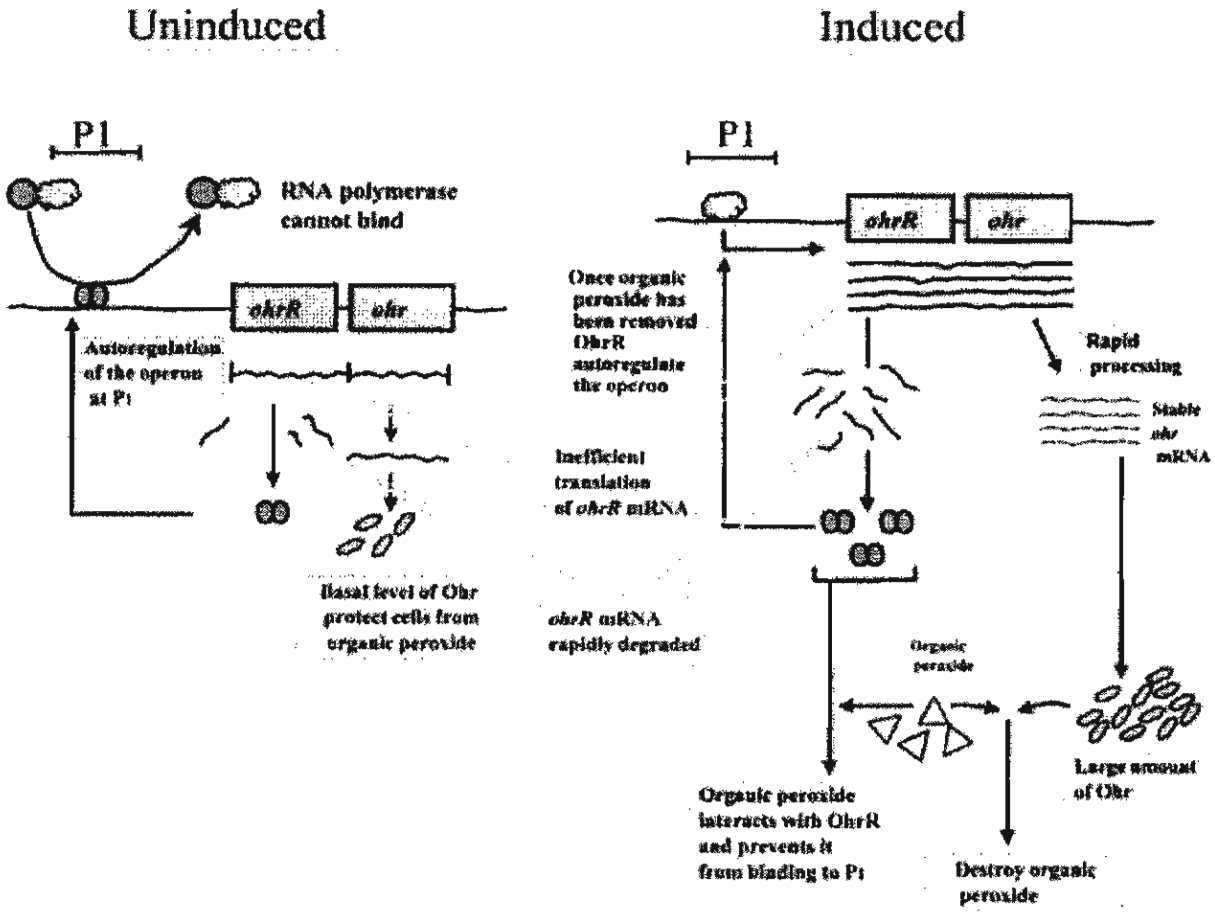

Fig. 5. A model for the *ohrR* regulation at transcriptional and post transcriptional levels

6. Organic peroxide is the in vivo inducer of OhrR

High level expression of *ohrR* from an expression vector results in strong repression of expression from P1 promoter. Transcription from P1 can be lifted by treatment with an organic peroxide (Sukchawalit *et al.*, 2001). This is a sensitive system with which to test the ability of various compounds to induce transcription from P1. *In vivo*, an organic peroxide is metabolized to the corresponding organic alcohol by alkyl hydroperoxide reductase (Poole and Ellis, 1996). However, the *in vivo* system cannot differentiate whether an organic peroxide moiety or its metabolite is acting as an inducer of the system. Thus, we tested the ability of organic alcohols to induce transcription from P1 promoter. Chloramphenicol acetyltransferase (Cat) levels in *X. campestris* pv. *phaseoli* pP1/pBBRohrR (Mongkolsuk *et al.*, 2002) treated with 100 μM *tert*-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CuOOH) and corresponding alcohols were determined and compared to untreated cells. As expected, tBOOH and CuOOH strongly induced *cat* expression from P1 as shown by high Cat levels while *tert*-butyl and cumic alcohols did not (Fig. 6A). The *in vivo* role of OhrR as an organic peroxide sensor was further investigated. We have previously shown that induction of *ohr* requires a functional *ohrR* (Mongkolsuk *et al.*, 2002). We hypothesized, therefore, that if an organic peroxide is responsible for the inactivation of OhrR and subsequent derepression of *ohr* expression, then high levels of an organic peroxide metabolizing enzyme, such as alkyl hydroperoxide reductase, would be expected to reduce the magnitude of tBOOH induced expression of *ohr*. In contrast, high level of enzymes not involved in organic peroxide metabolism, such as catalase, should not affect *ohr* induction. The levels of *ohr* mRNA in uninduced and tBOOH-induced *X. campestris* pv. *phaseoli* cultures having normal AhpC-AhpF, high AhpC-AhpF and high catalase levels were determined. The results show that a reduction in the level of the tBOOH induced *ohr* mRNA was detected only in cells having high levels of AhpC and AhpF (Fig. 6B). No changes were detected in the other two strains (Fig. 9B). In addition, there was a correlation between the level of *ohr* mRNA and the inducing concentration of tBOOH, suggesting that OhrR senses changes in the intracellular concentration of the organic peroxide.

Surprisingly, H_2O_2 weakly induced *cat* expression from P1 at 20-fold lower levels than those obtained on an organic peroxide induction (data not shown). Weak induction of gene expression from P1 by H_2O_2 raises a question as to whether H_2O_2 act as a physiological inducer of the OhrR regulon. Thus, we examined the expression *ohr*, a gene controlled by OhrR in response to peroxide treatment. *In vivo*, the low level of induction of *ohr* by H_2O_2 could be due to high catalase activity. Thus, *ohr* induction by H_2O_2 was examined in a *katA*

(KatA is the major catalase in *X. campestris* pv. *phaseoli* log-phase cells) *katE* [KatE is a growth-phase-regulated catalase (Vattanaviboon and Mongkolsuk, 2000)] double mutant and the parental strain. Northern blotting results showed that the levels of *ohr* mRNA induced with 100 and 150 μM H_2O_2 were higher in the *katA katE* mutant than in the parental strain (Fig. 7A). Nonetheless, these levels were more than 10-fold lower than the level attained after induction with organic peroxide. In the control experiment, the expression of an OxyR-regulated gene, *ahpC* was strongly induced by H_2O_2 in both strains (data not shown). The data suggest that low level H_2O_2 induction of *ohr* expression was partially due to elimination of H_2O_2 by catalase.

A possibility for lower induced levels of *ohr* by H_2O_2 is that OhrR is oxidized at different rate by H_2O_2 and tBOOH . The idea was tested by examining the kinetic of H_2O_2 and tBOOH induction of *ohr* expression. The results in Fig. 7B show that tBOOH treatment rapidly induced *ohr* expression and full induction was achieved after exposure to tBOOH for 10 min. Similarly, the peak of *ohr* mRNA level induced by H_2O_2 was also detected after the treatment for 10 min, however, this level was 20-fold less than the level attained by tBOOH treatment. These findings ruled out the idea that low level of *ohr* induction by H_2O_2 was due to the slow rate of OhrR oxidation.

Since, H_2O_2 was a much less efficient inducer than tBOOH, experiments were done to test the effects of treating cells with increasing doses of H_2O_2 on the induction of *cat* expression from the P1 promoter. Western analysis of Cat level clearly demonstrated that treatment of cells with H_2O_2 at concentrations between 100 to 500 μM resulted in corresponding increase in the amount of Cat specified by the P1 promoter (Fig. 7C). However, further increase in the concentration of H_2O_2 upto 5 mM did not result in further increase in the Cat level (Fig. 7C). Moreover, the H_2O_2 fully induced levels were 20-fold lowered than the Cat level in cells treated with 100 μM tBOOH. This clearly showed that the low level of OhrR oxidation by H_2O_2 was due to its inability to efficiently oxidize the protein. These data lead us to conclude that OhrR is not involved in sensing changes in H_2O_2 levels. The physiological importance of the H_2O_2 induction of the OhrR regulon remains unclear. Nonetheless, low level induction of the OhrR regulon might serve as an additional defense against low concentrations of organic peroxides, such as nucleic acid and lipid peroxides produced as a result of exposure to high concentrations of H_2O_2 (Kappus, 1987; Turton *et al.*, 1997)

The ability of other oxidants to induce *cat* expression from P1 was also investigated. A superoxide generator (menadione) and N-ethylmaleimide (NEM) did not cause induction, although these substances strongly induced expression of genes in the OxyR regulon (data not shown and Mongkolsuk *et al.*, 1997; Loprasert *et al.*, 2000). We also tested with other compounds (at concentration of 1 mM), such as salicylate, benzoate, butylate and hydroxytoluene which commonly induce expression of genes under MarR family of repressor (Alekshun and Levy, 1999). None of these compounds induced *cat* expression from P1 (data not shown). These findings suggest that both the peroxide and the organic moieties of organic peroxides are required for efficient interaction with and inactivation of OhrR.

7. Organic peroxide and H_2O_2 treatments inhibited OhrR binding to P1 in vitro

The putative mechanism of inactivation of OhrR and induction of gene expression is thought to involve organic peroxide, either directly or indirectly, resulting in modified OhrR by oxidation, rendering it inactive. An *in vivo* promoter assay cannot differentiate between direct and indirect modification of OhrR by an organic peroxide. We have used a DNA mobility shift assay to show that OhrR interacts specifically with the P1 promoter region and the location of the OhrR binding site was mapped by DNaseI footprinting experiment (Mongkolsuk *et al.*, 2002). The DNA mobility shift assay allowed us to test the *in vitro* effects of oxidants, such as CuOOH, tBOOH, H_2O_2 and other chemicals on the ability of OhrR to bind to P1. CuOOH, tBOOH or H_2O_2 treatment prevented OhrR from binding to the P1 fragment (Fig. 8A, B and C). The inactivation of OhrR by organic peroxides (tBOOH and CuOOH) occurred at concentrations more than 10-fold lower than the concentration of H_2O_2 needed for OhrR inactivation. Addition of other oxidants, such as menadione, NEM or related compounds such as *tert*-butyl and cumic alcohols to the binding reaction mixture did not interfere with OhrR binding to the P1 promoter region (data not shown). The *in vitro* experiment provides crucial data to support the hypothesis that organic peroxide and H_2O_2 interact directly with and probably oxidize OhrR, so rendering the protein inactive as a repressor. Next, we tested whether oxidation of OhrR by peroxide is a reversible process. We determined if a reducing agent, such as dithiothreitol (DTT), could reverse the inhibitory effect of an organic peroxide on OhrR binding to the P1 promoter. The results show that 10 mM DTT reversed the inhibitory effects of tBOOH on OhrR binding to the P1 promoter (Fig. 11D). DTT was also able to reverse the inhibitory effect of H_2O_2 (data not shown). These data supported the idea that tBOOH oxidizes OhrR to render it inactive and the oxidized protein could be reduced to give back its biological activity. It remains to be seen *in vivo* if oxidized

OhrR can be reactivated by any of the intrinsic cellular reducing systems. These data, coupled with the *in vivo* results favour the hypothesis that an organic peroxide is responsible for efficient oxidation of OhrR and, as a consequence, inactivation of the repressor OhrR.

8. C22 residue of OhrR is required for peroxide oxidation

A cysteine residue in proteins is often a target for oxidation as in the case of OxyR where oxidation of highly conserved C residues lead to formation of a disulphide bond (Zheng *et al.*, 1998). This converts OxyR to an activator (oxidized) form. Comparison of OhrR amino acid sequences from various organisms showed a highly conserved C residue (C22) located close to the amino terminus. In addition, amino acid sequences surrounding the C residue are also highly conserved suggesting that the region is important for OhrR function (Fig. 9A). While other C residues in the protein are not in highly conserved positions (data not shown). A site-directed mutagenesis that changed C22S was performed to examine the regulatory role of the conserved C residue. The mutated *ohrR*C22S was cloned in pBBR1MCS-5 (Kovach *et al.*, 1994) resulting in pBRRohrRC22S. The plasmid vector, pBBRohrR and pBBRohrRC22S were transformed into *Xp* P1lacZ (*X. campestris* pv. *phaseoli* mini-Tn5 P1lacZ, Km^R), a strain that has P1 promoter fused to *lacZ* in a mini-Tn5 vector and subsequently transposed into the chromosome. Analysis of β-galactosidase activity showed that both wild type OhrR and mutated OhrRC22S repressed the P1 promoter activity (Fig. 9B). This indicated that the C22 residue has no role in the binding of OhrR to its target site within the P1 promoter. In contrast, treatment with 100 μM tBOOH or CuOOH highly induced *lacZ* expression driving from the P1 promoter in cells harbouring pBBRohrR but not in cells harbouring pBBRohrRC22S (Fig. 9B). These data indicated that C22 of OhrR is important to the protein ability to sense organic peroxide and this residue is the target for oxidation by organic peroxide that probably changes the protein conformation and renders it inactive. Recent findings indicate that the conserved C residue in *Bacillus subtilis* OhrR is being oxidized by organic peroxide to a sulfenic acid intermediate that cause inactivation of the protein (Fuangthong and Helmann 2002).

The data presented in this report imply that the physiological role of OhrR is to sense and respond to changes in organic peroxide levels. Addition of organic peroxide causes inactivation by oxidation of OhrR leading to derepression of *ohr* expression. We have shown that high level of OhrR conferred increased resistance to organic peroxide killing (Mongkolsuk *et al.*, 2002). Oxidation of OhrR by an organic peroxide results in loss of the protein ability to bind to its target site. A recent finding s show that OhrR from *B. subtilis*

behave similarly (Fuangthong and Helmann, 2002). Efficient interaction with the inducer and subsequent oxidation of OhrR requires that the inducer has both peroxide and organic moieties. Replacing either of these entities renders the inducer ineffective or reduces its efficiency. It remains to be seen how OhrR differentiates between organic peroxide and H_2O_2. One explanation is that the OhrR peroxide sensing site is buried in a hydrophobic region which favours interaction with the organic moiety of an organic peroxide. Full oxidation of the protein might require that the ligand remains at the active site for a prolonged period of time. Alternatively, protein-ligand interaction with the organic moiety of the inducer may facilitate the oxidation reaction e.g. by aligning the peroxide group and its target. In contrast, since H_2O_2 can diffuse into the cell, the low level induction detected may be a result of H_2O_2 reacting in a non-directed way with the sensing site of OhrR. These possibilities will be investigated.

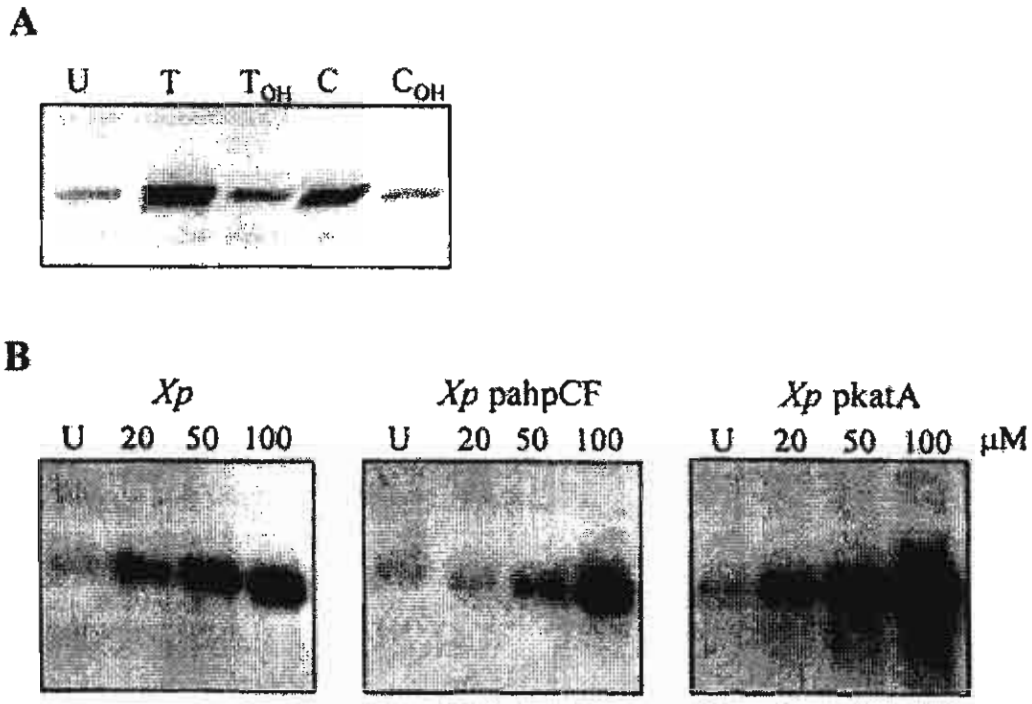

Fig. 6 Organic peroxide is the *in vivo* inducer of OhrR.

In A, Western analysis of Cat production by *X. campestris* pv. *phaseoli* harbouring pP1 and pBBRohrR in response to various inducers. *X. campestris* pv. *phaseoli* pP1/ pBBRohrR (Sukchawalit *et al.*, 2001) was treated with 1 mM *tert*-butyl alcohol (T_{OH}), 1 mM cumic alcohol(C_{OH}), 100 μM tBOOH (T) and 100 μM CuOOH (C). Cell collection, lysate preparation, gel electrophoresis and Western analysis of Cat were done as previously described (Loprasert *et al.*, 2000). Total protein (30 μg) was loaded into each lane. U represents an uninduced culture.

In B, Northern analysis of *ohr* expression in response to tBOOH treatments in *X. campestris* pv. *phaseoli* and strains having high AhpC-AhcF or catalase levels. *X. campestris* pv. *phaseoli* (*Xp*) strains with high catalase level (*Xp* pkatA) or high AhpC-AhpF levels (*Xp* pahpCF) were treated with 20, 50, 100 μM tBOOH for 10 min. Total RNA extraction and Northern blotting analysis of *ohr* mRNA were done as previously described (Mongkolsuk *et al.*, 1998). Total RNA (10 μg) was loaded into each well of a 2% agarose formaldehyde gel. U represents uninduced culture.

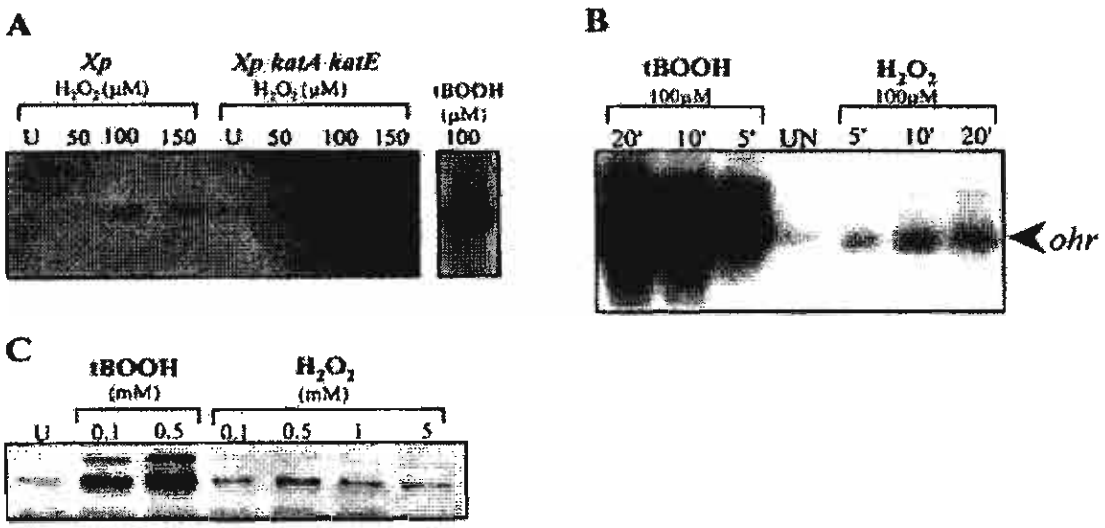

Fig. 7 H_2O_2 is not a physiological inducer of OhrR.

In A, Northern blot analysis of *ohr* expression in response to H_2O_2 in a *katA katE* mutant and the kinetic of induction. Cultures of *X. campestris* pv. *phaseoli* (*Xp*) and a *katA katE* mutant (*Xp katA katE*) were treated with increasing concentrations of H_2O_2. In addition, a *X. campestris* pv. *phaseoli* culture was induced with 100 μM tBOOH. In B, The kinetic of *ohr* induction in *X. campestris* pv. *phaseoli* in response to 100 μM tBOOH or H_2O_2 at 5, 10 and 20 min. The levels of *ohr* mRNA were determined using Northern blotting analysis as described in Fig. 1B legend except in tBOOH-treated cells, 5 μg of total RNA was loaded. U represents uninduced culture. In C, Western analysis of dose response of H_2O_2 treatment and derepression of P1 promoter. Log-phase cultures of *X. campestris* pv. *phaseoli* pP1/ pBBRohrR were induced with 0.1, 0.5 mM tBOOH or 0.1, 0.5, 1, 5 mM H_2O_2 for 15 min. U represents uninduced culture. Total protein (10 μg) was loaded into each lane and detection of Cat performed as described in the experimental procedures.

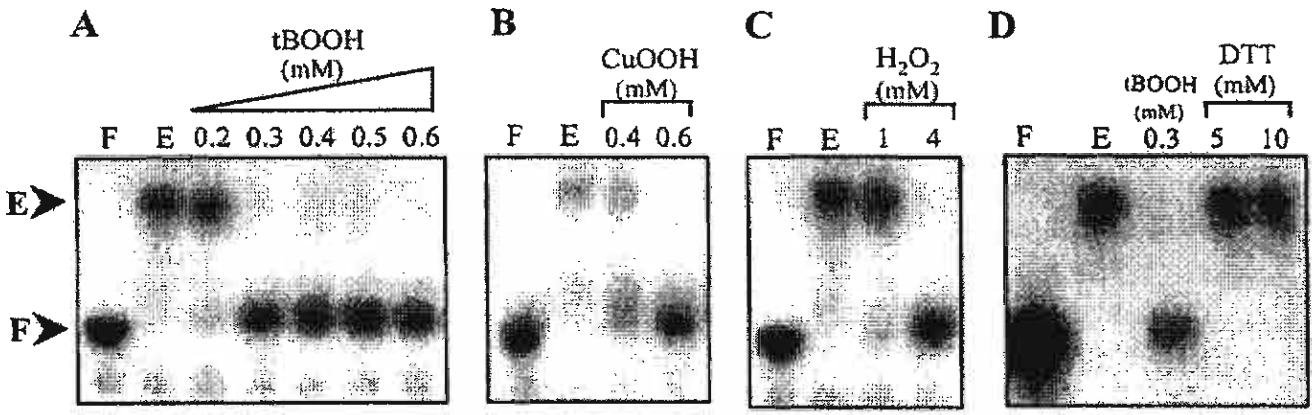

Fig. 8 The effect of organic peroxide and DTT on OhrR binding to the P1 promoter.

DNA band shift experiments using purified OhrR and radioactively labelled P1 promoter fragment were done as described in Mongkolsuk *et al.* (2002). The binding reaction consisted of 15 ng OhrR and labelled P1 probe. A, effect of adding increasing concentrations of tBOOH to the binding reaction; B, effect of adding CuOOH to the binding reaction; C, effect of adding H_2O_2 to the binding reaction; D, effect of addition of DTT to the binding reaction containing 0.3 mM tBOOH, DTT was added 1 min after addition of tBOOH to the binding reaction. F and E represent free and OhrR-bound probe respectively.

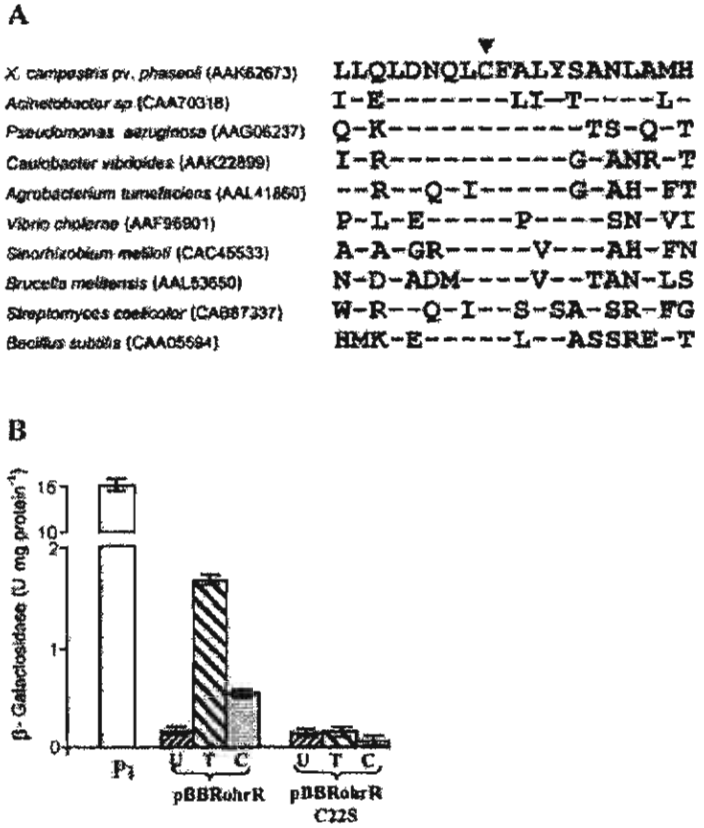

Fig. 9 The conservation of C22 in OhrR and the effect of OhrRC22S mutation on expression of *lacZ* from the P1 promoter.

A, conserved regions around C22 (▼) in OhrR from various gram-negative and gram-positive bacteria. - represents the same amino acid as in *X. campestris* pv. *phaseoli* OhrR. B, pBBRohrR and pBBRohrRC22S were transformed into *Xp* P1lacZ. P1 represented the *Xp* P1lacZ with a plasmid vector. tBOOH (T) or CuOOH (C) at final concentration of 100 μM was added to the culture of *Xp* P1lacZ harbouring pBBRohrR or pBBRohrRC22S and incubated for additional 15 min prior to cell harvest and enzyme assay. U represents uninduced culture. β-galactosidase was assayed as described in the experimental procedures

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OUTPUTS

1. Students graduated under this grant

Ph.D.

Nopmanee Chauvatcharin (Mahidol University)

M.Sc.

Chotirote Seeanukul (Mahidol University)

2. Students doing thesis under this grant

Ph.D.

Warunya Panmanee (Mahidol University)

Warawan Eiamphungporn (Mahidol University)

Peerakarn Bunjerdkij (Interuniversity, Mahidol University/ AIT/ CRI)

M.Sc.

Kaewkunya Nakjarung (Mahidol University)

3. Presentations

1. Seeanukun, C., Vattanaviboon, P. and Mongkolsuk, S. 2000. Cloning and molecular analysis of *Xanthomonas* peptide methionine sulfoxide reductase. The 12th Annual meeting of The Thai Society for Biotechnology. Kanjanaburi, Thailand, 1-3 Nov, 2000.

2. Vattanaviboon, P., Whangsuk, W. and Mongkolsuk, S. 2000. *OxyR* and *ahpCF* play an important role on menadione resistance in *Xanthomonas campestris* pv. *phaseoli*. The 12th Annual meeting of the Thai Society for Biotechnology. The Kanjanaburi, Thailand, 1-3 Nov, 2000.

3. Wiriyawutikorn, N., Vattanaviboon, P. and Mongkolsuk, S. 2000. *Xanthomonas campestris* pv. *phaseoli katA* monofunctional catalase: cloning, characterization and mutagenesis to examine its role against oxidative stress. The 12th Annual meeting of the Thai Society for Biotechnology. Kanjanaburi, Thailand, 1-3 Nov, 2000.

4. Wiriyawutikorn, N., Switala, J., Vattanaviboon, P, Loewen, P.C. and Mongkolsuk, S. Cloning and characterization of the gene encoding for the major catalase from *Xanthomonas campestris* pv. *phaseoli*. The 51st Annual Meeting of the Canadian Society of Microbiologist, 10-13 June 2001, Waterloo, Canada

5. Vattanaviboon, P., Komwacharapong, W., and Mongkolsuk, S. 2001. Menadione induced peroxide scavenging enzymes and cross protection against peroxide killing. BioThailand 2001 from research to market, Queen Sirikit Convention Center, Bangkok, Thailand, 7-9 November 2001.

4. Publications

1. **Atichartpongkul, S., S. Loprasert, P. Vattanaviboon, W. Whangsuk, J. D. Helmann, and S. Mongkolsuk.** 2001. Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression. Microbiology **147:**1775-82. **impact factor 2.846**

2. **Fuangthong, M., S. Atichartpongkul, S. Mongkolsuk, and J. D. Helmann.** 2001. OhrR is a repressor of ohrA, a key organic hydroperoxide resistance determinant in Bacillus subtilis. **Journal of Bacteriology 183:**4134-41. **impact factor 3.984**

3. **Sukchawalit, R., S. Loprasert, S. Atichartpongkul, and S. Mongkolsuk.** 2001. Complex regulation of the organic hydroperoxide resistance gene (*ohr*) from *Xanthomonas* involves OhrR, a novel organic peroxide-inducible negative regulator, and posttranscriptional modifications. **Journal of Bacteriology 183:**4405-12. **impact factor 3.984**

4. **Mongkolsuk, S., and J. D. Helmann.** 2002. Regulation of inducible peroxide stress responses. **Molecular Microbiology 45:**9-15. **impact factor 6.398**

5. **Mongkolsuk, S., W. Panmanee, S. Atichartpongkul, P. Vattanaviboon, W. Whangsuk, M. Fuangthong, W. Eiamphungporn, R. Sukchawalit, and S. Utamapongchai.** 2002. The repressor for an organic peroxide-inducible operon is uniquely regulated at multiple levels. **Molecular Microbiology 44:**793-802. **impact factor 6.398**

6. **Panmanee, W., P. Vattanaviboon, W. Eiamphungporn, W. Whangsuk, R. Sallabhan, and S. Mongkolsuk.** 2002. OhrR, a transcription repressor that senses and responds to changes in organic peroxide levels in *Xanthomonas campestris* pv. *phaseoli*. **Molecular Microbiology 45:**1647-54. **impact factor 6.398**

7. **Chauvatcharin, N., P. Vattanaviboon, J. Switala, P.C. Loewen and S. Mongkolsuk** 2003. Cloning and characterization of *katA*, encoding the major monofunctional catalase from *Xanthomonas campestris* pv. *phaseoli* and characterization of the encoded catalase KatA. **Current Microbiology** (in press) **impact factor 1.059**

Appendix

Reprints of Publications

1. **Atichartpongkul, S., S. Loprasert, P. Vattanaviboon, W. Whangsuk, J. D. Helmann, and S. Mongkolsuk.** 2001. Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression. Microbiology **147:**1775-82.

2. **Fuangthong, M., S. Atichartpongkul, S. Mongkolsuk, and J. D. Helmann.** 2001. OhrR is a repressor of ohrA, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. J Bacteriol **183:**4134-41.

3. **Sukchawalit, R., S. Loprasert, S. Atichartpongkul, and S. Mongkolsuk.** 2001. Complex regulation of the organic hydroperoxide resistance gene (*ohr*) from *Xanthomonas* involves OhrR, a novel organic peroxide-inducible negative regulator, and posttranscriptional modifications. J Bacteriol **183:**4405-12.

4. **Mongkolsuk, S., and J. D. Helmann.** 2002. Regulation of inducible peroxide stress responses. Mol Microbiol **45:**9-15.

5. **Mongkolsuk, S., W. Panmanee, S. Atichartpongkul, P. Vattanaviboon, W. Whangsuk, M. Fuangthong, W. Eiamphungporn, R. Sukchawalit, and S. Utamapongchai.** 2002. The repressor for an organic peroxide-inducible operon is uniquely regulated at multiple levels. Mol Microbiol **44:**793-802.

6. **Panmanee, W., P. Vattanaviboon, W. Eiamphungporn, W. Whangsuk, R. Sallabhan, and S. Mongkolsuk.** 2002. OhrR, a transcription repressor that senses and responds to changes in organic peroxide levels in *Xanthomonas campestris* pv. phaseoli. Mol Microbiol **45:**1647-54.

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Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression

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Xanthomonas campestris **Ohr (a protein involved in organic peroxide protection) and** ***Escherichia coli*** **OsmC (an osmotically inducible protein of unknown function) are related proteins. Database searches and phylogenetic analyses reveal that Ohr and OsmC homologues cluster into two related subfamilies of proteins widely distributed in both Gram-negative and Gram-positive bacteria. To determine if these two subfamilies are functionally distinct,** ***ohr*** **and** ***osmC*** **in** ***Pseudomonas aeruginosa*** **(a bacterium with one representative from each subfamily) were analysed. Only** ***ohr*** **mutants are hypersensitive to organic peroxide, and this phenotype can be restored by complementation with** ***ohr*** **but not** ***osmC*****. In addition, expression of** ***ohr*** **was highly induced only by organic peroxides, and not by other oxidants or stresses. In contrast,** ***osmC*** **was induced by ethanol and osmotic stress. A similar pattern of regulation was observed for Ohr and OsmC homologues in the Gram-positive bacterium** ***Deinococcus radiodurans*****, though uninduced expression was much higher and induction lower in this species. These data clearly support the conclusion that Ohr and OsmC define two functionally distinct subfamilies with distinct patterns of regulation.**

Keywords: *Pseudomonas aeruginosa*, *Deinococcus radiodurans*, organic peroxide resistance, osmotic stress

INTRODUCTION

Organic peroxides are highly toxic and can damage cellular macromolecules, including proteins, lipids and DNA. Furthermore, these compounds participate in free-radical reactions that generate more reactive organic radicals which thereby increases their toxicity (Halliwell & Gutteridge, 1984). In bacteria, organic peroxides are generated as by-products of aerobic metabolism (Gonzales-Flecha & Demple, 1997). In addition, pathogenic bacteria are exposed to reactive oxygen species, including organic peroxides, generated by the host as a part of the active defence response (Baker & Orlandi, 1995; Levine *et al.*, 1994). Thus, detoxification of organic peroxides is important for bacterial survival and proliferation in the host.

Abbreviations: Ahp, alkyl hydroperoxide reductase; tBOOH, *tert*-butyl hydroperoxide.

Bacteria have evolved complex systems to protect themselves from organic-peroxide toxicity. Alkyl hydroperoxide reductase (Ahp) is the best-characterized bacterial enzyme involved in the metabolism of organic peroxides (Poole, 1996; Niimura *et al.*, 1995). This enzyme consists of two subunits: catalytic subunit C (AhpC) and reductase subunit F (AhpF). AhpC reduces organic peroxides to the corresponding alcohols (Poole & Ellis, 1996). AhpC belongs to a large family of peroxidases (the AhpC/thiol-specific antioxidant family) found in organisms ranging from bacteria to man (Chae *et al.*, 1994a). Some organisms express multiple AhpC/thiol-specific antioxidant paralogues, presumably with distinct functions (regulation or cellular localization) (Baillon *et al.*, 1999; Bsat *et al.*, 1996; Hillas *et al.*, 2000).

In the bacterial phytopathogen *Xanthomonas campestris* pv. *phaseoli*, the defence against organic-peroxide toxicity is complex (Loprasert *et al.*, 1996). In addition to AhpC, there is a recently characterized novel

organic hydroperoxide resistance gene, *ohr* (Loprasert *et al.*, 1997; Mongkolsuk *et al.*, 1998a). *X. campestris ohr* mutants are sensitive to organic peroxides, but not to other oxidants (Mongkolsuk *et al.*, 1998a). In addition, *ohr* has a unique pattern of oxidant-induced expression; only organic peroxides induce high levels of expression (Mongkolsuk *et al.*, 1998a). This unusual pattern of induction distinguishes *ohr* from other known oxidative stress genes. Analysis of Ohr primary structure shows that it has homology to proteins with unknown functions from both Gram-positive and Gram-negative bacteria, and that it has moderate homology to an osmotically inducible protein (OsmC) from *Escherichia coli* (Gutierrez & Devedjian, 1991).

On the basis of sequence analysis of Ohr and OsmC homologues, we propose that these two proteins define two protein subfamilies. In this report, we focus on two organisms with one member of each subfamily: *Pseudomonas aeruginosa* and *Deinococcus radiodurans*. Genetic analyses in *P. aeruginosa*, and expression studies in both organisms, support the hypothesis that these proteins are functionally, as well as structurally, distinct.

METHODS

Bacterial strains, growth conditions and oxidant killing. *Xanthomonas* strains, *D. radiodurans* and *P. aeruginosa* PAO1 were grown in Silva–Buddenhagen medium (0·5% sucrose, 0·5% yeast extract, 0·5% peptone, 0·1% glutamic acid; pH 7·0) at 28 °C, TGY medium (0·1% glucose, 0·8% tryptone, 0·4% yeast extract; pH 7·2) at 32 °C and Luria–Bertani (LB) medium at 37 °C, respectively. Bacterial growth was monitored spectrophotometrically at OD_{600}.

Quantitative determinations of plating efficiency in the presence of various oxidants of *Pseudomonas* strains were performed as described previously (Hassett *et al.*, 2000; Ochsner *et al.*, 2000). Essentially, cells from exponential phase cultures were serially diluted and plated on LB agar containing various concentrations of *tert*-butyl hydroperoxide (tBOOH). The numbers of colonies at the different oxidant concentrations were counted after 24 h incubation at 37 °C. Percentage survival is defined as the percentage ratio between the c.f.u. growing on plates containing tBOOH and those growing on plates without tBOOH.

Alignment and phylogenetic analysis. Protein sequences related to Ohr and OsmC were retrieved from public sequence databases using the BLAST program (Altschul *et al.*, 1997). These amino acid sequences were aligned using the program CLUSTAL W, version 1.7 (Thompson *et al.*, 1994). A phylogenetic tree was constructed by the neighbour-joining method, using the TREE program from the phylogenetic analysis page of D. L. Robertson, E. Beaudoing & J. M. Claverie (http://igs-server.cnrss-mrs.fr/anrs/phylogenetics). The results were drawn using the program PHYLODENDRON, version 0.8d (D. G. Gilbert, Department of Biology, University of Indiana, USA; http://iubio.bio.indiana.edu).

Stress-induced expression of *ohr* and *osmC*. Exponential phase cultures (OD_{600} = 0·4) were divided into flasks and oxidants or other chemicals were added. The following concentrations of chemicals were used: 250 μM H_2O_2, 200 μM cumene hydroperoxide, 200 μM tBOOH, 100 μM menadione, 2% (w/v) sodium chloride and 4% (v/v) ethanol for *P. aeruginosa*; 250 μM H_2O_2, 100 μM tBOOH, 4% (w/v) sodium chloride and 4% (v/v) ethanol for *D. radiodurans*. Treated and untreated cultures were harvested after 20 min incubation at appropriate temperatures.

Cloning of *P. aeruginosa ohr* and *osmC*. Full-length *P. aeruginosa ohr* and *osm*C genes were cloned using PCR. Primers 5′ohrP (5′-TCAGACAGGTGACTCTC-3′), 3′ohrP (5′-AGTCGGAAGCTTCAGAC-3′), 5′osmCP (5′-CGACG-CGAGCGGATGTC-3′) and 3′osmCP (5′-AGCGTTCCGC-TCAGCCG 3′) were designed using sequence data obtained from the genome sequence of *P. aeruginosa* (Stover *et al.*, 2000). A primer pair, *P. aeruginosa* genomic DNA, the PCR reaction mix and 2 U *Pfu* polymerase were mixed and used to amplify either *ohr* or *osm*C genes, under the following conditions: denaturation at 96 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 2 min. The 450 bp *ohr* and 470 bp *osmC* PCR-generated fragments were cloned into pBBR1MCS-4 and pBBR1MCS-5, respectively (Kovach *et al.*, 1994), giving two recombinant plasmids, pBBRohrP and pBBRosmCP. The nucleotide sequences of both genes were determined using a BigDye terminator cycle sequencing kit on an automated DNA sequencer (ABI 310).

Construction of *ohr* and *osmC* mutants in *P. aeruginosa*. Mutants were constructed by insertional inactivation of *ohr* and *osm*C genes. Essentially, pBBRohrP was digested with *Sfi*I and *Sac*II. The ends of the 340 bp fragment containing the coding region of *ohr* were gap-filled by DNA polymerase, and the blunt-ended fragment was cloned into *Sma*I-digested pKnock-GM (Alexeyev, 1999) to give pKnock-ohr. Similarly, pBBRosmCP was digested with *Sal*I and *Bst*EII. The ends of the 240 bp DNA fragment containing part of the *osm*C coding region were gap-filled by DNA polymerase, and cloned into *Sma*I-digested pKnock-Ap (Alexeyev, 1999) to give pKnock-osmCP. The sequences of the cloned DNA in both recombinant plasmids were determined using an automated DNA sequencer (ABI 310). pKnock-ohrP and pKnock-osmCP were conjugated into *P. aeruginosa* PAO1 as described previously (Hassett *et al.*, 2000). Gentamicin-resistant and carbenicillin-resistant colonies will arise from homologous recombination of the in-coming recombinant plasmid with either *ohr* or *osm*C genes on the chromosome, depending on the fragment on the plasmid. Insertion of the plasmid into the chromosome is expected to inactivate the gene. Transconjugants containing pKnock-ohr and pKnock-osmC were selected with gentamicin (15 μg ml^{-1}) and carbenicillin (200 μg ml^{-1}), respectively. Putative mutants were screened by PCR using a universal sequence primer for a site located in pKnock vectors and either the 3′*ohr* or the 3′*osm*C primer. The expected insertions resulting in inactivation of *ohr* and *osmC* were confirmed by Southern analysis of genomic DNA extracted from the mutants and were probed with gene-specific probes (data not shown).

Northern analysis of *ohr* and *osmC* homologues. Total RNA was extracted from *P. aeruginosa* and *D. radiodurans* by using the hot acid phenol method performed as described previously (Mongkolsuk *et al.*, 1997). RNA samples were separated by electrophoresis in formaldehyde agarose gels and were then transferred by capillary action to pieces of nylon membrane. Total RNA (10 μg) was loaded into each well. Probes were prepared, and RNA hybridization and membrane washing were performed as described previously (Mongkolsuk *et al.*, 1997). *P. aeruginosa ohr* and *osm*C probes of 300 bp and 375 bp, respectively, were made from *Mlu*I-digested pKnock-ohrP and *Sfi*I–*Hin*dIII-digested pKnock-osmCP. The DNA fragments were separated on an agarose gel, extracted and then purified prior to being radioactively labelled using a

random prime DNA-labelling kit. *D. radiodurans ohr* and *osmC* probes were made using PCR. Primers corresponding to coding regions of either *ohr* (5′ohrD, 5′-TGCGGGCGA-GGGAATAG-3′, and 3′ohrD, 5′-GTGTCTTATTCGCGG-AC-3′) or *osmC* (5′osmCD, 5′-CAGCGAGCACACTGGGC-3′, and 3′osmCD, 5′-GCTTGAGCGACTCAGCC-3′) were designed using the *D. radiodurans* genome sequence (White *et al.*, 1999). PCR was performed with *D. radiodurans* genomic DNA and the gene-specific primers in the PCR reactions noted above, using the following conditions: denaturation at 96 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min for 35 cycles. The 445 bp *ohr* and 470 bp *osmC* PCR-generated fragments were gel-purified, and radioactively labelled probes were made using the random primer DNA-labelling kit.

RESULTS AND DISCUSSION

Analysis of Ohr and OsmC homologues

Analysis of Ohr homologues from various bacteria suggested that there were two groups of related proteins (Koonin *et al.*, 2000; Mongkolsuk *et al.*, 1998a; Volker *et al.*, 1998). Some homologues, such as Ohr from *Acinetobacter calcoaceticus* and *P. aeruginosa*, have high levels of identity (50% or more), while others, such as *E. coli* OsmC, have moderate levels of identity (around 20%) when compared with the Ohr from *X. campestris* pv. *phaseoli* (Mongkolsuk *et al.*, 1998a). Since the physiological role of these homologues is unknown, it is not yet clear whether these structural distinctions are of functional significance. To address this question, we have performed a phylogenetic analysis of Ohr homologues and initiated molecular genetic studies in two model systems containing homologues from both the Ohr and the OsmC subfamilies.

X. campestris pv. *phaseoli* Ohr and *E. coli* OsmC amino acid sequences were used to search the GenBank and bacterial genome databases for related proteins. Homologues of both proteins are widely distributed in both Gram-negative and Gram-positive bacteria, but no homologues were detected in eukaryotes. Amino acid alignments generated using CLUSTAL W (Thompson *et al.*, 1994) suggest that the Ohr/OsmC family can be divided into two subfamilies, each being defined by sequence motifs conserved only among Ohr (designated Oh regions) or only among OsmC (designated Os regions) homologues (Fig. 1). At present, we do not know the biological significance of these different motifs. A notable feature of the primary structure of Ohr and OsmC family members is the two highly conserved cysteine residues. C residues have been shown to be the active site of AhpC, an enzyme that metabolizes organic peroxide (Chae *et al.*, 1994b). The amino acid sequences around the second C residue are conserved within members of the Ohr and OsmC families but are very diverse between the two families. The conserved amino acid region around C-125 of the Ohr family members contains the sequence motif VCPY (Fig. 1). This region is not present in members of the OsmC family. The VCPY motif places the cysteine residue in an environment of abnormally strong nucleophilicity that makes it highly susceptible to reactive oxygen species (Lim *et al.*, 1994). The strongly nucleophilic regions in thiol-specific antioxidant proteins such as AhpC (Chae *et al.*, 1994b) and in the peroxide-scavenging protein ovothiol (Turner *et al.*, 1988) have been shown to be the catalytic sites for the breakdown of peroxides. This suggests that the C-125 residue in members of the Ohr family could participate in peroxide reduction. This idea is being investigated. The amino acid sequences were used to construct a phylogenetic tree (Fig. 2); it clearly shows that there are two separate groups of proteins, defined here as the Ohr and OsmC subfamilies.

Several bacteria produce either Ohr or OsmC. For example, *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Vibrio cholerae* and *Xylella fastidiosa* have only the Ohr homologue, whereas *E. coli* has only an OsmC homologue. In *Bacillus subtilis*, *Mycoplasma genitalium* and *Sinorhizobium meliloti*, proteins described as 'OsmC homologues' (Volker *et al.*, 1998) clearly belong to the Ohr subfamily (Fig. 2). Interestingly, *Mycoplasma genitalium* has no known proteins, other than the Ohr homologue, involved in peroxide detoxification (Fraser *et al.*, 1995). This suggests that in some bacteria Ohr might have a crucial role (or roles) in protecting against peroxide toxicity. Neither Ohr nor OsmC homologues were found in the genomes of several bacteria such as *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Neisseria meningitidis* and *Rickettsia prowazekii*. Overall, members of the Ohr family appear to be more widely distributed among diverse bacteria than members of the OsmC family.

Unexpectedly, several bacteria have homologues from both subfamilies. Several Gram-negative bacteria (*P. aeruginosa*, *Pseudomonas putida*) and a Gram-positive bacterium (*D. radiodurans*) have one member each from the OsmC and Ohr subfamilies. Other Gram-positive bacteria, such as *B. subtilis* and *Streptomyces coelicolor*, have one member of the OsmC family and two or more members of the Ohr family. Multiple Ohr homologues have not been identified in genomes from Gram-negative bacteria. At present, the functions of the multiple Ohr homologues are unknown but are the subject of further investigation.

Ohr and OsmC homologues have different physiological roles

The separation of Ohr and OsmC homologues into two subfamilies raises an important question: do these two subfamilies have distinct or overlapping functions? Bacteria such as *P. aeruginosa*, having one member each from the *ohr* and *osmC* subfamilies, offer an attractive model system for investigating this question. Using insertional inactivation, we generated mutants of the *P. aeruginosa ohr* and *osmC* genes. The *P. aeruginosa ohr* mutant, but not the *osmC* mutant, has a much reduced (more than 100 times lower) plating efficiency on agar containing 500 μM tBOOH when compared with the

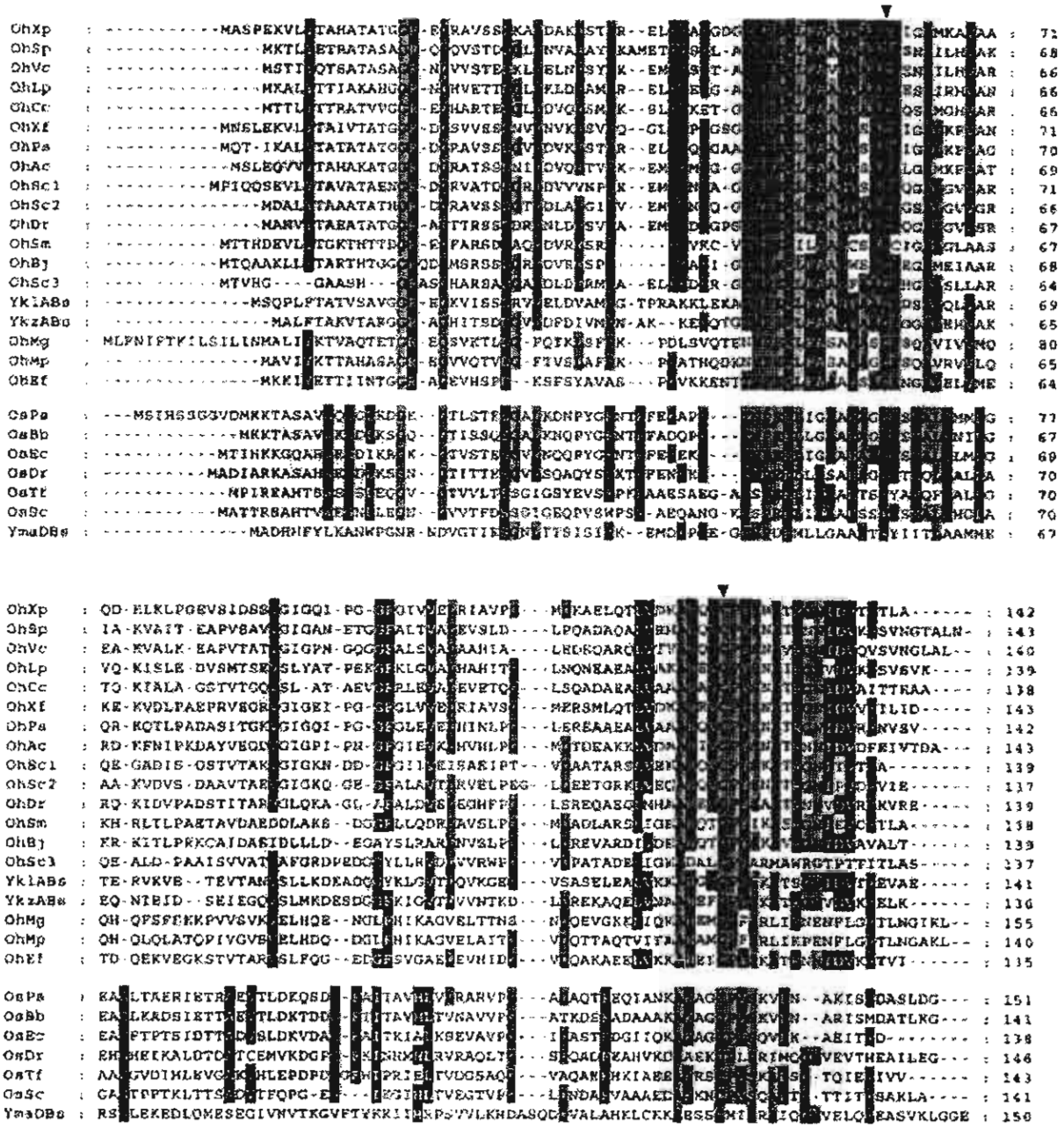

Fig. 1. Multiple amino acid sequence alignment of Ohr and OsmC homologues. Various homologues of Ohr and OsmC were aligned by using the CLUSTAL W program (Thompson *et al.*, 1994). The origins of these proteins are as follows: OhXp is from *Xanthomonas campestris* pv. *phaseoli* (AF036166); OhSp is from *Shewanella putrefaciens* (TIGR24 sputre6401)*; OhVc is from *Vibrio cholerae* (AE003853); OhLp is from *Legionella pneumophila* (CUCGC446 lpneumo WG.011079-R)*; OhCc is from *Caulobacter crescentus* (TIGR C.crescentus12574)*; OhXf is from *Xylella fastidiosa* (AE003849); OhPs is from *Pseudomonas aeruginosa* (PAGP 287 contig1)*; OhAc is from *Acinetobacter* (Y09102); OhSc1, OhSc2, OhSc3 and OsSc are from *Streptomyces coelicolor* (AL133423.1, AL163672.1, AL031515.1 and AL031515.1, respectively); OhDr and OsDr are from *Deinococcus radiodurans* (AE002025 and AE000513); OhSm is from *Sinorhizobium meliloti* (Stanford 382 smelil 423032c12)*; OhBj is from *Bradyrhizobium japonicum* (AAF78793.1); YklABs, YkzABs and YmaDBs are from *Bacillus subtilis* (AJ002571 and Z99113); OhMg is from *Mycoplasma genitalium* (U39732); OhMp is from *Mycoplasma pneumoniae* (MPAE000018); OhEf is from *Enterococcus faecalis* (TIGR1351 gef6391)*; OsPs is from *P. aeruginosa* (PAGP287 contig1)*;

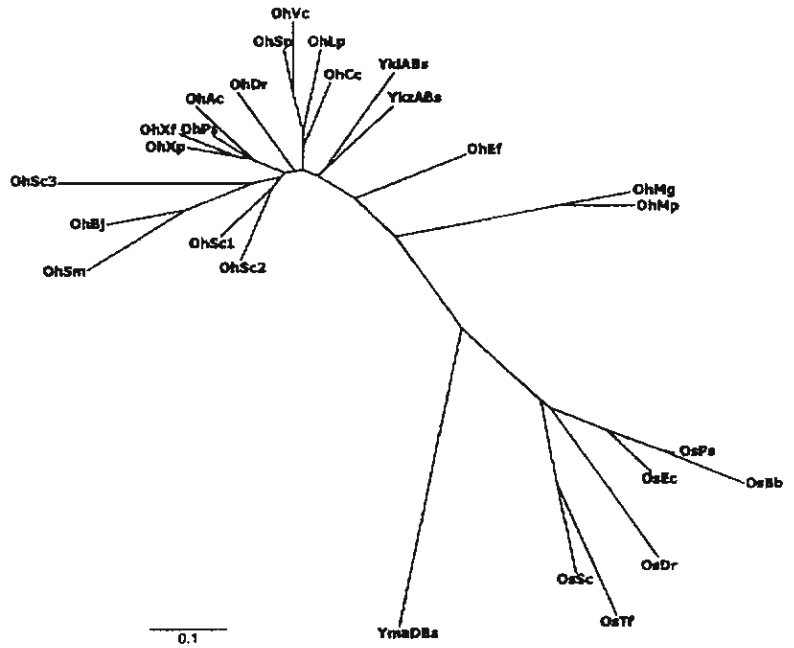

Fig. 2. An unrooted phylogenetic tree of Ohr and OsmC homologues. The tree was obtained by a neighbour-joining method by TREE phylogenetic analysis and was displayed using the PHYLODENDRON program (see Methods). Bar, 0·1 changes per site. Protein designations are the same as those in Fig. 1.

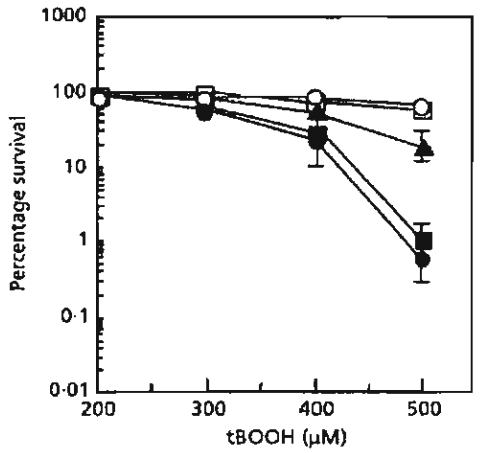

Fig. 3. Plating efficiencies of *P. aeruginosa ohr* and *osmC* mutants in the presence of oxidants. *P. aeruginosa* PAO1 (○), an *ohr* mutant (●), an *osmC* mutant (□), an *ohr* mutant harbouring pBBRohrP (▲) and an *ohr* mutant harbouring pBBRosmCP (■) were grown to exponential phase, serially diluted and plated on plates containing various concentrations of tBOOH. The experiments were performed independently four times, and the error bars represent standard error of the mean.

parent strain (Fig. 3). No changes in the plating efficiency in the presence of H_2O_2 or menadione for either mutant were observed (data not shown). Mutations in oxidative stress genes can lead to decreased aerobic growth rate and plating efficiency (Mongkolsuk *et al.*, 1998b; Hassett *et al.*, 2000). However, both mutants had the same growth rate as the parent strain in rich medium, and no deficiency in aerobic plating was detected (data not shown).

The *ohr* phenotype can be complemented by a plasmid containing the *ohr* gene (pBBRohrP), but not by a plasmid carrying the *osmC* gene (pBBRosmCP) (Fig. 3). The *ohr* mutant carrying pBBRohrP showed a 60-fold increase in plating efficiency in the presence of tBOOH when compared with the mutant harbouring the vector alone (Fig. 3), though this level was slightly lower than the tBOOH-resistance level attained by the parent strain. In the parental strain, *ohr* is expressed at high levels after exposure to tBOOH (Fig. 4); however, *ohr* expression in a moderate-copy-number expression vector (pBBR1MCS-4) might not be high enough to confer full protection against tBOOH toxicity in the *ohr* mutant. pBBRohrP did not raise tBOOH resistance in either the parental strain or the *osmC* mutant. These findings are similar to those presented in a previous

OsBb is from *Bordetella bronchiseptica* (Sanger518 bbronchi contig2522)*; OsEc is from *Escherichia coli* (X57433); OsTf is from *Thiobacillus ferrooxidans* (TIGR tferroxidans4156)*. Asterisks indicate data from unfinished genome sequences. The conserved regions found in either Ohr homologues (Oh regions) or OsmC homologues (Os regions) are shown by regions of black shading with white lettering. Grey shading with black lettering indicates identical amino acid residues found in both Ohr and OsmC (15 out of 26 sequences). ▼, Highly conserved C residues. The numbers at the ends of each line on the right-hand side refer to the numbers of amino acid residues.

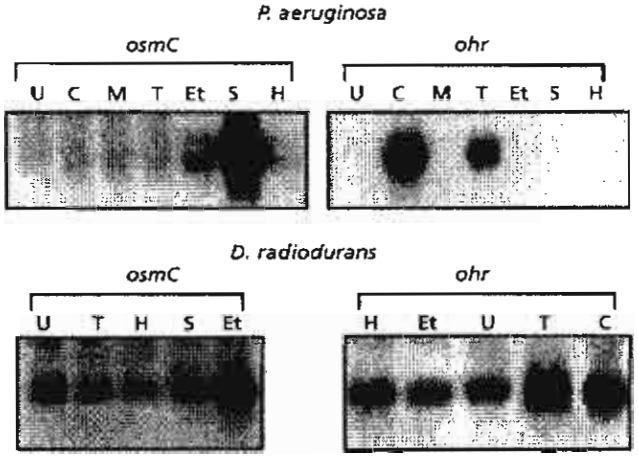

Fig. 4. Expression analysis of *ohr* and *osmC* in response to stresses in *D. radiodurans* and *P. aeruginosa*. *P. aeruginosa* and *D. radiodurans* cultures were grown and were either left untreated (U) or treated with oxidants (C, cumene hydroperoxide; H, H_2O_2; M, menadione; T, tBOOH) or subjected to osmotic stress (S, salt) and ethanol stress (Et) as described in Methods. RNA isolation, gel electrophoresis, blotting and hybridization were as described in Methods. Northern blots were probed with radioactively labelled *P. aeruginosa ohr* or *osmC* or *D. radiodurans ohr* or *osmC* probes.

report on *X. campestris* pv. *phaseoli*, in which the wild-type strain harbouring an *ohr* expression vector did not display increased resistance to tBOOH (Mongkolsuk *et al.*, 1998a).

We have tested several parameters, including aerobic growth, response to osmotic stress, and ethanol resistance, in the *P. aeruginosa osmC* mutant and found no significant changes in these parameters in comparison with the parent strain (data not shown). A similar analysis of *E. coli osmC* mutants failed to detect any physiological alterations (Gutierrez & Devedjian, 1991). The distinct phenotypes of the *ohr* and *osmC* mutants, and the inability of the *osmC* gene to complement the *ohr* mutant, support the idea that these genes play different roles in the cell.

ohr and *osmC* homologues have different expression patterns

X. campestris pv. *phaseoli ohr* has a unique expression pattern in that its expression is induced only by organic peroxide, and not by menadione or H_2O_2 (Mongkolsuk *et al.*, 1998a). In contrast, *E. coli osmC* is under both growth-phase (RpoS) and osmotic-stress regulation (Bouvier *et al.*, 1998; Gordia & Gutierrez, 1996). This suggests that members of the *ohr* and *osmC* subfamilies may have different patterns of stress-inducible expression.

We used Northern blotting experiments to determine the expression patterns of *ohr* and *osmC* homologues in response to osmotic and oxidative stresses in *P. aeruginosa* and *D. radiodurans* bacteria, each of which has one gene from each subfamily. In both organisms, *ohr* was strongly induced by low concentrations of organic peroxides (cumene hydroperoxide and tBOOH) (Fig. 4) but not by other oxidants such as menadione (not shown for *D. radiodurans*) or H_2O_2. Neither osmotic stress (a high salt concentration) nor ethanol induced expression of the *ohr* homologues. In contrast, expression of *osmC* homologues in both bacteria was induced by ethanol, while salt stress induced *osmC* expression only in *P. aeruginosa*; none of the oxidants tested induced the gene expression (Fig. 4). Thus, the patterns of *ohr* and *osmC* expression in *P. aeruginosa* and *D. radiodurans* are consistent with the known regulation of *X. campestris* pv. *phaseoli ohr* (Mongkolsuk *et al.*, 1998a) and *E. coli osmC* (Gutierrez & Devedjian, 1991). The *ohr* and *osmC* mRNAs in both bacterial species were each approximately 0·7 kb in length, indicating that these genes are transcribed as monocistronic mRNAs. Expression of both genes is different: *ohr* and *osmC* are induced by organic peroxide and osmotic stress, respectively. At present, well-characterized regulators of stress-induced gene expression such as OxyR, SoxRS and RpoS cannot account for the *ohr* and *osmC* patterns of expression, implying that these genes are regulated by novel regulators.

It was noticeable that basal levels of *ohr* and *osmC* from *P. aeruginosa* and *D. radiodurans* varied greatly, ranging from barely detectable amounts in the former to moderately high levels in the latter. In addition, the degree of induction varied significantly between these bacteria: *D. radiodurans* showed a lower magnitude of induction than *P. aeruginosa*. It remains to be seen if these differences in basal level expression and degree of induction are related to the ability of each bacterium to cope with organic peroxide stress or are simply indicative of the differences between Gram-negative and Gram-positive bacteria. It is remarkable that the patterns of stress-induced expression of *ohr* and *osmC* homologues are highly conserved in a diverse range of bacteria. This suggests that both genes might have important functions.

Concluding remarks

Members of the Ohr family are widely distributed in both Gram-negative and Gram-positive bacteria. Analysis of primary structure, the physiological characterization of mutants and expression patterns show that Ohr and OsmC proteins belong to different, but related, subfamilies. We have shown, in *P. aeruginosa* and *X. campestris* pv. *phaseoli* (Mongkolsuk *et al.*, 1998a), that mutations in *ohr* increase susceptibility to organic peroxides. This phenotype, coupled with the specific induction of *ohr* by organic peroxides, suggests that *ohr* represents a novel organic peroxide protection system. Recent results from Ochsner *et al.* (2001) confirm our finding that mutation in *P. aeruginosa ohr* results in

increased organic-peroxide sensitivity. More studies are needed to discover the physiological function of OsmC. The osmotically inducible expression of the gene suggests that it could have some kind of role in the bacterial osmotic-stress response. Recently, Conter *et al.* (2001) reported contradictory results that *E. coli osmC* mutants showed increase sensitivity to tBOOH but not to cumene hydroperoxide.

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OhrR Is a Repressor of *ohrA*, a Key Organic Hydroperoxide Resistance Determinant in *Bacillus subtilis*

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Bacillus subtilis **displays a complex adaptive response to the presence of reactive oxygen species. To date, most proteins that protect against reactive oxygen species are members of the peroxide-inducible PerR and σ^B regulons. We investigated the function of two *B. subtilis* homologs of the *Xanthomonas campestris* organic hydroperoxide resistance (*ohr*) gene. Mutational analyses indicate that both *ohrA* and *ohrB* contribute to organic peroxide resistance in *B. subtilis*, with the OhrA protein playing the more important role in growing cells. Expression of *ohrA*, but not *ohrB*, is strongly and specifically induced by organic peroxides. Regulation of *ohrA* requires the convergently transcribed gene, *ohrR*, which encodes a member of the MarR family of transcriptional repressors. In an *ohrR* mutant, *ohrA* expression is constitutive, whereas expression of the neighboring *ohrB* gene is unaffected. Selection for mutant strains that are derepressed for *ohrA* transcription identifies a perfect inverted repeat sequence that is required for OhrR-mediated regulation and likely defines an OhrR binding site. Thus, *B. subtilis* contains at least three regulons (σ^B, PerR, and OhrR) that contribute to peroxide stress responses.**

Elevated levels of reactive oxygen species (ROS) can damage proteins, DNA, and lipids and eventually lead to cell death. These ROS include hydrogen peroxide, superoxide anion, hydroxyl radical, and organic hydroperoxides. Bacteria have numerous enzymes to detoxify ROS (36), including catalases, superoxide dismutases, alkyl hydroperoxide reductase, and related peroxidases of the AhpC/thiol-specific antioxidant (TSA) family.

In *Bacillus subtilis*, there are several well-characterized systems that defend the cell against oxidants. Oxidatively stressed cells induce the synthesis of KatA, the major vegetative catalase (5, 15). A second catalase, KatB, is induced upon starvation or as part of the σ^B-dependent general stress response (17). A third catalase, KatX, is found in endospores (4, 30). *B. subtilis* also encodes a peroxide-inducible alkyl hydroperoxide reductase, encoded by the *ahpCF* operon (1, 7). Superoxide dismutase is encoded by the *sodA* gene (22, 23), which affects resistance to superoxide generating compounds and also participates in the maturation of the spore coat (21).

Alkyl hydroperoxide reductase (AhpCF) is the best-studied enzyme that can detoxify organic hydroperoxides (24) and is the founding member of the large AhpC/TSA family of peroxidases (11). The AhpC subunit reduces peroxides to the corresponding alcohols and it, in turn, is reduced by the AhpF flavoprotein (16, 25, 31, 32). Other members of the AhpC/TSA protein family can be reduced by thioredoxin and are referred to as thioredoxin-dependent peroxidases (TPx) (9, 10, 33). While most members of the AhpC/TSA family have two active site cysteine residues that are oxidized to a disulfide during each catalytic cycle, some related proteins have a single redox active cysteine (1 Cys peroxiredoxin proteins) and are reduced by an unknown electron donor. In addition to *ahpC*, *B. subtilis* contains three additional genes (*ytgI*, *ygaF*, and *ykuU*) that encode members of the AhpC/TSA family, but the functions of these genes have not yet been studied. A similar set of paralogs is found in yeast, which expresses five distinct members of the AhpC/TSA protein family which vary in subcellular localization (29).

Recently, a new type of organic hydroperoxide resistance (*ohr*) gene has been isolated from *Xanthomonas campestris* (27). The *ohr* mutant is more sensitive to organic hydroperoxides than is the wild type; however, it does not display sensitivity to hydrogen peroxide and superoxide generators (27). The Ohr protein is a member of a conserved family of proteins of largely uncharacterized function (OsmC/Ohr family [3]). Consistent with a role in organic peroxide detoxification, Ohr proteins have two conserved cysteine residues that are catalytically important, but Ohr proteins are not obviously homologous to the AhpC/TSA family of enzymes (3). There are two homologs of Ohr in *B. subtilis*; these homologs are encoded by the *yklA* and *ykzA* genes, but mutations in these genes have not been reported to have an effect on resistance to ROS (38).

In general, most enzymes that function in resistance to ROS are either inducible by oxidative stress or synthesized as part of a stationary-phase adaptative response. For example, *Escherichia coli* OxyR is a global peroxide regulator that can activate the expression of hydroperoxidase I (KatG), alkyl hydroperoxide reductase (AhpCF), a DNA-binding protein (Dps), and other resistance proteins (36). In *B. subtilis*, a similar peroxide stress response is regulated by PerR, a hydrogen peroxide- and metal ion-sensing repressor of the genes encoding KatA, AhpCF, MrgA (a Dps homolog), and heme biosynthesis enzymes (8). Interestingly, in both organisms, resistance to ROS

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is upregulated upon starvation. This stationary-phase induction of oxidant defenses is regulated by σ^S in *Escherichia coli* and by the general stress response regulator, σ^B, in *B. subtilis*.

We demonstrate here that the two *B. subtilis ohr* homologs, *yklA* and *ykzA*, are both involved in organic hydroperoxide resistance, and we therefore rename these genes *ohrA* and *ohrB*, respectively. In addition, we show that the intervening gene, *ohrR* (formerly *ykmA*), encodes an organic peroxide-sensing repressor (OhrR) for *ohrA*. In contrast, expression of *ohrB* is part of the σ^B-dependent general stress regulon (38).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All *E. coli* and *B. subtilis* strains were grown in Luria-Bertani (LB) medium with appropriate antibiotics (100 μg of ampicillin, 100 μg of spectinomycin, 10 μg of chloramphenicol, 8 μg of neomycin, and 1 μg of erythromycin per ml and 25 μg of lincomycin per ml for macrolide-lincosamine-streptogramin B [MLS] resistance) at 37°C with vigorous shaking.

Construction of *ohrA* and *ohrB* mutant strains. Previously, *yklA*::pMUTIN and *ykzA*::pMUTIN strains (BFS1816 and BFS1818) were described that contain insertional disruptions in each gene that result in transcriptional fusions to *lacZ* (38). Chromosomal DNA from BFS1816 *(ohrA-lacZ)* or BFS1818 *(ohrB-lacZ)* was transformed into CU1065 with selection for MLS resistance to generate strains HB574 and HB575, respectively. The presence of *lacZ* at the desired site was confirmed by PCR.

The *ohrB* gene was cloned into *Bam*HI and *Eco*RV-digested pBCSK (Stratagene) as a 593-bp PCR product extending 162 bp upstream and 21 bp downstream of the *ohrB* reading frame, generating plasmid pBC-zA. To create an *ohrA ohrB* double mutant, plasmid pMF2 was constructed by subcloning a 189-bp *Sph*I-*Eco*RI fragment of *ohrB* from pBC-zA into pGEM-cat at the *Sph*I-*Eco*RI sites. pMF2 was transformed into HB574 with selection for chloramphenicol resistance to generate HB2003. The presence of the *ohrB*::pMF2 disruption was confirmed by PCR of chromosomal DNA.

To introduce an *ahpC* mutation into the *ohrA* (HB574), *ohrB* (HB575), and *ohrA ohrB* (HB2003) mutant backgrounds, chromosomal DNA containing *ahpC*::Tn*10* (*ahpC1603*) (from strain HB6506 [7]) was transformed into HB574, HB575, and HB2003 to create HB2008, HB2009, and HB2010, respectively.

Construction of an *ohrR* (*ykmA*) mutant. The region of the *B. subtilis* chromosome containing the *ohrA*, *ohrR*, and *ohrB* genes was amplified by PCR to generate plasmid pYK15. A region extending from the *Pst*I site internal to *ohrA* to the *Sph*I site internal to *ohrB*, and therefore containing the entire *ohrR* gene, into pGEM-3zf to generate pGEM-mA. To construct an *ohrR* mutant, a kanamycin cassette from pDG792 (19) was subcloned into the *Bcl*I site internal to *ohrR* in pGEM-mA, generating pMF1. An *ohrR* mutant, HB2000, was constructed by transformation of linearized-pMF1 into CU1065 with selection for kanamycin resistance. HB2001 and HB2002 were generated by transforming *ohrR::kan* into HB574 and HB575, respectively. All strains were checked by PCR.

Construction of *ohrA-cat-lacZ* and *ohrR-cat-lacZ* fusions in SPβ. To construct an *ohrA-cat-lacZ* fusion, the *ohrA* promoter was amplified by PCR with primers 495 and 529. A *Bam*HI site was introduced into primer 529, and this PCR fragment contains internal *Hin*dIII sites. After *Bam*HI-*Hin*dIII digestion, this fragment was cloned into pJPM122 after digestion with *Bam*HI-*Hin*dIII to generate pMF3. To generate pMF4 containing an *ohrR-cat-lacZ* operon fusion, the *ohrR* promoter was amplified by PCR with primers 497 and 530 and cloned into pJPM122 as described above. pMF3 and pMF4 were transformed into strain ZB307A to transfer the promoter-*cat-lacZ* fusions into the SPβc2Δ2::Tn*917*::pBSK10Δ6 prophage by double cross over recombination. Using phage transduction, the operon fusions were transferred to CU1065 to generate HB2012 (SPβ *ohrA-cat-lacZ*) and HB2011 (SPβ *ohrR-cat-lacZ*) and into the *ohrR* mutant strain to generate HB2014 and HB2013.

RNA isolation and Northern hybridization. Cells were grown to mid log phase (optical density at 600 nm of $[OD_{600}] = 0.4$). Oxidants and chemicals used for induction were 100 μM cumene hydroperoxide (CHP), 100 μM *tert*-butyl hydroperoxide, 100 μM H_2O_2, 4% ethanol, or 4% NaCl. After 15 min of treatment, the cells were placed immediately on ice and centrifuged at 10,000 rpm at 4°C. Total RNA was isolated using RNAwiz RNA isolation kit (Ambion). Then, 10 μg of total RNA was loaded onto a 1% formaldehyde gel. The separated RNA was then transferred to a nylon membrane and hybridized with radiolabeled probe at 42°C overnight in ULTRAhyb solution (Ambion). The *ohrA* probe was prepared by *Hin*fI digestion of the PCR product generated from primers 531 and 496. A 314-bp *Hin*fI fragment containing the *ohrA* coding region was purified from an agarose gel and labeled with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase. The *ohrB* probe was prepared from an internal 200-bp *Sph*I-to-*Eco*RI fragment isolated from pBC-zA. The *ohrR* probe was prepared from *Hin*fI digestion products of the PCR fragment generated from primers 527 and 536. This PCR product contains the coding region of *ohrR*, which has two internal *Hin*fI restriction sites. *Hin*fI fragments were labeled by the fill-in method with [α-^{32}P]dATP. Membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) for 5 min at 42°C, followed by two washes with 0.1× SSC–0.1% SDS for 15 min at 42°C.

Primer extension. RNA was prepared using a hot phenol extraction protocol. A total of 10 μg of RNA was annealed with the ^{32}P-labeled oligonucleotide PE (Table 1). Primer extension reactions were performed using the Ready-To-Go You-Prime First-Strand Beads Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

β-Galactosidase assays. Cells were grown overnight in LB medium containing appropriate antibiotic(s) and then diluted 1:100 in the same medium. Samples of 1 ml were harvested at an OD_{600} of ca. 0.4 and assayed for β-galactosidase essentially as described earlier (26).

Disk diffusion assay. Cell were grown overnight in LB medium containing appropriate antibiotic(s) and then diluted 1:100 in the same medium. Then, 100 μl of cells at an OD_{600} of ca. 0.4 were mixed with 3 ml of LB containing 0.75% agar and poured onto plates containing 15 ml of LB agar with appropriate antibiotic(s). Next, 6-mm paper disks containing 10 μl of the indicated chemical were placed on top. Plates were incubated overnight at 37°C, and the clear zones were measured. The chemicals used included 0.4 M CHP, 0.2 M *tert*-butyl hydroperoxide, 1.6 M hydrogen peroxide, or 0.5 M paraquat.

Selection and characterization of mutants derepressed for *ohrA-cat-lacZ*. Approximately 10^4 cells of log-phase HB2012 were plated on LB agar containing 8 μg of neomycin, 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and between 2 and 5 μg of choramphenicol per ml. Blue colonies were recovered, and elevated expression of β-galactosidase activity was confirmed after growth in liquid medium. For each resulting strain, a transducing lysate was prepared and the SPβ *ohrA**-*cat-lacZ* fusions were transferred to CU1065. Transductants that retained elevated β-galactosidase activity (4 of 12) were judged to contain *cis*-acting mutations. The *ohrA* promoter region was amplified from each transductant using a primer specific to the 5′ region of the *cat* gene (primer 366) and a primer annealing upstream of the insert (primer 535). The resulting PCR products were used directly as templates for sequencing. One strain chosen for further characterization was designated HB2031. HB2031 chromosomal DNA was transformed into the *ohrR* mutant HB2000 to generate HB2044.

RESULTS

The *B. subtilis* OhrA (formerly YklA) and OhrB (formerly YkzA) proteins are homologs of *E. coli* OsmC (38), an osmotically inducible envelope protein of unknown function (6, 18, 20). However, they are much more similar to *X. campestris* Ohr, a protein that protects cells against organic hydroperoxides (27). Previously, *ohrB* was shown to be under σ^B control and respond to general stresses, whereas *ohrA* transcription was found to be elevated in minimal medium (38).

Overlapping roles of *ohrA* and *ohrB* in organic hydroperoxide resistance. Alkyl hydroperoxide reductase (AhpCF) reduces organic hydroperoxides to their corresponding alcohols. However, in previous studies we were unable to demonstrate an organic hydroperoxide-sensitive phenotype for an *ahpC*::Tn*10* mutant strain (7). Indeed, the most striking phenotype of this disruption mutant was an elevated resistance to H_2O_2 due to derepression of the PerR regulated *katA* gene. These results suggest that other gene products may also contribute to organic peroxide resistance.

Disk diffusion assays were used to determine if OhrA and OhrB protect cells against ROS and to determine if these functions are redundant with AhpCF. Mutation of *ohrA*, but

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristics	Relevant mutation(s)	Reporter	Reference, source, or derivation
Strains				
B. subtilis				
CU1065	W168 *attSPβ trpC2*			37
ZB307A	W168 SPβ2Δ2::Tn*917*::pBSK10Δ6			40
BFS1816	168 wild-type *ohrA-lacZ*	*ohrA*	*ohrA*	38
BFS1818	168 wild-type *ohrB-lacZ*	*ohrB*	*ohrB*	38
HB574	CU1065 *ohrA-lacZ*	*ohrA*	*ohrA*	This work
HB575	CU1065 *ohrB-lacZ*	*ohrB*	*ohrB*	This work
HB1703	CU1065 *ahpC*::Tn*10* (1603)	*ahpC*		7
HB2000	CU1065 *ohrR*::*kan*	*ohrR*		This work
HB2001	HB574 *ohrR*::*kan*	*ohrR, ohrA*	*ohrA*	This work
HB2002	HB575 *ohrR*::*kan*	*ohrR, ohrB*	*ohrB*	This work
HB2003	HB574 *ohrB*::pGEMCAT	*ohrA, ohrB*	*ohrA*	This work
HB2006	ZB307A SPβ*c2Δ2*::Tn*917*::ϕ*(ohrR'-cat-lacZ)*		*ohrR*	This work
HB2007	ZB307A SPβ*c2Δ2*::Tn*917*::ϕ*(ohrA'-cat-lacZ)*		*ohrA*	This work
HB2008	HB574 *ahpC*::Tn*10*	*ohrA, ahpC*	*ohrA*	This work
HB2009	HB575 *ahpC*::Tn*10*	*ohrB, ahpC*	*ohrB*	This work
HB2010	HB2003 *ahpC*::Tn*10*	*ohrA, ohrB, ahpC*	*ohrA*	This work
HB2011	CU1065 SPβ*c2Δ2*::Tn*917*::ϕ*(ohrR'-cat-lacZ)*		*ohrR*	This work
HB2012	CU1065 SPβ*c2Δ2*::Tn*917*::ϕ*(ohrA'-cat-lacZ)*		*ohrA*	This work
HB2013	HB2000 SPβ*c2Δ2*::Tn*917*::ϕ*(ohrR'-cat-lacZ)*	*ohrR*	*ohrR*	This work
HB2014	HB2000 SPβ*c2Δ2*::Tn*917*::ϕ*(ohrA'-cat-lacZ)*	*ohrR*	*ohrA*	This work
HB2031	CU1065 SPβ*c2Δ2*::Tn*917*::ϕ*(ohrA*-cat-lacZ)*		*ohrA*	This work
HB2044	HB2000 SPβ*c2Δ2*::Tn*917*::ϕ*(ohrA*-cat-lacZ)*	*ohrR*	*ohrA*	This work
HB6506	HB1000 *ahpC*::Tn*10*(1603)	*ahpC*		7
E. coli				
DH5α	ϕ80*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^-, m_K^+) *supE44 relA1 deoR* Δ*(lacZYA-argF)*U169			Lab stock
GM 2163	F⁻ *ara-14 leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 supE44 hisG4 rpsL1* (Str^r) *xyl-5 mtl-1 dam*13::Tn*9* (Cm^r) *dcm-6 mcrB1 hsdR2* (r_K^-, m_K^+) *mcrA*			NEB
Plasmids				
pGEM-cat	pGEM-3zf(+)-cat-1 (carrying Cm^r gene)			39
pGEM-mA	pGEM-3zf(+) with *PstI-SphI* containing *ohrA'-ohrR-ohrB*			This work
pBC-zA	pBCSK (Stratagene) containing *ohrB*			This work
pJPM122	*cat-lacZ* operon fusion vector for SPβ			34
pDG792	pMTL23 containing Kan^r cassette			19
pMF1	pGEM-mA containing the *BamHI-BglII* Kan^r cassette (1.6 kb) from pDG792 at *BclI* site in *ohrR*	*ohrR*		This work
pMF2	pGEM-cat containing intergenic *SphI-EcoRI* fragment of *ohrB*			This work
pMF3	pJPM122 with *ohrA* promoter			This work
pMF4	pJPM122 with *ohrR* promoter			This work
Primers				
366	5′-ACTCTCCGTCGCTATTGTAACCAG-3′			Lab stock
495 (forward)	5′-CGGGATCCTAGCGGGGTAATGTTCAATG-3′			This work
496 (reverse)	5′-CCGAATTCAAAAGCGGTTGACATTCCAG-3′			This work
497 (forward)	5′-CGGGATCCTGTATTGCTTTGTCATCTCC-3′			This work
519 (reverse)	5′-CGGGATCCAAATCAAGAACACCGTCATC-3′			This work
527 (forward)	5′-GGTGAACACCATGGAAAATAAATT-3′			This work
528 (reverse)	5′-CCGGATCCGTTGCTGAATAAATAAA-3′			This work
529 (reverse)	5′-CGGGATCCAATGACCTTTCCTTCTCTTC-3′			This work
530 (reverse)	5′-CCCAAGCTTAAATCAAGAACACCGTCATC-3′			This work
531 (forward)	5′-CGGGATCCTATATTGGGGGAATGAAAAA-3′			This work
535	5′-GTACATATTGTCGTTAGAAC-3′			This work
536 (reverse)	5′-AATGTCAACCGCTTTTTCT-3′			This work
PE	5′-AACGCGGTCTGATCAAATGA-3′			This work

not *ohrB* or *ahpC*, leads to significantly increased sensitivity to CHP (Fig. 1A) and *tert*-butyl hydroperoxide (data not shown). The *ohrA ohrB* double mutant displays much greater sensitivity to CHP than either single mutant, suggesting that both proteins are involved in CHP detoxification and that lack of one can be partially compensated for by the presence of the other. In contrast, AbpCF does not appear to play a significant role in CHP resistance, a finding consistent with our previous studies.

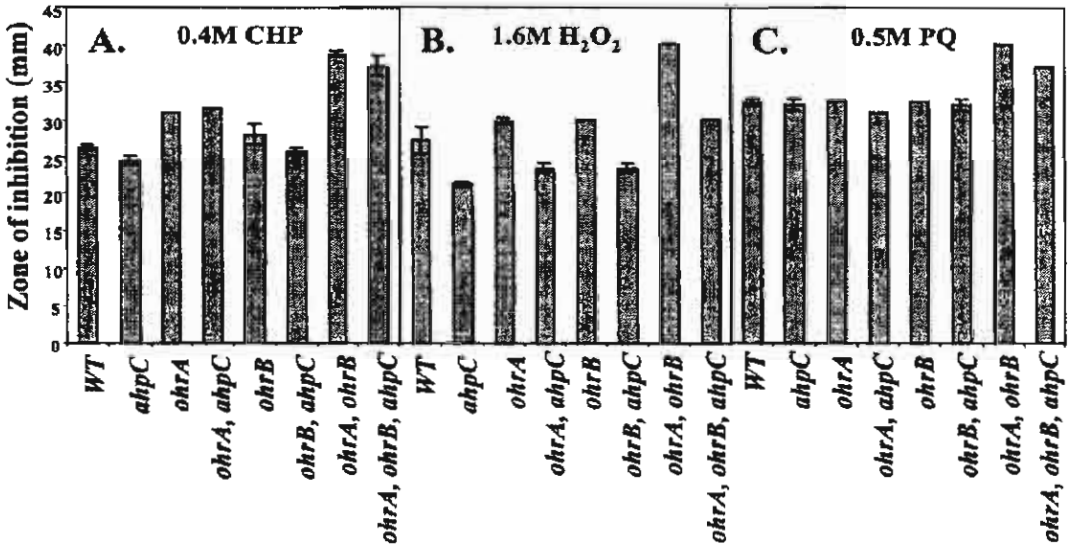

FIG. 1. Roles of OhrA, OhrB, and AhpCF in protection against ROS. The sensitivity of each indicated strain was measured as a zone of growth inhibition in a disk diffusion assay. Filters contained either 0.4 M CHP (A), 1.6 M H_2O_2 (B), or 0.5 M paraquat (C). The data shown are representative of three experiments. The error bars indicate the standard deviations from duplicate samples. PQ, paraquat.

In all four strains containing an *ahpC* mutation, resistance to CHP is not significantly altered relative to the control strain (Fig. 1A). Thus, even in the absence of both OhrA and OhrB, AhpCF still does not play a measurable role in CHP resistance. These strains all lack AhpCF function since, as reported previously (7), mutation of *ahpC* leads to derepression of catalase and a consequent increase in H_2O_2 resistance (Fig. 1B). In addition to greatly increased sensitivity to CHP, the *ohrA ohrB* double mutant also displays a striking sensitivity to both H_2O_2 (Fig. 1B) and the superoxide-generating compound, paraquat (Fig. 1C).

Transcriptional regulation of *ohrA* and *ohrB*. Northern blot analysis of CU1065 RNA isolated after exposure to various stresses demonstrates that *ohrA* is strongly induced by *tert*-butyl hydroperoxide and CHP, but not by H_2O_2, ethanol, or salt (Fig. 2A). In contrast, *ohrB* is strongly induced by ethanol or salt (Fig. 2B), a result consistent with the data of Volker et al. (38). It is also weakly inducible by *tert*-butyl hydroperoxide and CHP (Fig. 2B).

The regulation of *ohrA* by organic peroxides was also confirmed in primer extension experiments. A major *ohrA* transcript was found in cells induced with *tert*-butyl hydroperoxide and corresponds to a candidate σ^A-dependent promoter (Fig. 3). This inducible transcript corresponds to the transcript previously described for the *ohrA* gene (38). The constitutive signal corresponding to an apparent start site further upstream may be due to readthrough transcripts from the upstream *proBA* operon: this signal may result from reverse transcriptase pausing or termination at the base of the *proBA* terminator stem-loop. Readthrough from this upstream operon is consistent with the observation that *ohrA* expression is enhanced in minimal medium (38).

The induction of *ohrA* by organic peroxides was also confirmed using transcriptional reporter fusions (Table 2 and Fig. 4). With the pMUTIN derived transcriptional fusion, *ohrA-lacZ* expression can be induced ~100-fold by either CHP or *tert*-butyl hydroperoxide (Fig. 4). Similar regulation is also seen when a 219-bp region containing the *ohrA* promoter is used to generate a *lacZ* fusion inserted ectopically in SPβ (Table 2). This suggests that all necessary *cis*-regulatory elements are present within this DNA fragment.

Although AhpCF, at the levels present under these growth conditions, does not contribute significantly to protection against the killing action of CHP (Fig. 1A) or *tert*-butyl hydroperoxide (data not shown), AhpCF can reduce these compounds in vivo. This is apparent since the *ohrA* promoter can be induced by CHP and *tert*-butyl hydroperoxide at lower concentrations in strains carrying an *ahpC* mutation (Fig. 4). Note that these experiments were performed using the pMUTIN derived *ohrA-lacZ* fusion, so all strains are also mutant for *ohrA*.

OhrR is a repressor of *ohrA*. The *ohrA* and *ohrB* genes are transcribed in the same direction and are separated by *ohrR* (formerly *ykmA*), which is transcribed in the opposite direction and encodes a member of the MarR family of transcriptional repressors (Fig. 5). This proximity makes OhrR a good candidate for a regulator of *ohrA* and/or *ohrB*. In addition, an OhrR family member is known to repress *ohr* expression in *X. campestris* (S.M., unpublished data).

To determine if OhrR is a transcriptional regulator of *ohrA* and/or *ohrB*, β-galactosidase activity was measured in wild-type (HB2012) and *ohrR* mutant (HB2014) cells harboring an *ohrA-cat-lacZ* transcriptional fusion carried at SPβ (Table 2). The >100-fold upregulation of *ohrA* in the *ohrR* mutant was also confirmed in strains constructed using the pMUTIN integrational vector (which are additionally mutant for *ohrA*). The β-galactosidase activity in cells harboring *ohrA-lacZ* and an *ohrR* mutation (HB2001) was very high (~2,500 U) compared

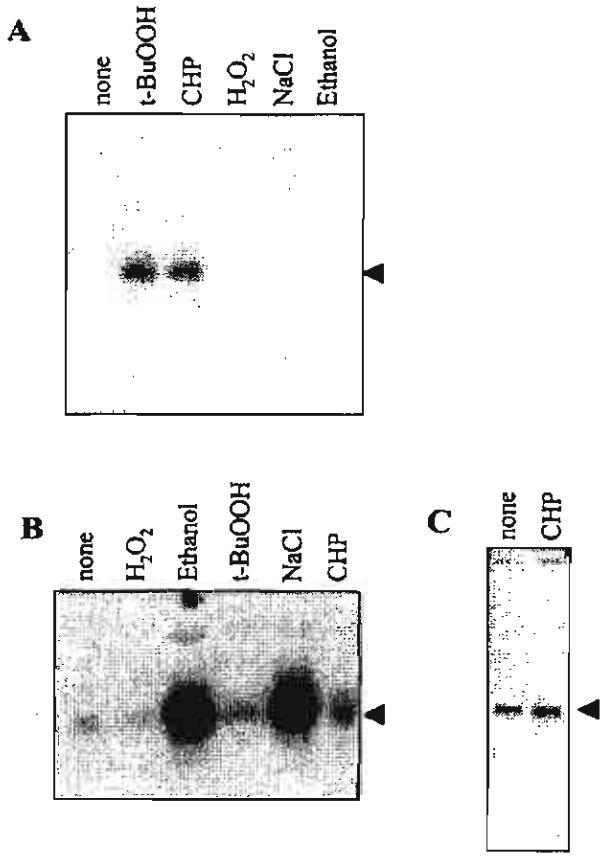

FIG. 2. Northern analysis *ohr* region genes. Expression of *ohrA* (A), *ohrB* (B), and *ohrR* (C) was measured using 10 μg of total RNA from each sample separated on a 1% formaldehyde gel. RNA was transferred to a nylon membrane and hybridized with a radiolabeled DNA fragment containing the coding region of each gene. Arrows indicate the major transcript of each gene. Cells were either uninduced (none) or were treated with 100 μM CHP, 100 μM *tert*-butyl hydroperoxide (t-BuOOH), 100 μM H_2O_2, 4% ethanol, or 4% NaCl for 15 min as indicated.

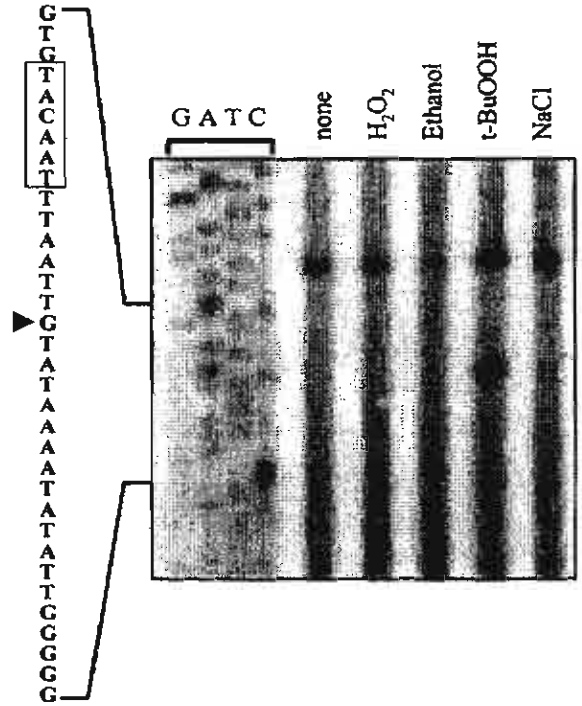

FIG. 3. Primer extension analysis of the *ohrA* promoter. Cells were grown and treated as described for Fig. 2 prior to RNA isolation. The major alkyl peroxide responsive transcriptional start point for the *ohrA* gene corresponds to position −27 relative to the start codon, in agreement with previously published start site mapping data (38). The origin of the larger band is not clear, but may be due to readthrough transcription from the upstream *proAB* operon.

to cells harboring *ohrA-lacZ* alone (HB574) (~6 U). In contrast, mutation of *ohrR* did not greatly affect the level of expression of the *ohrB-lacZ* fusion, which is very low in growing cells (1 to 2 U). These data demonstrate that mutation of *ohrR* is sufficient for derepression of *ohrA*, but not *ohrB*.

There is no significant increase in *ohrR-cat-lacZ* activity in *ohrR* versus wild-type cells (Table 2), suggesting that OhrR is not autoregulated. Moreover, expression of the *ohrR-cat-lacZ* fusion did not respond to CHP treatment (Table 2), a finding consistent with the slight response to CHP (1.3-fold induction) observed in the Northern analysis of *ohrR* mRNA (Fig. 2C).

Putative binding site of OhrR. Inspection of the *ohrA* promoter region reveals possible binding motifs for OhrR. The *ohrA* promoter region contains one perfect inverted repeat (TACAATT-AATTGTA) and an adjacent imperfect repeat with three mismatches (Fig. 6A). Alternatively, this region may be viewed as an 11-bp direct repeat.

To determine if these sequence motifs are important for OhrR-mediated repression, we selected for mutant strains that were derepressed for *ohrA-cat-lacZ* expression and characterized the resulting *cis*-acting mutants. Two independent mutants (*ohrA**) contained the identical 15-bp deletion (Fig. 6B). These mutations likely arose from unequal crossing over between the two 11-bp direct-repeat elements noted above. Remarkably, this deletion also removes the native −10 element of the *ohrA* promoter but replaces this region with another sequence that closely matches the −10 consensus, thereby likely generating a new σ^A-dependent promoter.

To determine if this altered promoter retains sequences that bind OhrR, the *ohrA*-cat-lacZ* fusion from one representative strain (HB2031) was transduced into the *ohrR* mutant to gen-

TABLE 2. β-Galactosidase activity of *ohrA* and *ohrR* transcription fusion in wild-type and *ohrR* backgrounds

Strain	Genotype		Mean β-Galactosidase activity ± SD (Miller unit)[a]	
	Mutation	Reporter	Uninduced	100 μM CHP
HB2012	None	*ohrA*	3.44 ± 0.09	90.47 ± 1.35
HB2014	*ohrR*	*ohrA*	513.04 ± 19.58	520.31 ± 16.43
HB2011	None	*ohrR*	3.65 ± 0.19	3.73 ± 0.12
HB2013	*ohrR*	*ohrR*	4.27 ± 0.14	4.46 ± 0.24
HB2031	None	*ohrA**	128.55 ± 4.12	ND
HB2044	*ohrR*	*ohrA**	230.37 ± 31.22	ND

[a] ND, not done.

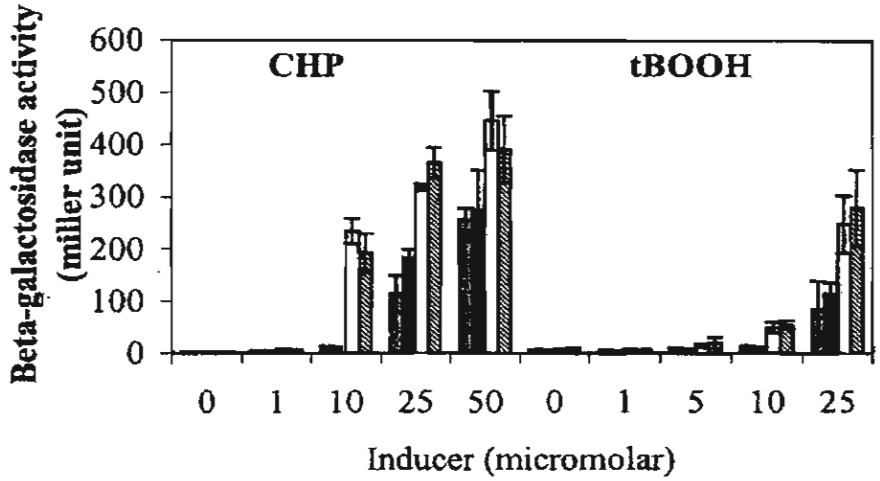

FIG. 4. Effect of an *ahpC* mutation on induction of *ohrA* by organic hydroperoxides. β-Galactosidase activities were assayed in various mutants (bars: gray, *ohrA*, HB574; black, *ohrA ohrB*, HB2003; white, *ohrA ahpC*, HB2008; cross-hatched, *ohrA ohrB ahpC*, HB2010). Cells were grown to mid-log phase, and various concentrations of CHP or *tert*-butyl hydroperoxide (tBOOH) were added to the cultures for 15 min at 37°C with shaking. The data shown are representative of triplicate determinations.

erate strain HB2044. Comparison of β-galactosidase activity in the wild-type and *ohrR* mutant cells indicates that OhrR still exerts a small, but reproducible, repressive effect on this promoter (Table 2). This result is consistent with models in which OhrR binds to the inverted repeat sequences noted above and suggests that the imperfect inverted repeat, which is retained in the mutant promoter region, may be sufficient for mediating some repression by OhrR.

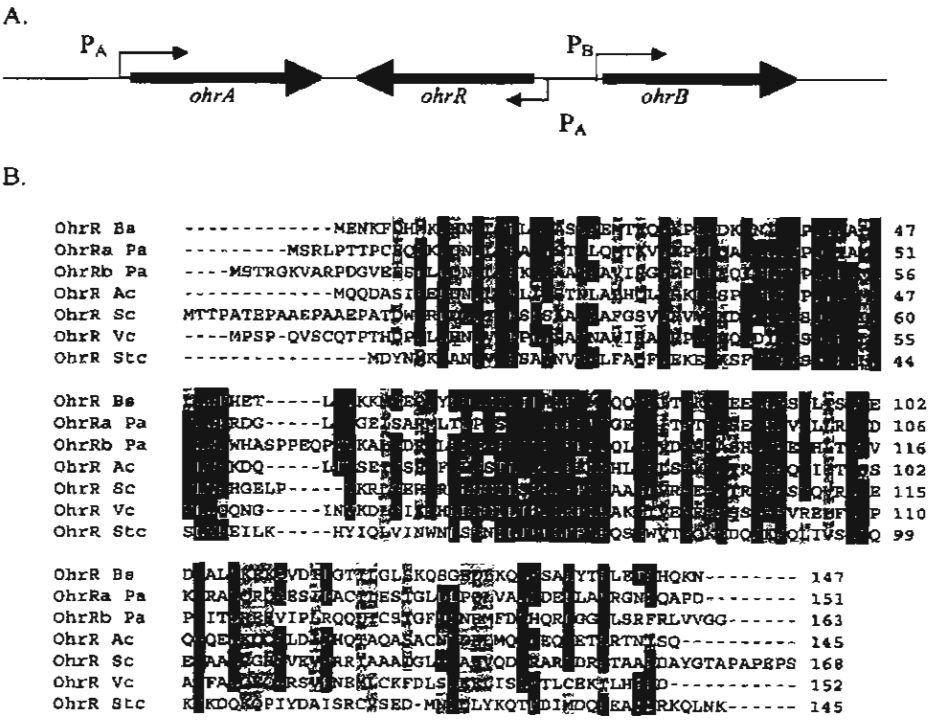

FIG. 5. *ohrR* encodes a MarR-like repressor of *ohrA*. (A) Schematic of the *ohrA ohrR ohrB* region. P_A indicates a σ^A-dependent promoter element; P_B indicates a σ^B-dependent promoter. (B) alignment of OhrR with other closely related MarR family members. The abbreviations used are as follows (strain; GenBank accession number): OhrR Bs (*B. subtilis*; E69857), OhrRa Pa (*P. aeruginosa* PAO1; D83290), OhrRb Pa (*P. aeruginosa* PAO1; G83292), OhrR Ac (*Acinetobacter* sp. strain ADP1; CAA70318), OhrR Sc (*Streptomyces coelicolor*; CAB87337); OhrR Vc (*Vibrio cholerae* group O1 strain N16961; B82389), and OhrR Stc (*Staphylococcus sciuri* strain ATCC 29062). The amino acid sequences were aligned (using CLUSTALW) and conserved residues highlighted using the BoxShade utility.

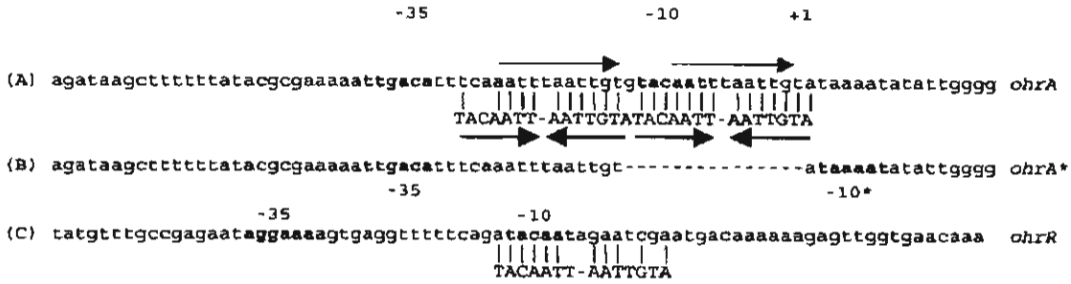

FIG. 6 Genetic identification of sequences required for OhrR-mediated repression. The perfect inverted repeat is indicated in capital letters with matching bases identified by a vertical line. (A) In the *ohrA* promoter, there are two adjacent inverted repeats. The first is imperfect; the second is a perfect inverted repeat (thick arrows). This region also contains two 11-bp direct repeats (thin arrows). The −10 and −35 regions are shown in boldface. (B) The sequence of the mutant promoter region (*ohrA**) is shown with a dashed line to indicate the 15-bp deletion. A new −10 element is created by the deletion. (C) A related, imperfect inverted repeat is found overlapping the *ohrR* promoter region.

DISCUSSION

Cells have evolved numerous overlapping mechanisms to protect against the ravages of ROS (35, 36). In the case of organic hydroperoxides, the best-studied defensive enzyme is alkyl hydroperoxide reductase, encoded by the *ahpCF* operon. However, bacterial cells contain additional activities that are important in protection against organic peroxides, including other peroxiredoxins and, as described here, members of the Ohr family. The role of Ohr in defense against oxidative stress was first described in *X. campestris* pv. phaseoli (27), and recent results indicate a similar function in *Pseudomonas aeruginosa* (28). Ohr proteins are not obviously homologous to known peroxidases, but it is reasonable to speculate that these proteins may enzymatically detoxify peroxides. Although Ohr expression is clearly regulated, the mechanisms controlling Ohr expression have yet to be described.

We have shown that the both OhrA and OhrB contribute to organic hydroperoxide resistance. Unlike PerR regulated genes, which can be induced by either organic hydroperoxides or H_2O_2 (7, 8, 12, 13), *ohrA* responds specifically to organic hydroperoxides, and this regulation requires OhrR. Consistent with previous studies, *ohrB* expression responds to heat, ethanol, and salt stress as part of the σ^B-dependent general stress response (Fig. 2A) (38). However, OhrB also has a role in organic hydroperoxide resistance, as shown by the increased CHP sensitivity of the *ohrA ohrB* double mutant (Fig. 1).

The relationship between the Ohr proteins and AhpCF is complex. Interestingly, only *ohrA* is under the control of OhrR. It is possible that OhrA plays the primary protective role when cells are exposed to organic hydroperoxides and OhrB is involved in detoxification of organic hydroperoxides produced during general stress. It is also possible that OhrA, OhrB, and the Ahp/TSA family members have distinct, albeit overlapping, substrate selectivities. Introduction of an *ahpC* mutation into the *ohrA*, *ohrB*, or *ohrA ohrB* strains did not increase sensitivity to organic hydroperoxides (Fig. 1), suggesting that AhpCF does not play a major role in protecting cells against the killing action of these organic hydroperoxides. The lack of a protective role for AhpCF in the present studies may result from the use of logarithmically growing cells (in which *ahpCF* is expressed at a low level) and the use of defined organic peroxides as the stressor. AhpCF and other genes repressed by PerR are known to be induced upon entry into stationary phase, upon starvation for iron and manganese, or in response to peroxides (7, 8, 14). In stationary-phase cells or under conditions in which both H_2O_2 and organic peroxides are generated, AhpCF levels would be elevated and could thereby contribute to oxidative defenses. Indeed, *perR* mutant cells have elevated resistance to CHP that depends on the *ahpC* gene (8). It is curious that AhpCF overproduction (in a *perR* mutant) leads to a CHP-resistant phenotype, whereas OhrA overproduction (in an *ohrR* mutant) does not, although OhrA is now sufficiently abundant as to be visible by Coomassie blue staining of whole-cell lysates (data not shown). Similarly, Ohr overproduction in *X. campestris* did not increase resistance to organic hydroperoxides (27).

The presence of two Ohr paralogs with distinct regulation is reminiscent of other genes involved in oxidative defense in *B. subtilis*. The *katA* gene is induced by ROS by virtue of its regulation by PerR, while the *katB* and *katX* genes are part of the σ^B regulon (4, 5, 8, 17, 30). Similarly, PerR represses expression of the Dps homolog encoded by *mrgA* (12), while a second Dps homolog encoded by the *dps* gene is regulated by σ^B (2).

Our genetic analysis defines a 15-bp region required for OhrR-mediated repression of the *ohrA* gene. This region includes a perfect inverted repeat, TACAATT-AATTGTA, which likely defines the OhrR binding site. Related imperfect inverted repeat sequences (three mismatches) are found in the *ohrA* and the *ohrR* promoter regions. Analysis of the *ohrA** mutant suggests that an imperfect inverted repeat element may still allow some residual regulation by OhrR (Table 2). However, the imperfect inverted repeat overlapping the *ohrR* promoter does not appear to mediate repression, since we found no evidence for *ohrR* autoregulation (Table 2).

OhrA and OhrB are representative of a large family of conserved proteins found throughout the Bacterial domain (3). Our data lend further support to the suggestion that these proteins function in protecting cells against organic peroxides. Moreover, since *ohr* homologs are often found closely associated with an *ohrR*-like gene (3), the mechanism of regulation described here may also be conserved. Thus, OhrR is a novel type of organic peroxide-sensing transcription factor and rep-

resents a third regulator (together with PerR and σ^B) involved in oxidative stress responses in *B. subtilis*.

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Complex Regulation of the Organic Hydroperoxide Resistance Gene (*ohr*) from *Xanthomonas* Involves OhrR, a Novel Organic Peroxide-Inducible Negative Regulator, and Posttranscriptional Modifications

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Analysis of the sequence immediate upstream of *ohr* revealed an open reading frame, designated *ohrR*, with the potential to encode a 17-kDa peptide with moderate amino acid sequence homology to the MarR family of negative regulators of gene expression. *ohrR* was transcribed as bicistronic mRNA with *ohr*, while *ohr* mRNA was found to be 95% monocistronic and 5% bicistronic with *ohrR*. Expression of both genes was induced by *tert*-butyl hydroperoxide (tBOOH) treatment. High-level expression of *ohrR* negatively regulated *ohr* expression. This repression could be overcome by tBOOH treatment. In vivo promoter analysis showed that the *ohrR* promoter (P1) has organic peroxide-inducible, strong activity, while the *ohr* promoter (P2) has constitutive, weak activity. Only P1 is autoregulated by OhrR. *ohr* primer extension results revealed three major primer extension products corresponding to the 5′ ends of *ohr* mRNA, and their levels were strongly induced by tBOOH treatment. Sequence analysis of regions upstream of these sites showed no typical *Xanthomonas* promoter. Instead, the regions can form a stem-loop secondary structure with the 5′ ends of *ohr* mRNA located in the loop section. The secondary structure resembles the structure recognized and processed by RNase III enzyme. These findings suggest that the P1 promoter is responsible for tBOOH-induced expression of the *ohrR-ohr* operon. The bicistronic mRNA is then processed by RNase III-like enzymes to give high levels of *ohr* mRNA, while *ohrR* mRNA is rapidly degraded.

During bacterial interactions with hosts, bacteria are exposed to host defense responses, including increased concentrations of reactive oxygen species (ROS), such as H_2O_2, organic peroxide, and superoxide anion (5, 14). In addition, normal aerobic respiration produces significant levels of ROS (10, 11). ROS are toxic to biological systems and must be removed rapidly. Among different ROS, organic peroxides are highly toxic, partly due to the abilities of these compounds to participate in free radical reactions which generate reactive organic radicals by reacting with membranes and other macromolecules (11).

Bacteria have evolved complex systems for sensing, protection, and regulation against organic peroxide toxicity. Alkyl hydroperoxide reductase is the best-characterized enzyme system involved in metabolizing toxic organic peroxides to the less toxic organic alcohols (7, 24, 25). In *Escherichia coli*, the gene for the catalytic subunit, *ahpC*, has an interesting pattern of expression. Its expression is regulated by OxyR, a global peroxide sensor and transcriptional regulator (30, 32), and is highly inducible by various oxidants (16, 19, 27). In *Xanthomonas campestris* pv. phaseoli, *ahpC* is differentially regulated by OxyR. Reduced OxyR represses while oxidized OxyR activates *ahpC* expression (15, 16, 19). The mechanism for protection against organic peroxides in *X. campestris* pv. phaseoli is complex. In addition to the *ahpC* and catalase peroxidase systems, an organic hydroperoxide resistance (*ohr*) gene also provides protection against organic peroxide toxicity (20). Inactivation of *ohr* in *Xanthomonas* and several other bacteria results in increased susceptibility to organic peroxide toxicity (4, 9, 20, 22, 26).

ohr has unique patterns of oxidative stress-induced expression, unlike other genes involved in protection against oxidative stress. In several bacteria, *ohr* expression is highly induced by treatment with low concentrations of organic peroxides (4, 9, 20, 22). In contrast, exposure to other oxidants or stresses does not induce *ohr* expression (2, 9, 20, 22). The regulator of *ohr* expression has not been identified, but atypical patterns of gene expression suggest that a novel regulator may be involved in the process. Since *ohr* is widely distributed among diverse groups of gram-positive and gram-negative bacteria (4), understanding the regulatory mechanisms is important. Analyses of primary structures of Ohr homologues, alterations in the physiological properties of their mutants, and patterns of expression of the genes together suggest that Ohr probably belongs to a novel family of proteins involved in organic peroxide protection (4). At present, the biochemical mechanism of Ohr-mediated protection is not known.

In this communication, we identify a negative regulator of *X. campestris* pv. phaseoli *ohr*, OhrR. *ohrR* is located upstream of and forms an operon with *ohr*. The gene product, OhrR, func-

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tions as a negative regulator of *ohr* expression. Transcriptional analysis of both genes suggests that expression of *ohr* is regulated from a distant *ohrR* promoter and also involves an RNA processing step.

MATERIALS AND METHODS

Culture conditions and oxidant treatments. *Xanthomonas* strains were grown aerobically in Silva-Buddenhagen medium (0.5% sucrose, 0.5% yeast extract, 0.5% peptone, 0.1% glutamic acid [pH 7.0]) at 28°C. *tert*-Butyl hydroperoxide (tBOOH)-induced *ohr* expression was achieved by the addition of 200 μM tBOOH to *Xanthomonas* log-phase cultures (19). The induction times for Western and primer extension experiments were 30 and 15 min, respectively.

Phylogenetic analysis. A phylogenetic tree was constructed by the neighbor-joining method using the Tree program from the phylogenetic analysis page at http://igs-server.cnrss-mrs.fr/anrs/phylogenetics. The results were drawn using the program PHYLODENDRON (version 0.8d 1999; Department of Biology, University of Indiana [http://iubio.bio.indiana.edu]).

Northern analysis of *ohr*, *ohrR*, and *ahpC*. Total RNAs from uninduced and tBOOH-induced cultures of *X. campestris* pv. phaseoli were purified using the hot phenol method (16, 17). Ten micrograms of purified RNA was loaded into each lane of formaldehyde agarose gels, and RNA samples were electrophoretically separated. Separated RNA samples were transferred to nylon membranes. The membranes were exposed to various probes using prehybridization, hybridization, and high-stringency washing conditions as previously described (16, 19). *ohrR*-specific probes were prepared from plasmid pohrR digested with *Sac*I and *Kpn*I. The 250-bp fragment was purified from an agarose gel. *ohr*- and *ahpC*-specific probes were prepared from plasmids pohr and pahpC, respectively, as previously described (19, 20). The gene-specific DNA fragments were radioactively labeled using a random primer kit and [α-^{32}P]dCTP.

RT-PCR of *ohrR-ohr* mRNA. Reverse transcription (RT) of *ohrR-ohr* mRNA was performed to confirm the bicistronic transcriptional organization of these genes. Briefly, RNA was isolated from tBOOH-induced *X. campestris* pv. phaseoli cultures using the hot acid-phenol method (19). Purified RNA was treated with 10 U of RNase-free DNase for 30 min to remove contaminating DNA. Primer ohr5′ (5′GCATCGGCCTCTTCGTTGGAC3′) was mixed with 10 μg of RNA, and 200 U of cloned Moloney murine leukemia virus reverse transcriptase was added. The mixture was incubated at 42°C for 60 min. Then, 5 μl of the reaction mixture was added to a PCR containing primers ohr5′ and ohrR3′ (5′GTCGAGCGCCTTGTCCGAGGA3′). PCR was performed using previously described conditions for 35 cycles, and PCR products were analyzed in an agarose gel (19).

Western analysis of Ohr and Cat. Cell lysates were prepared from *X. campestris* pv. phaseoli cultures as previously described (20). Twenty micrograms of protein was loaded into each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated protein samples were transferred to nitrocellulose membranes by electroblotting (20). Immunological reactions with an anti-Ohr or an anti-Cat antibody were done as described by Mongkolsuk et al. (19, 20). The antibody reactions were detected using an alkaline phosphatase-conjugated goat anti-rabbit antibody. Subsequent detection of alkaline phosphatase activity was done using a kit from Promega in accordance with the instructions of the manufacturer.

Construction of pBBRohrR and pBBRohrRBs. pohr (20) was digested with *Sfi*I. The ends of the fragment were filled in with DNA polymerase I, and the fragment was redigested with *Sac*I. The 550-bp fragment containing *ohrR* was purified from an agarose gel and ligated into pBBR1MCS-5 (13) digested with *Sma*I and *Sac*I to give pBBRohrR. A frameshift mutation in *ohrR* was created by digesting pBBRohrR with *Bst*EII located in the coding region, the ends of the fragment were filled in with DNA polymerase I, and the ends were religated. This procedure gave pBBRohrRBs.

Construction of *X. campestris* pv. phaseoli *ohrR1*, *ohrR2*, and *ohrR3* mutants. The 65-bp *Pst*I-*Sac*II and 211-bp *Pst*I fragments from restriction enzyme-digested pohr were electrophoretically separated from other DNA fragments using a 1.5% agarose gel. The purified DNA fragments were recovered from the gel and cloned into similarly digested vector pUC18tet. This procedure gave pUCohrR1 and pUCohrR2. pohr was digested with *Pst*I-*Hin*cII, and the 615-bp fragment was purified by electrophoresis and recovered from an agarose gel. The purified fragment was cloned into similarly digested pUC18Km to give pUCohrR3 (see Fig. 3). pUCohrR1, pUCohrR2, and pUCohrR3 were electroporated into *X. campestris* pv. phaseoli using previously described conditions (21), resulting in *XpohrR1*, *XpohrR2*, and *XpohrR3*, respectively. Transformants with pUCohrR1 and pUCohrR2 were selected for Tet^r (15 μg/ml), while transformants with pUCohrR3 were selected for Km^r (15 μg/ml). Genomic DNA was isolated from these transformants and digested with appropriate restriction enzymes. After electrophoretic separation, the DNA fragments were hybridized with *ohrR* and pUC18 as probes (data not shown) to confirm proper integration of the plasmid into the *X. campestris* pv. phaseoli chromosome.

Construction of pP1 and pP2. pohr (20) was digested with *Acc*65I, the ends of the fragment were filled in with DNA polymerase I, and the blunt-ended DNA was redigested with *Bam*HI. The 615-bp fragment containing P1 was separated by electrophoresis, purified from the agarose gel, and cloned into *Bgl*II-*Sma*I-digested promoter probe vector pUFR027cat-km (28). This procedure generated pP1 and placed the *ohrR* promoter in front of a promoterless *cat* gene. pP2 was constructed by digesting pohr (20) with *Not*I, filling in the ends of the fragment with DNA polymerase I, and redigesting the blunt-ended DNA with *Sac*I. The 145-bp fragment containing P2 was recovered from the agarose gel after electrophoretic separation and cloned into *Sac*I-*Sma*I-digested pUFR027catKm to give pP2.

***ohr* primer extension.** RNA was extracted as described above for Northern analysis (16, 17). In addition, purified RNA samples were treated with 10 U of RNase-free DNase for 30 min. Primer ohrP1 (5′GTCGAGCGCCTTGTCCGA GGA3′), located 70 bp from the translation initiation codon of *ohr*, was radioactively labeled using T4 polynucleotide kinase and [^{32}P]ATP. Briefly, 10 μg of DNase I-treated RNA was added to a reverse transcriptase reaction mixture. The reaction was started by the addition of 200 U of Moloney murine leukemia virus reverse transcriptase. Products of the reaction were analyzed on sequencing gels. The sequence ladders were made using an fmol sequencing kit, *ohrR1*-labeled primer, and pohr (20) as the template.

Nucleotide sequence accession number. The nucleotide sequence of *ohrR* has been deposited in GenBank under accession number AF036166.

RESULTS

***ohr* is not regulated by OxyR.** In *Xanthomonas* spp., *Pseudomonas aeruginosa*, *Deinococcus radiodurans*, and *Bacillus subtilis*, *ohr* expression is strongly induced by exposure to organic peroxides (tBOOH and cumene hydroperoxide [CuOOH]) but not by exposure to other oxidants and stresses (4, 9, 20, 22). This pattern of induced expression appears to be conserved in various bacteria and is unique to members of the *ohr* family (4). Understanding the regulatory mechanisms of *ohr* is likely to be generally important due to the wide distribution of *ohr* homologues among gram-negative and gram-positive bacteria (4, 9, 20, 22, 26). OxyR, a peroxide sensor and transcriptional regulator, is a potential regulator for organic peroxide-inducible expression of *ohr*. For *Xanthomonas* spp., it has been shown that OxyR-regulated genes are highly induced by tBOOH, suggesting that it may also be involved in sensing organic peroxides (16, 19). First, we tested whether OxyR is involved in the regulation of *ohr*. Total RNAs isolated from uninduced and tBOOH-induced cultures of *X. campestris* pv. phaseoli and an *oxyR* mutant (21) were probed with radioactively labeled *ohr* or *ahpC* gene-specific probes. *ahpC* expression was used as a positive control for an OxyR-regulated gene (19). The results of Northern analysis showed that *ohr* expression was highly induced by tBOOH to similar levels in both the *oxyR* mutant and the parent strain (Fig. 1). As expected, *ahpC* expression was highly induced by tBOOH only in the parent strain (Fig. 1). The data prove that OxyR is not the regulator of *ohr*.

Identification of a putative *ohr* regulator, *ohrR*. A search for a tBOOH-responsive regulator of *ohr* was initiated. During the analysis of *ohr* homologues in bacteria for which genome sequences have been completed, such as *B. subtilis*, *P. aeruginosa*, and *D. radiodurans*, we noticed that adjacent to the *ohr* homologues, there were open reading frames (ORFs) encoding proteins with moderate amino acid sequence identities to mem-

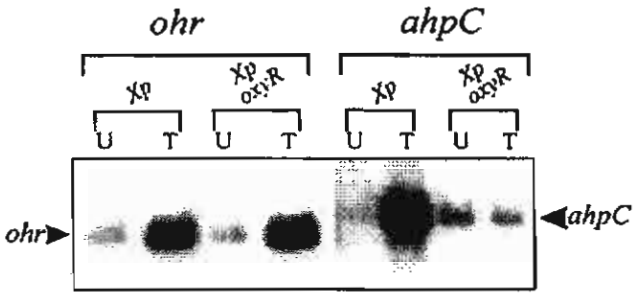

FIG. 1. OxyR-independent tBOOH induction of *ohr*. Northern analysis of *ohr* and *ahpC* expression in uninduced (U) or tBOOH-induced (T) cultures of *X. campestris* pv. phaseoli (*Xp*) and an *oxyR* mutant (*Xp oxyR*) is shown. The arrowheads to the left and right indicate the positions of *ohr* and *ahpC* mRNAs, respectively.

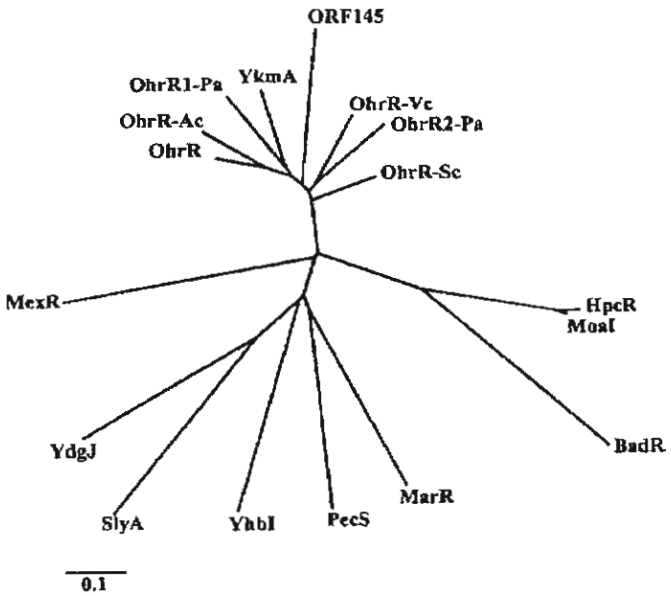

FIG. 2. Phylogenetic tree for OhrR and other members of the MarR family. Analysis and construction of the tree were performed as described in Materials and Methods. Proteins, GenBank accession numbers (in parentheses), and organisms are as follows: BadR (U75363), *Rhodopseudomonas palustris*; HpcR (S56952), *E. coli*; MarR (P27245), *E. coli*; MexR (U23763), *P. aeruginosa*; MoaI (D63524), *Klebsiella aerogenes*; OhrR (AF036166), *X. campestris* pv. phaseoli (this study); OhrR-Ac (Y09102), *Acinetobacter* sp.; OhrR1-Pa (D83290) and OhrR2-Pa (G83292), *P. aeruginosa*; ORF145 (Y13052), *Staphylococcus sciuri*; OhrR-Vc (B82389), *V. cholerae*; OhrR-Sc (AL163672), *S. coelicolor*; PecS (P42195), *Erwinia chrysanthemi*; SlyA (P40676), *Salmonella enterica* serovar Typhimurium; YdgJ (D69783), YhbI (Z99108), and YkmA (E69857), *B. subtilis*. The bar indicates genetic distance.

bers of a family of negative regulators of gene expression including *E. coli* MarR (1, 2). These ORFs were candidates for regulators of *ohr*. Thus, additional sequencing upstream and downstream of *X. campestris* pv. phaseoli *ohr* was undertaken. Analysis of the nucleotide sequence immediately upstream of *X. campestris* pv. phaseoli *ohr* revealed an ORF encoding a 17-kDa peptide with 18% identity to *E. coli* MarR. We designated this ORF *ohrR*. The amino acid sequence of OhrR was used to search databases. These searches revealed two groups of related proteins. One group contains closely related proteins of unknown functions with amino acid identities ranging from 32 to 54% in both gram-positive and gram-negative bacteria. The genes for most members of this group are located adjacent to *ohr* homologues. We have designated these unknown proteins OhrR homologues. The second group has less identity to OhrR (18 to 22%). Members of this group include *E. coli* MarR and other known negative regulators of gene expression (1, 2, 8).

The amino acid sequences of both groups of homologues were used to construct a phylogenetic tree (Fig. 2). Analysis of the tree supported the idea that OhrR homologues belong to a larger and more diverse MarR family of transcriptional repressors. The highly conserved MarR amino acid sequence motif D-X-R-X_5-L/I-T-X_2-G, where X represents any amino acid (2), was found in all OhrR homologues. In addition, it was possible to extend the highly conserved MarR motif to L/M-X_3-G-X_3-R-X_5-D-X-R-X_5-L-T-X_2-G by comparing members of the OhrR and MarR families. At present, the function of the conserved motif has not been clearly established.

The *ohrR-ohr* gene order in various *Xanthomonas* strains was determined by PCRs using a primer set located in the 3′ region of *ohrR* and the 5′ region of *ohr* and genomic DNAs from various *Xanthomonas* strains. Analysis of DNA fragments generated by the PCRs showed that the *ohrR-ohr* gene organization was conserved among all the *Xanthomonas* strains tested (data not shown). The availability of bacterial genome and gene sequences in various databases allowed us to determine whether the *ohrR-ohr* gene organization was also conserved in other bacteria. The analysis revealed that in *Acinetobacter calcoaceticus*, *D. radiodurans*, *P. aeruginosa*, *Vibrio cholerae*, *Streptomyces coelicolor*, and *X. campestris* pv. phaseoli, *ohrR* is located immediately upstream of *ohr* (Fig. 3A). The organization in *B. subtilis* is slightly different, in that *ohrR* (*ykmA*) is located between two *ohr* homologues, *yklA* and *ykzA* (9, 31). *P. aeruginosa* is an exception; it has two different copies of *ohrR*, one copy (*ohrR*1-Pa) located upstream of *ohr* and another copy (*ohrR*2-Pa) located downstream of a glutathione peroxidase gene (*gpx*).

Transcriptional organization of *ohrR-ohr*. Next, we examined the transcriptional organization of *X. campestris* pv. phaseoli *ohrR* and *ohr*. Northern analysis showed that *ohr* is transcribed as a 0.5-kb monocistronic mRNA (Fig. 1). *ohrR* was used to probe RNA isolated from tBOOH-induced cultures. The results showed that the *ohrR* probe hybridized to a 1.0-kb mRNA (Fig. 3B). This mRNA is much longer than the coding region of *ohrR* but is similar in size to the expected bicistronic *ohrR-ohr* mRNA. However, this explanation contradicted the results of the *ohr* Northern analysis (Fig. 1). To clarify the issue, Northern experiments using the *ohr* probe were repeated. Longer exposure for the *ohr* Northern hybridization revealed an additional positive reaction of *ohr* mRNA with 1.0-kb as well as 0.5-kb mRNA species (Fig. 3B). The former corresponded to the length of the expected bicistronic *ohrR-ohr* mRNA. More than 90% of *ohr* mRNA was monocistronic, while the remainder corresponded to the *ohrR-ohr* bicistronic form (20).

To confirm the identity of the putative operonic *ohrR-ohr* mRNA, it was analyzed by RT-PCR. A PCR primer set located

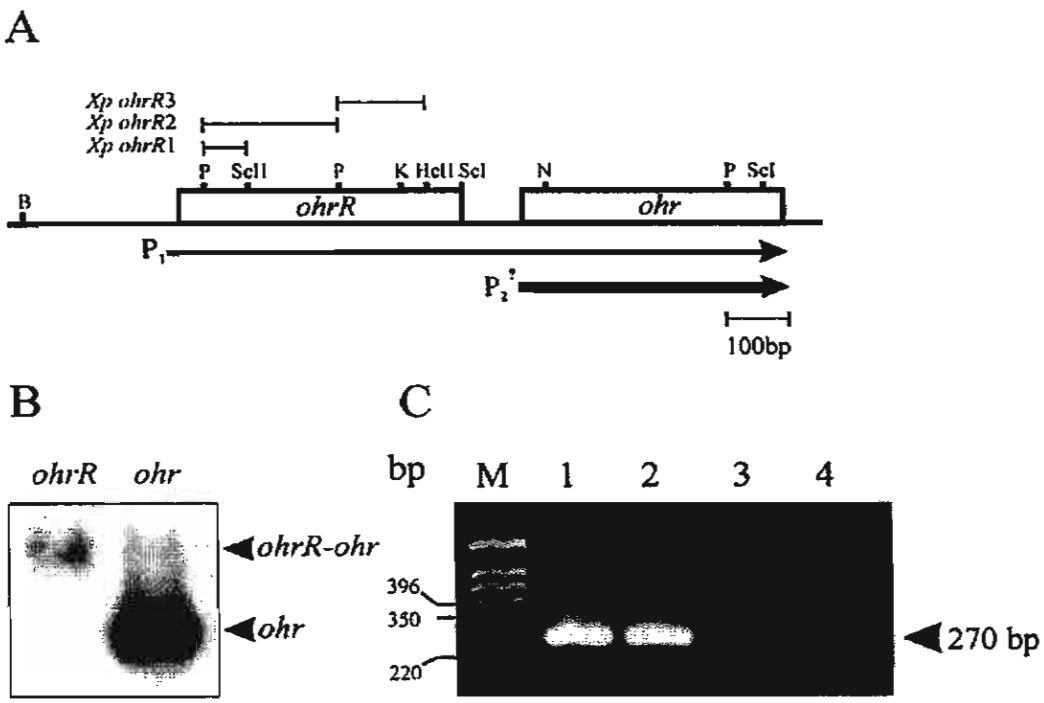

FIG. 3. Gene order and transcriptional organization of *ohrR-ohr*. (A) The bars above the map of *ohrR-ohr* indicate the locations and sizes of the fragments used in the construction of *ohrR* mutants (*Xp* designations). The sizes and directions of the arrows represent the amounts and directions of transcription, respectively. Hc, *Hin*cII; K, *Kpn*I; N, *Not*I; P, *Pst*I; ScI, *Sac*I; ScII, *Sac*II. (B) Northern analysis of *ohrR* and *ohr*. Ten micrograms of RNA samples from tBOOH-induced cultures were separated in formaldehyde-agarose gels, and the RNA was transferred to nylon membranes. The membranes were hybridized separately to radioactively labeled *ohrR* or *ohr* probes. The arrowheads indicate monocistronic *ohr* mRNA and bicistronic *ohrR-ohr* mRNA. (C) RT-PCR of RNA samples from tBOOH-induced *X. campestris* pv. phaseoli cultures. RNA extraction and DNase I treatment were done as described in Materials and Methods. The conditions for PCR and the primers used are described in Materials and Methods. Lane M, molecular weight markers; lane 1, PCR of a positive control DNA sample; lane 2, RT-PCR of an RNA sample from tBOOH-induced *X. campestris* pv. phaseoli cultures; lane 3, the same RNA sample and PCR conditions as in lane 2 except that the RT step was omitted; lane 4, PCR of reagents (negative control).

3′ of the *ohrR* and 5′ of the *ohr* coding regions was added to cDNA obtained by RT of total RNA from a tBOOH-induced culture. Analysis of DNA fragments from the PCRs showed the expected 270-bp fragment when the cDNA and a control *Xanthomonas* genomic DNA were used as templates (Fig. 3C). The 270-bp fragment was not detected in PCRs with the same RNA sample but with the RT step omitted (Fig. 3C).

Effect of OhrR on *ohr* expression. OhrR belongs to a family of negative regulators of gene expression (Fig. 2); thus, we investigated its effect on *ohr* expression. pBBRohrR was electroporated into *X. campestris* pv. phaseoli, and the levels of *ohr* expression in the transformants were determined. Northern analysis clearly showed that high-level expression of *ohrR* resulted in more than a 10-fold reduction in uninduced *ohr*

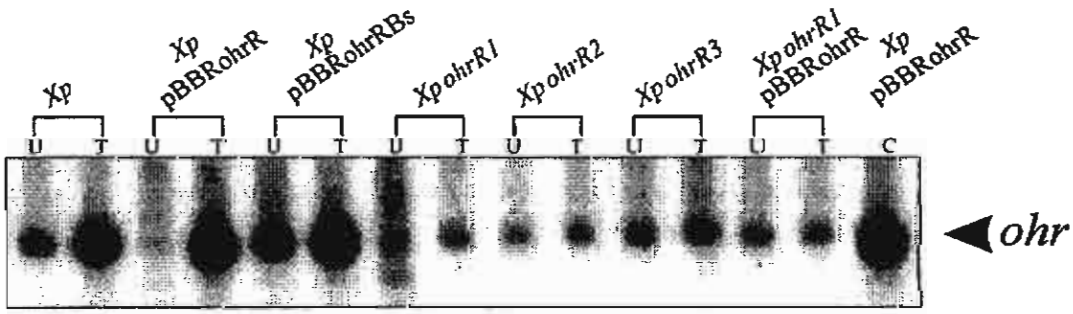

FIG. 4. Northern analysis of the effects of *ohrR* on *ohr* expression. Northern blotting of various *X. campestris* pv. phaseoli cells (*Xp* designations) was performed as described in Materials and Methods. The Northern blot was probed with radioactively labeled *ohr*. U, uninduced; T, tBOOH induced; C, CuOOH induced.

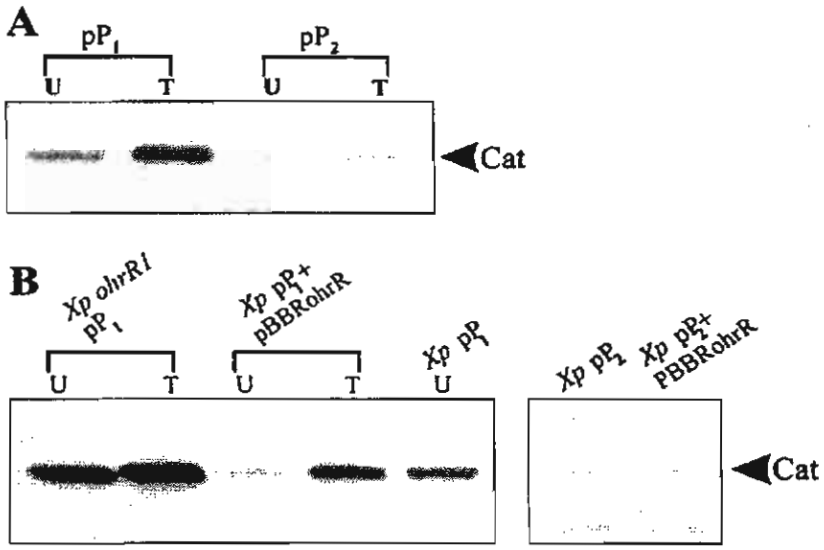

FIG. 5. In vivo *ohrR* and *ohr* promoter analysis. Cat levels were determined by Western immunoblotting performed as described in Materials and Methods. Forty micrograms of total protein was loaded in each lane. U and I, lysates prepared from uninduced and tBOOH-induced cultures, respectively. (A) Analysis of in vivo promoter activities of *ohrR* (pP1) and *ohr* (pP2). (B) Effects of OhrR on pP1 and pP2. Western analysis of Cat levels in various strains (*X. campestris* pv. phaseoli [*Xp*] and *ohrR* mutant [*Xp ohrR1*] harboring pP1 or pP2 and with or without pBBRohrR) is shown.

mRNA levels (Fig. 4), while *ohr* expression was fully induced by exposure to tBOOH or CuOOH in both nontransformed *X. campestris* pv. phaseoli and the strain harboring pBBRohrR (Fig. 4). In contrast, Northern analysis of *X. campestris* pv. phaseoli carrying pBBRohrRBs, an *ohrR* frameshift mutant of pBBR1MCS-5, showed no repression of *ohr* expression (Fig. 4). The data provide strong evidence for the role of OhrR as a negative regulator of *ohr* expression.

Expression from *ohrR* and *ohr* promoter fusions. Northern analysis of *ohrR* and *ohr* expression suggested that *ohrR* and *ohr* should have weak and strong inducible promoters, respectively. Thus, the promoter activities of both genes were examined in vivo. Plasmids pP1 and pP2, containing the *ohrR* and *ohr* promoters, respectively, in front of the reporter gene, *cat*, in a promoter probe vector were transformed into *X. campestris* pv. phaseoli. Western analysis of Cat levels in the pP1- and pP2-containing strains gave unexpected results: pP1 directed tBOOH-inducible high Cat levels, whereas pP2 directed constitutive low Cat levels (Fig. 5A). These results suggest that P1 is responsible for the tBOOH-inducible expression of both *ohrR* and *ohr*, whereas *ohr* has a weak promoter, conclusions that contradict those drawn from the Northern analysis.

OhrR is involved in autoregulation and tBOOH-induced expression from the *ohrR* promoter. The effects of OhrR on *ohr* expression (Fig. 4) and the results of in vivo analysis of *ohrR* and *ohr* promoter activities (Fig. 5A) raised several questions regarding *ohr* repression and derepression mechanisms. Accordingly, experiments were undertaken to determine the consequence of high-level expression of *ohrR* on the P1 and P2 promoters. *X. campestris* pv. phaseoli harboring pP1 or pP2 was transformed with pBBRohrR, and Cat levels in the transformants were determined. The results showed that uninduced Cat levels in cells harboring pP1 and pBBRohrR were severalfold lower than those in cells harboring pP1 alone (Fig. 5B). In contrast, the repression of *cat* expression by pBBRohrR was relieved by tBOOH treatment; similar Cat levels were detected in tBOOH-induced cultures of *X. campestris* pv. phaseoli cells harboring pP1 and pBBRohrR or the vector alone (Fig. 5A and B). As expected from these results, the frameshift mutation in *ohrR* (pBBRohrRBs) eliminated the repression of P1 (data not shown). pBBRohrR had no effect on pP2 (data not shown).

We extended these observations by examining the promoter activities specified by pP1 and pP2 in an *ohrR* mutant. Densitometer analysis of Cat levels specified by pP1 in an uninduced *ohrR* mutant were similar to tBOOH-induced levels in the parent strain harboring the plasmid. The Cat levels in the mutant were at least fourfold higher than the levels in the uninduced parent strain. Moreover, the expression of *cat* from the promoter was not inducible by tBOOH in the *ohrR* mutant (Fig. 5B). These findings were the first indication that *ohrR* was required for organic peroxide-induced expression of the *ohrR-ohr* system. P2 promoter activity was not affected by mutations in *ohrR* (data not shown).

Inducible expression of *ohr* might involve RNA processing of *ohrR-ohr* transcripts. Northern analysis identified a stable 0.5-kb *ohr* transcript that is presumably processed from a longer, 1-kb bicistronic *ohrR-ohr* transcript. Primer extension experiments were done to locate the 5′ end of abundant *ohr* mRNA and also to determine if tBOOH exposure influenced the amounts of primer extension products. Three primer extension products were recovered (Fig. 6). The amounts of these products increased 10-fold when RNA from tBOOH-induced cultures were used (Fig. 6). The locations of primer extension products are shown in Fig. 6. Analysis of nucleotide sequences in the region showed that a stem-loop secondary structure could form upstream of *ohr* with the 5′ ends of *ohr*

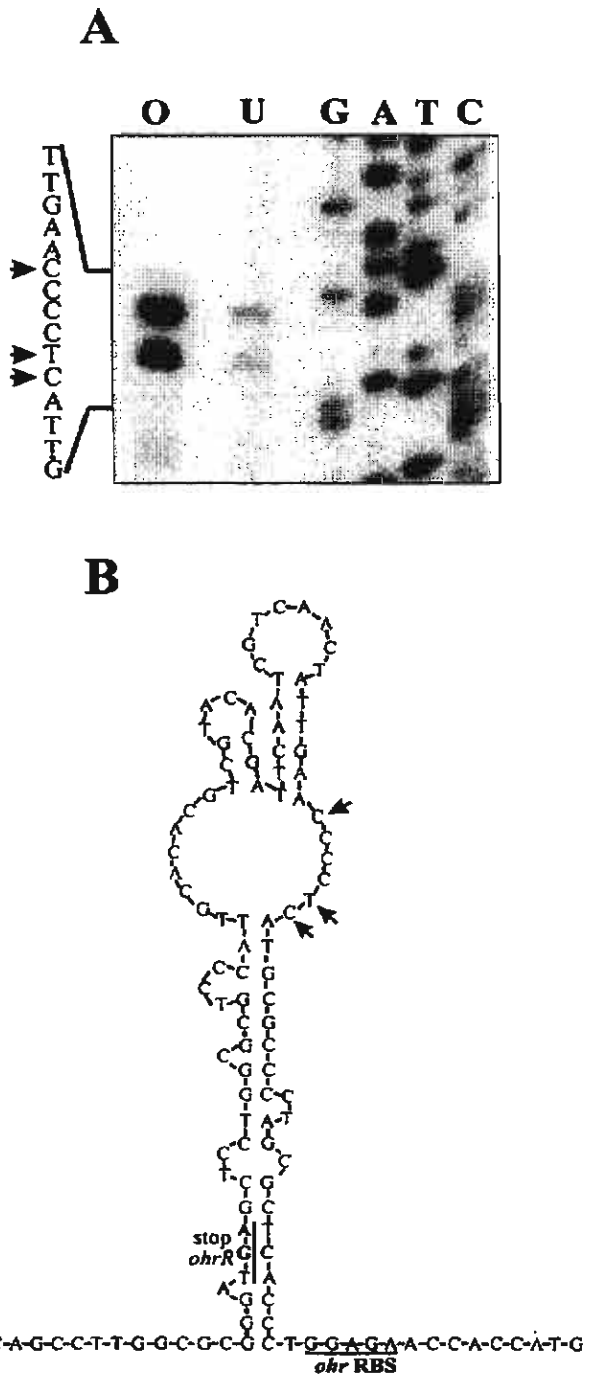

FIG. 6. Primer extension analysis of *ohr* mRNA and proposed processing sites of bicistronic *ohrR-ohr* mRNA.(A) Primer extension was performed with 10 μg of RNA isolated from uninduced (U) or tBOOH-induced (O) *X. campestris* pv. phaseoli cultures. G, A, T, and C are the sequence ladder. (B) Stem-loop secondary structure of the region around the putative RNA processing sites of the *ohrR-ohr* bicistronic mRNA. RBS, ribosome binding site. The arrows mark the locations of primer extension products in panel A and the locations of putative RNA processing sites in panel B.

mRNA located in the loop section. This structure could be involved in the processing of bicistronic *ohrR-ohr* mRNA (Fig. 6).

Mutations in *ohrR* are polar. In initial studies to determine the physiological roles of *ohrR* in organic peroxide resistance, insertional inactivation of the gene was generated using pUCohrR1 (Fig. 3A). In theory, inactivation of a putative negative regulator of *ohr* should result in a higher level of expression of *ohr* and thus a higher level of resistance to tBOOH. Accordingly, using the zone-of-growth-inhibition technique, the tBOOH and CuOOH resistance levels in *XpohrR1* mutant strain and the parent strain were determined and found to be 25 and 18 mm for tBOOH and 27 and 19 mm for CuOOH, respectively. The results indicate that the mutant was more susceptible to tBOOH and CuOOH. Two additional disruptants of *ohrR*, generated with pUCohrR2 and pUCohrR3, gave similar results (data not shown). Moreover, pBBRohrR was unable to complement the phenotype. No alterations in the levels of resistance to H_2O_2, a superoxide generator (menadione), were observed in *ohrR* mutants (data not shown). The tBOOH-sensitive phenotype and the inability to complement the *ohrR* mutation suggest that the integration of pUCohrR1, pUCohrR2, and pUCohrR3 is polar on *ohr*. Northern experiments were done to determine the effect of *ohrR* mutations on *ohr* expression. The results showed lower constitutive expression of *ohr* in all three mutants (Fig. 4), consistent with the idea that transcription of the gene normally initiated from P1 terminates in some parts of the integrated plasmids. The low constitutive *ohr* mRNA levels in the *ohrR* mutant strains (Fig. 5) may have been due to transcription initiation from a plasmid promoter. Low *ohr* expression levels in *ohrR* mutants accounted for the reduced tBOOH-resistant phenotype of the mutants.

DISCUSSION

Organic peroxide-inducible expression of *ohr* expression in an *oxyR* mutant demonstrated that the process is independent of the global peroxide sensor and regulator OxyR (Fig. 1). This finding was the first clear indication of the existence of an additional regulatory system(s) that responds to organic peroxides. Identification of *ohrR* upstream of *ohr* suggested that it might encode a putative negative transcriptional regulator. The phylogenetic analysis (Fig. 2) revealed that OhrR homologues comprise a group of highly conserved and widely distributed proteins found in both gram-positive and gram-negative bacteria. The gene order *ohrR-ohr* also shows a high degree of conservation. The analyses of transcriptional organization of *X. campestris* pv. phaseoli *ohrR-ohr* by Northern blotting and RT-PCR show that these genes are coregulated and have an atypical transcriptional organization. *ohrR* mRNA was found as a bicistronic message with *ohr*, while *ohr* mRNA was found in both bicistronic and monocistronic forms. The monocistronic form of *ohr* mRNA has been observed in diverse bacteria, such as *B. subtilis* (9, 31), *D. radiodurans*, and *P. aeruginosa* (4, 22). Determination of *ohrR* and *ohr* transcriptional organization is seen as crucial to an understanding of the complex regulation of the expression of both genes in *Xanthomonas*.

OhrR is a negative regulator of *ohr* expression. Identification of OhrR as a member of the *E. coli* MarR family of

negative regulators of gene expression suggested that OhrR probably functions in a fashion similar to that of other MarR family members. However, some members of the MarR family, such as BadR (8) and SlyA (6), have been shown to act as positive regulators of their target genes; one member of the family, MexR, can act as both a negative regulator and a positive regulator (23). Nonetheless, the majority of MarR family members are transcriptional repressors (2). The working assumption that OhrR is a negative regulator of *ohr* was supported by the finding that the high-level expression of *ohrR* resulted in the repression of *ohr* expression (Fig. 4). The loss of repression as a consequence of a frameshift mutation in *ohrR* further supported the role of OhrR as a negative regulator (Fig. 4). Similar observations have been made for other bacteria, where high-level expression of MarR family members results in the repression of their target genes (1, 2, 8, 18).

A unique feature of MarR family members is the aromatic ligands recognized by these proteins. Although these are structurally diverse, all of them contain at least an aromatic ring (1, 2). It is believed that these ligands bind to the negative regulators and inactivate them, hence allowing increased expression of the target genes (1, 2). We showed that the repression of *ohr* by OhrR can be relieved by exposing the cells to CuOOH and tBOOH, presumably by inactivation of OhrR by these ligands (Fig. 4). Hence, OhrR probably recognizes tBOOH, a nonaromatic compound, as a ligand. In *Xanthomonas*, tBOOH and CuOOH (an aromatic compound) induce *ohr* expression equally well (20). Alternatively, organic peroxides might directly oxidize OhrR, leading to inactivation of the protein. Experiments are in progress to purify OhrR to examine the effect of tBOOH binding on OhrR function.

***ohr* expression probably involves processing of a bicistronic transcript.** *ohr* primer extension experiments showed three major primer extension products corresponding to three 5′ ends of the mRNA. All three primer extension products showed 10-fold increases in expression when RNA samples from tBOOH-treated cultures were used as templates (Fig. 6). Accordingly, we searched the sequences upstream of the 5′ ends of *ohr* mRNA for a possible P2 promoter. Examination of the sequences upstream of the three major primer extension products identified the sequences TTGCAC and GATTCA, which show five of six matches to the *Xanthomonas* promoter consensus sequence at −35 and −10, respectively (12). However, these putative promoter sequences are separated by only 11 bp and so are unlikely to function as an efficient promoter in vivo. Analysis of *ohr* primer extension results failed to show a constitutive primer extension product, although analysis of the P2 promoter in vivo revealed weak constitutive activity. This could have been due to a very low expression level that even the primer extension technique was unable to detect for the transcription start site. Alternatively, the weak P2 activity could have been an artifact from the cloning of the P2 promoter fragment into the promoter probe vector.

An alternative explanation for the Northern blot and primer extension results is that *ohrR-ohr* is transcribed as a two-gene operon from the *ohrR* promoter (P1) as the bicistronic mRNA is processed. The *ohrR-ohr* intercistronic region (98 bp; Fig. 6B) is unusually long, suggesting that the region could be involved in the regulatory process. Examination of the sequence surrounding the 5′ ends of *ohr* reveals that the nucleotide sequence in this region has the potential to form a stem-loop secondary structure with the three sites defining the 5′ ends of *ohr* mRNA located in the loop (Fig. 6B). The potential secondary structure of the mRNA sequence at this point is similar to the RNase III processing site (3). RNase III recognizes stem-loop structures and usually cleaves the mRNA in the internal loop (3). In *E. coli*, RNase III processing has been shown to affect the rate of mRNA degradation and to increase or decrease the levels of gene expression (29). Thus, it is likely that the *ohrR-ohr* mRNA is processed by an RNase III-like enzyme(s). We propose that processing results in the production of the 0.5-kb *ohr* mRNA and the rapid degradation of *ohrR* mRNA (Fig. 3B). The inability to detect the monocistronic form of *ohrR* mRNA supports this idea and also suggests that the processed *ohrR* mRNA is less stable than *ohr* mRNA. This would reduce the level of translation of *ohrR* mRNA and hence reduce the production of OhrR. Furthermore, OhrR levels would be kept low by autoregulation of the *ohrR* promoter by OhrR. Thus, in uninduced cells OhrR would be maintained at low levels.

The characteristics of P1, namely, organic peroxide inducibility and strong activity, fit the observed effects of organic peroxides on *ohrR* and *ohr* expression. In addition, the lack of a strong inducible promoter in front of *ohr* favors the idea that P1 is responsible for the organic peroxide-inducible expression of both *ohrR* and *ohr* (Fig. 3B). This explanation can be extended to account for the polar effects of *ohrR* insertional mutations on *ohr* expression. The physical separation of the *ohrR* promoter from *ohr* by insertion of pUCohrR1, pUCohrR2, and pUCohrR3 into *ohrR* prevented the organic peroxide induction of *ohr*.

OhrR is required for tBOOH-induced expression from P1. A question arises as to whether OhrR is required for tBOOH induction of *ohrR-ohr*. *ohrR* promoter activity (P1) was constitutively high in an *ohrR* mutant (Fig. 5B), indicating that OhrR is not involved in the activation of operon expression. P1 could be repressed by OhrR (Fig. 5B), implying that OhrR is required to maintain the uninduced operon at low levels. This repression could be alleviated by exposure to tBOOH. These results strongly suggest that tBOOH-induced expression of the operon is due to derepression of P1. The derepression mechanism involving the inactivation of OhrR by tBOOH is likely to be the major step in organic peroxide-induced *ohr* expression. However, at present we cannot conclusively rule out that another, activating transcription factor also is involved in the induction process. The possibility is being investigated.

Model for *ohr* and *ohrR* tBOOH-inducible expression. Considering all the available data, we propose a model for *ohr* regulation by OhrR and induction of the genes by organic peroxides. *ohrR* and *ohr* are transcribed from the strong organic peroxide-inducible P1 promoter. Then, the bicistronic 1.0-kb *ohrR-ohr* mRNA is processed at sites upstream of the ribosome binding site for *ohr* by an RNase III-like enzyme to give a 0.5-kb *ohr* mRNA, while *ohrR* mRNA is rapidly degraded. In uninduced cells, a low level of OhrR keeps P1 repressed, resulting in low levels of both OhrR and Ohr. The expression of *ohrR* is autoregulatory. Upon exposure to organic peroxides, binding of the ligand (organic peroxides) to OhrR leads to inactivation of the protein and prevents it from binding to P1. This process derepresses the expression of the

operon and results in high-level expression of *ohrR-ohr*. The bicistronic *ohrR-ohr* mRNA is processed to give high levels of *ohr* mRNA and, in turn, high levels of Ohr and increased organic peroxide resistance. Concomitantly, the higher level of OhrR also produced is neutralized by the binding of the ligand to the protein. When organic peroxides have been removed, OhrR activity is restored and expression of the operon is once again repressed.

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MicroReview

Regulation of inducible peroxide stress responses

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Summary

Bacteria adapt to the presence of reactive oxygen species (ROS) by increasing the expression of detoxification enzymes and protein and DNA repair functions. These responses are co-ordinated by transcription factors that regulate target genes in response to ROS. We compare three classes of peroxide-sensing regulators: OxyR, PerR and OhrR. In all three cases, peroxides effect changes in the redox status of cysteine residues, but the molecular details are distinct. OxyR is converted into a transcriptional activator by the formation of a disulphide bond between two reactive cysteine residues. PerR is a metalloprotein that functions as a peroxide-sensitive repressor. Oxidation is modulated by metal ion composition and may also involve disulphide bond formation. OhrR represses an organic peroxide resistance protein and mediates derepression in response to organic peroxides. Peroxide sensing in this system requires a single conserved cysteine, which is oxidized to form a cysteine–sulphenic acid derivative.

Introduction

The evolution of oxygenic photosynthesis ≈ 2.4 billion years ago led to one of the greatest threats ever to have challenged the microbial world: the accumulation of ever increasing levels of oxygen gas in the atmosphere. Although this global change allowed the evolution of more efficient forms of respiration using oxygen as a terminal electron acceptor, it also created a strong selective pressure for enzymes that could protect cells against reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide. As a result, organisms either became restricted to niches free from oxygen (anaerobes) or acquired protective mechanisms including superoxide dismutase (SOD), catalase and alkyl hydroperoxide reductase (Ahp) (Storz and Zheng, 2000; Touati, 2000).

Most aerobes have multiple, often overlapping pathways for detoxifying ROS. These multiple enzymes may differ in their time of expression, subcellular localization or regulation. For example, many bacteria induce the expression of catalase or other protective enzymes during the transition from growth to stationary phase, presumably as an adaptation to protect the genome and other essential cellular components against oxidation during a possibly prolonged non-growing phase. In addition, protective enzymes may be specifically induced by exposure to low levels of oxidants (Storz and Imlay, 1999). In this review, we emphasize recent insights into the mechanisms of peroxide sensing for three families of proteins that activate inducible peroxide stress responses (Fig. 1). A common theme is the use of a highly redox-active cysteine residue to sense the presence of peroxides, but the molecular details of cysteine activation are likely to be distinct.

The OxyR family

Escherichia coli OxyR was the first peroxide-sensing transcription factor to be well characterized (Storz and Zheng, 2000; Zheng and Storz, 2000). The OxyR regulon includes genes involved in peroxide metabolism and protection (*katG*, *ahpC*, *ahpF*, *dps*), redox balance (*gor*, *grxA*, *trxC*) and important regulators such as *fur* and the small RNA *oxyS* (Zheng *et al.*, 2001a,b).

The molecular basis for the redox regulation of OxyR has been elucidated by an elegant combination of structural, biochemical, genetic and physiological studies. Early experiments indicated that OxyR senses oxidants directly: reduced OxyR binds to two adjacent major grooves separated by one helical turn, while in its oxidized form, it binds four adjacent major groove regions and activates transcription by direct contact with RNA polymerase. The initial reaction of OxyR with H_2O_2 is postulated to occur at Cys-199, leading to the formation of an unstable Cys–sulphenic acid (Cys–SOH) intermediate (Zheng *et al.*, 1998). The high reactivity of Cys-199 with peroxides is probably caused by the ionization of this residue to the thiolate anion, which is stabilized by proximity to a conserved arginine residue (Arg-266; Fig. 1A)

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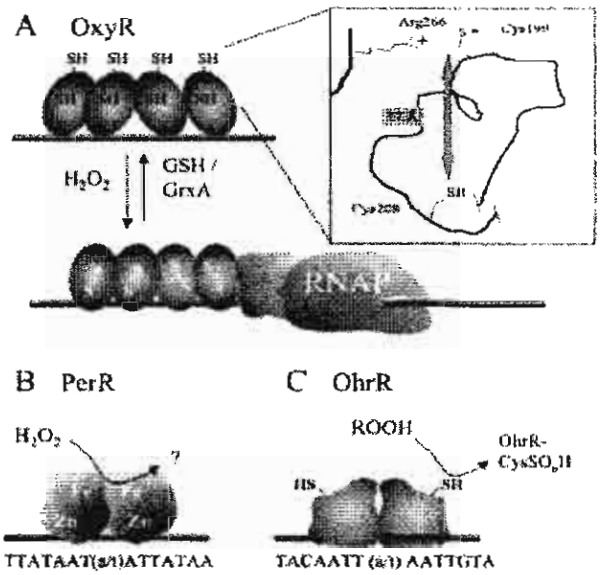

Fig. 1. Three classes of bacterial peroxide sensors.
A. OxyR is a tetrameric DNA-binding protein that can be reversibly oxidized to form a disulphide linking Cys-199 and Cys-208 (*E. coli* numbering). The three-dimensional structure of the OxyR regulatory domain in its reduced state (shown schematically in the inset; dark lines represent portions of the protein backbone) led to the remarkable finding that these two Cys residues are separated by ≈ 17 Å (Choi *et al.*, 2001). Cys-199 is postulated to be activated for reaction with H_2O_2 by its proximity to the positively charged side-chain of Arg-266. Oxidation leads to a refolding of the regulatory domain, and the consequent structural changes alter the interactions of OxyR with DNA, and therefore with RNA polymerase (RNAP).
B. PerR is shown in its active (DNA-binding) form, containing one zinc and one iron atom per monomer (Herbig and Helmann, 2001), bound to a consensus *per* box as found in *B. subtilis* and *S. aureus* (Chen *et al.*, 1995; Horsburgh *et al.*, 2001; Herbig and Helmann, 2002). Upon reaction, the protein is oxidized, leading to derepression of target genes. Oxidation apparently affects one or more Cys residues, postulated to include residues functioning as metal ligands, but the molecular details are not yet clear.
C. The OhrR protein is shown bound to its target site as defined for *B. subtilis* (Fuangthong *et al.*, 2001). Oxidation of OhrR upon exposure to organic peroxides leads to oxidation of the single cysteine residue (Cys-15), leading to the formation of a Cys–SOH (and Cys–SO_2H) derivative and a concomitant loss of DNA-binding activity (Fuangthong and Helmann, 2002).

(Choi *et al.*, 2001). Once oxidized, Cys-199 reacts with Cys-208 to form an intramolecular disulphide bond. As these two cysteine residues are separated by ≈ 17 Å in the reduced state, oxidation is necessarily accompanied by a refolding of a central domain in the OxyR protomer (Choi *et al.*, 2001).

The activation of OxyR by H_2O_2 is both sensitive and transient. Physiological studies indicate that aerobically growing *E. coli* maintain cytoplasmic H_2O_2 concentrations near 20 nM, primarily because of the peroxidase activity of Ahp (Costa Seaver and Imlay, 2001a). Indeed, the OxyR regulon is activated by as little as 100 nM intracellular H_2O_2, and growth is inhibited when levels reach 2 μM (Gonzalez-Flecha and Demple, 1997; Costa Seaver and Imlay, 2001b). As H_2O_2 decomposition is faster than diffusion across the cytoplasmic membrane, the addition of 10 μM external H_2O_2 is needed to achieve growth-inhibiting levels inside the cell (Costa Seaver and Imlay, 2001b). If growing cells are pulsed with 100 μM H_2O_2, OxyR is rapidly oxidized (<30 s) and then re-reduced with a half-time of 5 min (Aslund *et al.*, 1999). This transient activation reflects the direct oxidation of OxyR followed by reduction by glutaredoxin 1 (GrxA) and glutathione (GSH) (Zheng *et al.*, 1998). As a result, peroxide activation of OxyR in *grxA* and *gor* (glutathione reductase) mutants is prolonged. *In vivo* experiments from various bacteria show that high concentrations of superoxide generators (e.g. >0.3 mM paraquat) and S-nitrosothiols also induce the expression of OxyR-regulated genes (Mongkolsuk *et al.*, 1997; Storz and Zheng, 2000; Zheng *et al.*, 2001b). However, it is not clear whether these compounds oxidize OxyR directly or are acting indirectly by, for example, altering cellular thiol/disulphide homeostasis.

Although OxyR is identified primarily as an H_2O_2 sensor, it can also respond to disulphide stress resulting from defects in the systems that function to maintain an intracellular reducing environment (Aslund *et al.*, 1999). In wild-type cells, the redox potential of OxyR (–185 mV) is higher than that of the *E. coli* cytoplasm (–280 mV), and OxyR is reduced. However, mutant strains with alterations in both the glutathione- and thioredoxin-dependent thiol reduction systems exhibit a partial activation of OxyR even in otherwise unstressed conditions. This activation is correlated with changes in cellular redox status, as monitored by the ratio between reduced and oxidized glutathione.

These results have led to an appreciation of the distinction between peroxide stress and disulphide stress (Aslund *et al.*, 1999). Disulphide stress can be specifically elicited in cells by treatment with the thiol-specific oxidant, diamide, whereas peroxide stress has more far-reaching effects on cellular metabolism. Further evidence for the distinction between peroxide stress and disulphide stress has emerged from analysis of the *Streptomyces coelicolor* σ^R regulon (Paget *et al.*, 2001a). Induction of the σ^R regulon with diamide activates transcription of thioredoxin and thioredoxin reductase, thereby acting to restore oxidized thiols in the cell to their appropriate reduced state (Paget *et al.*, 1998). The activity of σ^R is regulated by RsrA, the cognate anti-σ factor (Kang *et al.*, 1999), which is a zinc metalloprotein that binds σ^R to form an inactive complex. Oxidation of RsrA leads to the formation of disulphide bonds and the concomitant release of Zn(II). Thus, it is likely that the role of RsrA as a sensor of oxidative stress involves one or more reactive cysteine residues co-ordinated to Zn(II) (Paget *et al.*, 2001b).

Regulators such as *oxyR* are widely distributed in most Gram-negative (both aerobes and anaerobes) and some Gram-positive bacteria. The C199 and C208 residues involved in H_2O_2 sensing are absolutely conserved, suggesting a common redox-sensing mechanism (Storz and Zheng, 2000). However, not all aspects of OxyR regulation are similar to *E. coli*: in *Xanthomonas campestris*, exposure to oxidant results in changes in both the level and activity of OxyR (Mongkolsuk *et al.*, 1997).

The PerR family

The ferric uptake repressor (Fur) superfamily of metalloregulatory proteins includes several small, dimeric, DNA-binding proteins that respond to metal ions including Fur itself (iron sensor), Zur (zinc sensor) and PerR (peroxide sensor) (Bsat *et al.*, 1998; Gaballa and Helmann, 1998; Escolar *et al.*, 1999). PerR was identified as the major regulator of the inducible peroxide stress response in *Bacillus subtilis* and is the prototype for a group of related peroxide-sensing repressors found in both Gram-positive and Gram-negative bacteria (Herbig and Helmann, 2002).

The *B. subtilis perR* gene was discovered as the repressor of the metalloregulated gene *mrgA*, which is strongly repressed by several different metal ions including manganese and iron (Chen *et al.*, 1995; Bsat *et al.*, 1998). The link between *mrgA* and the peroxide stimulon emerged from two findings: *mrgA* encodes a homologue of the *E. coli* peroxide-inducible DNA-binding protein, Dps (Almirón *et al.*, 1992), and MrgA is a major peroxide-inducible protein identified by amino-terminal sequencing (Hartford and Dowds, 1994). The PerR regulon, defined using both genome-scale searches for PerR binding sites (Per boxes) and transcriptional profiling, includes *mrgA*, the major vegetative catalase (*katA*), alkyl hydroperoxide reductase (*ahpCF*), the haem biosynthesis operon (*hemAXCDBL*), *fur*, *perR* and a zinc uptake system (*zosA*) (Fuangthong *et al.*, 2002; Herbig and Helmann, 2002; Gaballa and Helmann, 2002).

Like other Fur family members, PerR contains two metal binding sites per monomer (Herbig and Helmann, 2001): one site binds Zn(II) and may play a largely structural role, whereas the second site binds a regulatory metal (Fig. 1B). *In vivo*, either Mn(II) or Fe(II) function as co-repressors for the regulation of *mrgA* and *katA* (Chen *et al.*, 1995). However, the metal ion content of the growth medium has striking effects on peroxide induction: in Mn(II)-supplemented medium, these genes are tightly repressed, and peroxide induction is inefficient. In contrast, in medium with added iron, peroxide induction of PerR regulon genes leads to levels comparable with those in *perR* null mutant strains (Herbig and Helmann, 2001; Fuangthong *et al.*, 2002). As purified after over-expression in *E. coli*, PerR contains both zinc and iron and can be readily dissociated from DNA by low levels of H_2O_2. The addition of Mn(II), which binds competitively with Fe(II), yields a form of the protein that is not readily dissociated from DNA by peroxide (Herbig and Helmann, 2001). Together, these results suggest that PerR in the ferrous form may mediate most peroxide induction. Ongoing studies are continuing to investigate the correlation between the various metallated forms of PerR and peroxide responsiveness.

Several different mechanisms could account for peroxide sensing by PerR-type regulators. One class of mechanism posits that H_2O_2 reacts directly with the regulatory metal ion (e.g. Fe(II)), thereby leading to metal dissociation and concomitant derepression. In this case, the localized production of hydroxyl radical (generated via reaction of peroxide with bound ferrous ion) might also oxidize one or more amino acids near the metal binding site. This model would not necessarily require the formation of a disulphide bond. A second class of mechanism can be envisaged, in which H_2O_2 reacts with one or more cysteine residues, leading to disulphide bond formation as observed for OxyR. In general, cysteine thiolates co-ordinated to metal ions are attractive candidates for a redox centre. In this mechanism, redox activity of the metal centre is not necessarily involved in peroxide sensing. This type of mechanism has been documented for the redox regulation of the zinc-binding chaperone, Hsp33 (Graumann *et al.*, 2001).

Recent results lead us to favour a mechanism for *B. subtilis* PerR in which a reactive cysteine residue that is co-ordinated to the regulatory metal ion reacts with H_2O_2 leading to disulphide bond formation and concomitant loss of metal ion. Purified PerR binds Per boxes *in vitro*, and binding is sensitive to peroxides, but restored by thiol reductants (Herbig and Helmann, 2001). All described PerR homologues have a highly conserved CxxC motif near their carboxy-terminus, and these cysteine residues are critical for repressor function: mutations that change Cys to Ser abolish repressor function. However, PerR mutants that retain the ability of the amino acid to serve as a metal ligand (e.g. C139H or C139D) retain repressor function, but display little if any response to peroxide addition (M. Fuangthong, S. Soonsanga and J. D. Helmann, unpublished studies). Reaction of PerR with H_2O_2 probably leads to disulphide bond formation, as judged by chemical modification experiments and the ability of oxidized PerR to be reactivated by thiol reductants. The use of a metal-co-ordinated cysteine residue for peroxide sensing is consistent with the finding that the identity of the regulatory metal strongly affects peroxide responsiveness, but the molecular basis for this relationship is not yet resolved (Herbig and Helmann, 2001; Fuangthong *et al.*, 2002).

PerR and PerR-like regulators have now been described in a wide variety of organisms including *Staphylococcus aureus*, *Streptococcus pyogenes* and the Gram-negative pathogen *Campylobacter jejuni* (van Vliet *et al.*, 1999; King *et al.*, 2000; Horsburgh *et al.*, 2001). The *S. aureus* PerR regulon is very similar to that in *B. subtilis*: PerR mediates repression of catalase, alkyl hydroperoxide reductase, PerR, Fur and thioredoxin in response to Mn(II). In this organism, iron does not appear to be a co-repressor for the PerR regulon (Horsburgh *et al.*, 2001). Interestingly, *fur* is not peroxide inducible in either *S. aureus* or *B. subtilis*, indicating that not all members of the PerR regulon are members of the peroxide stimulon (Horsburgh *et al.*, 2001; Fuangthong *et al.*, 2002). In *S. pyogenes*, a catalase-negative bacterium, PerR co-ordinates an adaptive response to peroxide stress, but the targets of PerR regulation have not yet been identified (King *et al.*, 2000). Like *B. subtilis*, *C. jejuni perR* was identified during analysis of proteins induced by iron starvation (van Vliet *et al.*, 1998; 1999). Two classes of proteins were defined: those regulated by Fur and those regulated by PerR (e.g. AhpCF, KatA). Thus, the *C. jejuni* PerR apparently recognizes Fe(II) as a co-repressor. In *S. coelicolor*, a PerR-like regulator called CatR mediates the peroxide induction of CatA, one of three catalases in this organism (Hahn *et al.*, 2000a). Regulation of *catA* by metal ions has not been observed, however. In this case, it was postulated that peroxide sensing may involve the cysteine ligands to an associated Zn(II) ion.

There are several other examples of Fur-like regulators associated with peroxide stress genes. In *Mycobacterium* spp., the *furA* gene is upstream of, and perhaps co-transcribed with, *katG* and expression is induced by H_2O_2 (Milano *et al.*, 2001; Pym *et al.*, 2001; Zahrt *et al.*, 2001). Expression of *katG* is derepressed in a *furA* mutant, consistent with a role for FurA as a repressor of catalase–peroxidase expression (Pym *et al.*, 2001). In *S. coelicolor*, the *catC* catalase–peroxidase is co-transcribed with *furA*, which serves as a metal-sensing repressor for the *furA–catC* operon, but this operon does not appear to be inducible by H_2O_2 (Hahn *et al.*, 2000b). Finally, in *Desulfovibrio vulgaris*, a peroxide stress operon encodes a *fur* homologue together with rubrerythin, which functions in oxidative stress resistance (Lumppio *et al.*, 1997; 2001). It is likely that this operon is also regulated by this Fur homologue, but the details are not yet elucidated. It seems clear that PerR-like regulators are associated with peroxide stress genes in many bacteria and control gene expression in response to metal ions, H_2O_2 or both. The complexity of this situation is evident from the observation that the metal selectivity of repression and the ability of target genes to be induced by peroxide differ markedly among various PerR regulons, and even among the different members of the *B. subtilis* PerR regulon (Fuangthong *et al.*, 2002).

It is interesting to ponder the evolutionary origins of PerR. We propose that this family of regulators has evolved from an ancestral Fur-like regulator by selection for sensitivity to physiological levels of peroxides. These regulators still retain the need for metal ion cofactors, presumably to enhance the reactivity of one or more associated cysteine residues. In contrast, Fur functions as an Fe(II)-specific sensor and has presumably evolved to be more selective towards metal ions and less reactive to H_2O_2. Nevertheless, Fur may still retain some sensitivity to ROS: in *S. enterica* var. *typhimurium*, flavohaemoglobin is induced by nitric oxide in a Fur-dependent manner (Crawford and Goldberg, 1998). In addition, the *E. coli fhu* operon, encoding iron uptake functions, has been found to be dually regulated by both OxyR and Fur. In this case, activated OxyR mediates repression of this operon in response to oxidative stress. Remarkably, in an *oxyR* mutant, this operon is induced by high concentrations of H_2O_2 (1 mM), perhaps as a result of inactivation of bound Fur protein (Zheng *et al.*, 2001a). In summary, the Fur- and PerR-like regulators may form a continuum of regulators that vary from peroxide-sensing repressors that require a metal ion for DNA binding to metal-sensing repressors that may also display some sensitivity to ROS.

The OhrR family

The *ohr* (organic hydroperoxide resistance) gene was identified in *X. campestris* by virtue of its ability to restore organic peroxide resistance to an *E. coli ahpC* mutant (Mongkolsuk *et al.*, 1998). This *ohr* family of antioxidant proteins is found in a wide variety of bacteria, but their molecular mechanism of action is poorly characterized (Atichartpongkul *et al.*, 2001). Expression of *X. campestris ohr* is strongly and selectively induced by organic peroxides, and this regulation is independent of OxyR and instead requires a novel member of the MarR family of repressor proteins, OhrR (Fig. 1C) (Sukchawalit *et al.*, 2001).

Bacillus subtilis contains two linked *ohr* homologues: *ohrA* and *ohrB*. Expression of *ohrB* is controlled by σ^B, the general stress response σ factor. Like other general stress response genes, *ohrB* is induced by heat shock, entry into stationary phase and various oxidants and organic alcohols (Volker *et al.*, 1998). In contrast, *ohrA* is selectively induced by organic peroxides (Fuangthong *et al.*, 2001). Regulation of *ohrA* is mediated by OhrR, encoded by a convergent gene. OhrR binds to a pair of inverted repeat sequences overlapping the *ohrA* promoter site (Fig. 1C) and thereby blocks transcription initiation (Fuangthong *et al.*, 2001; Fuangthong and Helmann, 2002).

Insights into the mechanism of peroxide sensing by OhrR have emerged from recent genetic and biochemical studies (Fuangthong and Helmann, 2002). OhrR and related proteins in other bacteria have a single, conserved cysteine residue. Mutant strains expressing OhrR proteins with either a Cys-15Ser or a Cys-15Ala substitution are unable to induce *ohrA* expression in response to organic peroxides. *In vitro*, purified OhrR protein binds tightly to its cognate operator site, and binding is abolished by treatment with peroxides but can be restored by the thiol reductant, dithiothreitol. These results suggest that peroxide sensing requires oxidation of Cys-15 (Fuangthong and Helmann, 2002).

In contrast to the systems described above, *in vitro* oxidation of OhrR does not lead to disulphide bond formation and, instead, leads to the formation of a Cys–SOH (sulphenic acid) derivative. Biochemical experiments using both chemical modification and mass spectrometry have demonstrated that oxidized OhrR contains a Cys–SOH (and further oxidized) residue *in vitro*, and the formation of these derivatives is correlated with loss of DNA-binding activity. It remains to be determined whether or not a similar mechanism occurs *in vivo*: oxidized Cys residues are quite reactive and, *in vivo*, the subsequent formation of mixed disulphides, perhaps with free cysteine or other low-molecular-weight thiols, may be favoured. The formation of Cys–sulphenic acids has also been proposed as a mechanism for peroxide sensing for eukaryotic transcription factors (Claiborne *et al.*, 1999).

Interestingly, the *ohr* system is conserved in a variety of Gram-negative and some Gram-positive genomes. In addition to *X. campestris* and *B. subtilis*, *ohr* homologues been identified in *Pseudomonas aeruginosa* (Ochsner *et al.*, 2001), *Enterococcus faecalis* (Rince *et al.*, 2001) and *Actinobacillus pleuropneumoniae* (Shea and Mulks, 2002). Although the details of regulation in these systems have not yet been reported, the frequent linkage to an *ohrR* homologue suggests that this peroxide-sensing repressor is widespread (Sukchawalit *et al.*, 2001).

Concluding remarks

The need to co-ordinate gene expression effectively with changing environmental conditions has led to the evolution of remarkable 'biosensors' that function as both chemical sensors and transcription factors. The three families of protein reviewed here, OxyR, PerR and OhrR, all function *in vivo* to sense peroxides, and all three systems use redox-reactive cysteine residues. This probably reflects the ease with which proteins can modulate the redox activity of cysteine by alterations in pKa. In OxyR, the cysteine residue thought to be the initial target of oxidation is activated by proximity to a conserved arginine residue, which probably increases the proportion of reactive thiolate anion. In PerR, we propose that co-ordination of a reactive cysteine to a bound regulatory metal plays a similar role. Finally, OhrR has a single cysteine residue required for peroxide sensing via the formation of a Cys–sulphenic acid derivative. We speculate that it may also be activated by proximity to positively charged amino acids, but no structure is yet available to clarify this issue. Remarkably, all three classes of peroxide-sensing regulatory protein are broadly distributed in the Bacteria with representatives in both Gram-positive and Gram-negative lineages. Intriguingly, in *S. coelicolor*, members of all three classes of peroxide-sensing proteins are present. The relationships between the multiple peroxide stress and metal homeostasis regulons in bacteria present a challenging physiological puzzle for future investigation.

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Note added in proof

Recent results from Kim *et al.* (*Cell* **109:** 383–396) challenge the notion that disulphide bond formation is required for redox regulation by OxyR. These authors propose that modification of OxyR to the Cys-199 sulphenic acid derivative is sufficient to account for the observed *in vivo* regulation by peroxides. Moreover, they suggest that various modified forms of OxyR (S-OH, S-NO, and S-glutathionylation) may have distinct biological roles. The role of cysteine oxidation in the regulation of OhrR activity has been recently documented in *Xanthomonas campestris* (Panmanee *et al.*, *Mol Microbiol*, in press).

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The repressor for an organic peroxide-inducible operon is uniquely regulated at multiple levels

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Summary

ohrR **encodes a novel organic peroxide-inducible transcription repressor, and we have demonstrated that *ohrR* is regulated at the transcriptional and the post-transcriptional levels. Primer extension results show that *ohrR* transcription initiates at the A residue of the ATG translation initiation codon for the *ohrR* coding sequence. Thus, the gene has a leaderless mRNA. The *ohrR* promoter (P1) has high homology to the consensus sequence for *Xanthomonas* promoters, which is reflected in the high *in vivo* promoter activity of P1. Deletion of a 139 bp fragment containing the P1 promoter showed that the sequences upstream of −35 regions were required for neither the promoter activity nor OhrR autoregulation. *In vitro*, purified OhrR specifically binds to the P1 promoter. DNase I footprinting of OhrR binding to the P1 revealed a 44 bp region of protection on both DNA strands. The protected regions include the −35 and −10 regions of P1. We suggest that OhrR represses gene expression by blocking RNA polymerase binding to the promoter. There are two steps in the post-transcriptional regulation of *ohrR*, namely differential stability and inefficient translation of the mRNA. The bicistronic *ohrR–ohr* mRNA was highly labile and underwent rapid processing *in vivo* to give only stable monocistronic *ohr* mRNA and undetectable *ohrR* mRNA. Furthermore, the *ohrR* mRNA was inefficiently translated. We propose that, in uninduced cells, the concentration of OhrR is maintained at low levels by the autoregulation mechanism at the transcriptional levels and by the *ohrR* mRNA instability coupled with inefficient translation at the post-transcriptional level. Upon exposure to an organic peroxide, the compound probably interacts with OhrR and prevents it from repressing the P1 promoter, thus allowing high-level expression of the *ohrR–ohr* operon. The rapid processing of bicistronic mRNA gives highly stable *ohr* mRNA and corresponding high levels of Ohr, which remove an organic peroxide. Once the peroxide has been removed, the autoregulation mechanism feeds back to inhibit the expression of the operon.**

Introduction

Xanthomonas is a soil bacterium that causes diseases in plants. In the environment, *Xanthomonas* is exposed to reactive oxygen species (ROS) from a variety of sources (Baker and Orlandi, 1995; Gonzalez-Flecha and Demple, 1997). These ROS (e.g. superoxide, H_2O_2 and organic peroxide) are highly toxic to biological systems (Halliwell and Gutteridge, 1984). Bacteria have evolved multiple protective pathways to ensure their detoxification. Bacterial defence against organic peroxide is a complex process involving several structural and regulatory genes (Storz and Imlay, 1999).

Alkyl hydroperoxide reductase is the best characterized organic peroxide detoxification enzyme in bacteria (Poole, 1996; Poole and Ellis, 1996). *ahpC* encodes the catalytic subunit of the enzyme and is regulated by OxyR, the global regulator of peroxide stress response (Storz and Imlay, 1999). *ahpC* expression is induced by exposure of bacteria to various oxidants (Bsat *et al.*, 1997; Loprasert *et al.*, 1997; Rocha and Smith, 1999; Storz and Imlay, 1999). Inactivation of *ahpC* results in pleiotropic changes in oxidative stress response, suggesting that it involves processes other than organic peroxide detoxification (Bsat *et al.*, 1996; Rocha and Smith, 1999; Mongkolsuk *et al.*, 2000; Seaver and Imlay, 2001).

We discovered a novel family of genes in *Xanthomonas* designated *ohr* that are involved in organic peroxide resistance (Mongkolsuk *et al.*, 1998). *ohr* homologues have subsequently been found in both Gram-positive and Gram-negative bacteria (Atichartpongkul *et al.*, 2001; Fuangthong *et al.*, 2001; Ochsner *et al.*, 2001; Rince

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et al., 2001), and inactivation of the homologues in these bacteria leads to reduced resistance to organic peroxide but not to other oxidants (Mongkolsuk *et al.*, 1998; Atichartpongkul *et al.*, 2001; Fuangthong *et al.*, 2001; Ochsner *et al.*, 2001). *ohr* has an unique expression pattern; its expression is induced only by exposure of bacteria to organic peroxide, a feature highly conserved among diverse bacteria. Moreover, *ohr* expression is independent of OxyR (Sukchawalit *et al.*, 2001). Recently, we identified a regulatory gene *ohrR* that belongs to a family of organic peroxide-sensing transcription repressor genes in an operon with *ohr* (Fig. 1A; Fuangthong *et al.*, 2001; Sukchawalit *et al.*, 2001). In both Gram-positive and Gram-negative bacteria, organic peroxide-induced expression of *ohr* depends on a functional *ohrR* (Fuangthong *et al.*, 2001; Sukchawalit *et al.*, 2001). However, regulation of *ohr* by *ohrR* in *Xanthomonas* is complex. Current evidence suggests that induction of *ohr* expression by organic peroxide requires a functional OhrR, a strong upstream *ohrR* promoter (P1) and an RNA processing step (Sukchawalit *et al.*, 2001). Here, we have characterized the *ohrR* promoter (P1) and show that the repressor, OhrR, interacts specifically with its promoter to autoregulate itself. Post-transcriptional control of *ohrR* was also investigated and found to occur at the level of differential mRNA stability and inefficient translation of the mRNA. These mechanisms maintain a low intracellular concentration of OhrR.

Results and discussion

Primer extension and identification of the P1 promoter

We have identified a DNA fragment upstream of *ohrR* containing a promoter that is responsible for regulated *ohrR* and *ohr* expression (Sukchawalit *et al.*, 2001). Primer extension was performed on RNA samples from uninduced and *tert*-butyl hydroperoxide (tBOOH)-induced samples to locate the *ohrR* transcription start site. The results show that the major primer extension products corresponded to the transcription start site located at the A residue of the translation initiation codon, ATG of *ohrR* (Fig. 2). Examination of *ohrR* sequence shows that the gene has no ribosome binding site (RBS) upstream of the translation initiation codon. Thus, transcription and translation of the gene initiate from the same site, resulting in a leaderless mRNA.

Sequence analysis of the region upstream of the *ohrR* transcription initiation site revealed sequence motifs with high homology to the consensus promoter sequence for

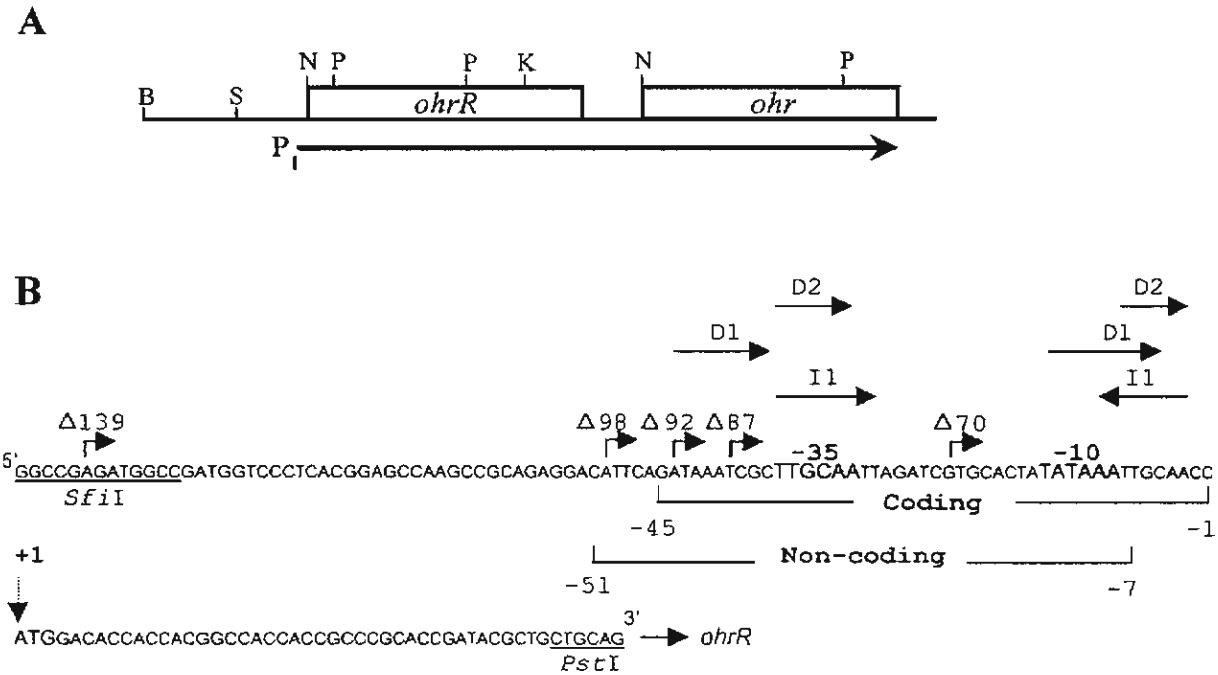

Fig. 1. *ohrR–ohr* operon and summary of various important transcriptional regulatory elements in P1.
A. *ohrR–ohr* operon. P1 and arrowhead indicate promoter and direction of transcription. B, *Bam*HI; K, *Kpn*I; N, *Nco*I; P, *Pst*I; and S, *Sfi*I.
B. The locations of various deletions from the 5′ of P1, the direct repeats D1 and D2, an inverted repeat I1, the –35 and –10 regions of the promoter are shown. The DNase I-protected regions resulting from OhrR binding to P1 of coding and non-coding strands are marked by brackets, and numbers at each end of the brackets indicate the location of the beginning and end of the protected region with respect to the transcription start site (+1). Bold ATG is the *ohrR* translation initiation codon.

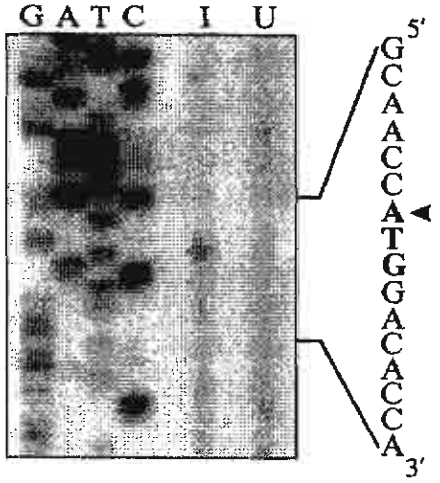

Fig. 2. Primer extension analysis of *ohrR*. Primer extension was done on RNA samples isolated from uninduced (U) and tBOOH (I)-induced cultures. The sequence ladder (G, A, T, C) was done according to *Experimental procedures*. The location of the *ohrR* transcription start site is shown by the arrow, and the bold ATG indicates the translation initiation codon of *ohrR*.

Xanthomonas (Katzen *et al.*, 1996). The sequences TTGCAA and TATAAA have five out of six and six out of six matches to the TTGTNN and G/TATNAA sequences for the –35 and –10 regions of the consensus promoter sequence for *Xanthomonas* respectively. The –35 and –10 regions of *ohrR* promoter are separated by 15 bp (Fig. 1B). Thus, the *ohrR* promoter should have a high promoter activity, an assumption supported by the *in vivo* analysis of P1 promoter activity (Fig. 3; Sukchawalit *et al.*, 2001). No other transcription start sites or sequences resembling the consensus sequence for a *Xanthomonas* promoter were found in the 139 bp P1 fragment. The *ohrR* promoter is designated P1.

Analysis of primer extension products performed on RNA samples isolated from uninduced and tBOOH-induced *Xanthomonas campestris* pv. *phaseoli* cultures show that the tBOOH treatment clearly increased *ohrR* transcription severalfold over the uninduced levels (Fig. 2). This suggests that tBOOH-inducible expression of *ohrR* and *ohr*, detected by Northern and Western experiments (Mongkolsuk *et al.*, 1998; Sukchawalit *et al.*, 2001), results primarily from increased transcription initiation from P1. These findings also suggest that OhrR represses gene expression by preventing RNA polymerase binding to the promoter.

In vivo deletion analysis and autoregulation of P1 by OhrR

We performed sequential deletions from the 5′ end of the 139 bp fragment containing P1 to identify regions important for the promoter activity. The locations of various deletions are summarized in Fig. 1B. The deleted fragments were cloned upstream of a promoterless chloramphenicol acetyltransferase gene (*cat*) in pUFRcat2-Km, a low-copy-number, broad-host-range promoter probe vector (Mongkolsuk *et al.*, 1993), and transformed into *X. campestris* pv. *phaseoli*. Western analysis of Cat levels showed that deletions upstream of the proposed –35 region had no effect on P1 promoter activity, whereas a deletion that removed the –35 region abolished the promoter activity (Fig. 3A). The data are therefore consistent with our proposed locations for the –35 and –10 regions of P1.

Next, the OhrR autoregulation of P1 was investigated. pBBRohrR (*ohrR* in an expression vector; Sukchawalit *et al.*, 2001) was transformed into cells harbouring various deletions of P1 fragments in the promoter probe vector, and Cat levels were determined by Western analysis. High-level expression of *ohrR* from an expression vector strongly repressed the P1 promoter activity (Fig. 3B). This confirms the role of OhrR as a negative autoregulator of P1. Furthermore, the Western results show that, in all strains harbouring plasmids with deletions upstream of the –35 region of P1, OhrR could strongly repress the promoter activity (Fig. 3B). Removal of half the direct repeat sequence ATAAATCGC (deletion no. 87) had no effect on OhrR repression of P1.

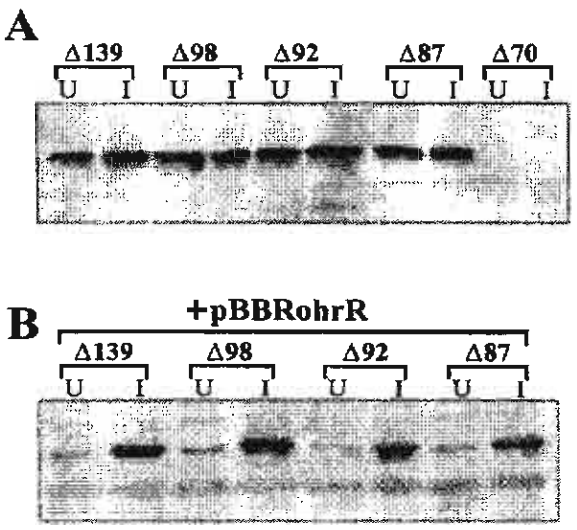

Fig. 3. Deletion analysis of a 139 bp DNA fragment containing P1 and the OhrR operator. Western analysis of Cat specified by pP1-cat and various deleted P1 fragments cloned in the pUFRcat2-Km promoter probe vector.
A. *X. campestris* pv. *phaseoli* harbouring various P1 deleted plasmids.
B. *X. campestris* pv. *phaseoli* harbouring pBBRohrR and various P1 deleted plasmids. The locations of various deletions are shown in Fig. 1B. Total protein (10 μg) was loaded into each lane. U, uninduced; I, tBOOH induced.

The autoregulation mechanism is a common feature among genes coding for transcriptional repressors including several *marR* family members (Martin *et al.*, 1995; Poole *et al.*, 1996; Xiong *et al.*, 2000). The mechanism allows fine tuning of the repressor concentration and prevents excessive synthesis of the repressor. Nonetheless, *ohrR* autoregulation is not highly conserved in other bacteria. In *Bacillus subtilis*, OhrR regulates *ohrA* (an *ohr* homologue) but not itself; *B. subtilis ohrR* appears to be regulated by sigma factor A (SigA) (Fuangthong *et al.*, 2001). This illustrates a different strategy that diverse bacteria can use to regulate an important transcriptional modulator. It remains to be seen how *ohrR* is regulated in other bacteria.

OhrR binding to P1

Next, we assessed the binding of purified OhrR to a 293 bp fragment containing the P1 in the DNA bandshift assay. The results of the DNA bandshift assay show that OhrR binds specifically to P1 (Fig. 4). The OhrR binding to P1 was abolished by unlabelled competing P1 fragment but not by unrelated DNA sequence (Fig. 4). Moreover, the substitution of a protein unrelated to OhrR did not produce mobility shift of P1 (Fig. 4). The addition of increasing concentrations of OhrR to the P1 fragment did not produce additional species of slower migrating bands, suggesting that there was no co-operative binding of OhrR to the operator, and probably only one binding site for the protein was present within the P1 fragment (Fig. 4).

The location of the OhrR binding site within the P1 promoter fragment was determined by DNase I protection assay (Fig. 5). Analysis of the footprint patterns shows

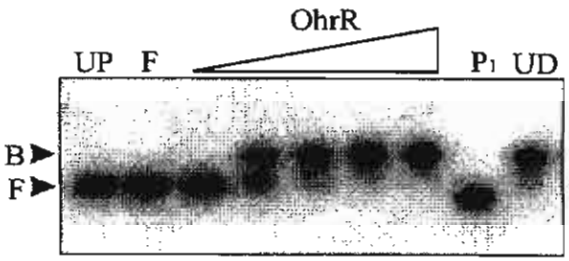

Fig. 4. DNA bandshift assay for binding of OhrR to P1. Purified OhrR was added to 293 bp of radioactively labelled P1 fragment in the binding buffer and separated in a polyacrylamide gel performed as described in *Experimental procedures*. The binding reaction consisted of labelled P1 fragment and 400 ng of OhrR. UP is the addition of 2 μg of unrelated protein (BSA) to the binding reaction; F is free P1 probe; the addition of increasing concentrations of OhrR (100, 200, 400, 800 and 1200 ng) to labelled P1 probe; P1 is the addition of 2 μg of unlabelled P1 DNA to the binding reaction; UD, the addition of 3 μg of unrelated DNA (pUC18 plasmid) to the binding reaction. The positions of free (F) and bound (B) P1 probe are shown on the left.

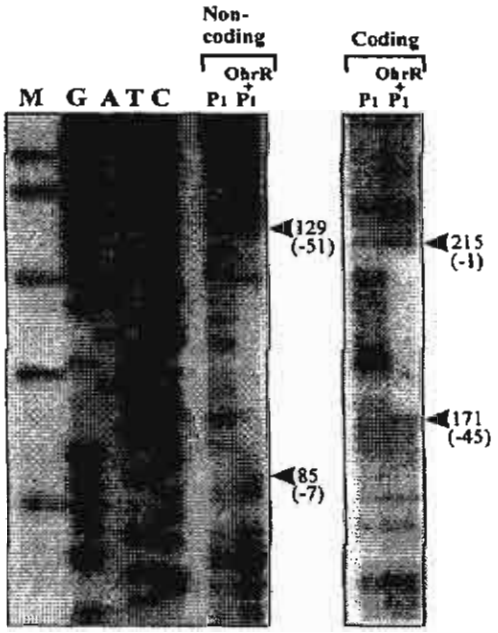

Fig. 5. DNase I protection assay to locate OhrR binding site to P1. The DNase I protection assay for the binding of OhrR to P1. P1 represents the DNA fragments treated with DNase I; P1 + OhrR represents the binding of OhrR to the DNA fragments before DNase I treatment. The labelling of non-coding and coding strands was done as described in *Experimental procedures*. The arrows indicate the size of the fragments in bp, and the numbers in the brackets indicate the position of the protected regions with respect to the +1 transcription initiation site. G, A, T and C are the sequence ladder. M is radioactively labelled ϕX174 *Hin*fI molecular weight markers.

that OhrR binding to P1 produced a DNase I-protected region of 44 bp extending from −1 to −45 on the coding strand and from −7 bp to −51 bp on the non-coding strand (Fig. 5). The DNase I-protected regions completely overlap the −35 and −10 regions of P1. Thus, the binding of OhrR prevents RNA polymerase from binding to the promoter, resulting in repression of gene expression.

Analysis of the DNA sequence within the OhrR protected region reveals several features that could constitute the OhrR operator site. There are several atypical AT-rich regions of two direct repeats ATAAATCG (D1) separated by 22 bp, TTGCAA (D2) separated by 21 bp and an inverted repeat TTGCAATT-AATTGCAA (I1) separated by 17 bp surrounded by normal GC-rich *Xanthomonas* DNA (Fig. 1B). These elements are all located in close proximity to the −35 and −10 regions of P1, and binding of OhrR to these sites would prevent RNA polymerase from binding to the promoter (Fig. 1B). The analysis of P1 deletion showed that half the ATAAATCG (D1) direct repeat could be removed (deletion no. 87) without altering the ability of OhrR to repress *cat* expression (Fig. 3),

indicating that this motif was not crucial for the binding of OhrR to P1. In the *B. subtilis* system, the proposed sequence for the putative binding site for OhrR located in front of the *ohrA* also has AT-rich regions of overlapping inverted and direct repeats (Fuangthong *et al.*, 2001). The importance of these elements as putative OhrR binding sites is suggested by analysis of a non-inducible *ohrA* mutant with a deletion that removes half the inverted and direct repeats (Fuangthong *et al.*, 2001). OhrR from *Xanthomonas* and *B. subtilis* also shares high levels of homology at the amino acid sequence level, suggesting that they might recognize similar DNA motifs as the binding site. Comparison of the AT-rich regions of P1 and the *ohrA* promoter revealed a region with a high degree of similarity. Based on the analysis of the sequence alignment of these regions (data not shown), we proposed the putative OhrR operator site to be an inverted repeat TTnCAATT-(16/17)-AATTGnAA. The site consists of AT-rich inverted repeats separated by a relatively long space of 16–17 bp. In *X. campestris* pv. *phaseoli*, the putative OhrR operator consisted of a perfect inverted repeat separated by 17 bp (I1) located between the –35 and 3′ of –10 regions of the P1 promoter (Fig. 1B). Results of both the DNA footprinting of OhrR binding to P1 and the *in vivo* deletion analysis suggest that the binding site of OhrR is located within the –35 and –10 regions of P1 (Figs 3 and 1B). In *B. subtilis*, the OhrR operator has an inverted repeat containing three mismatches and separated by 16 bp, and the operator overlaps the –35 and –10 regions of the *ohrA* promoter (Fuangthong *et al.*, 2001). OhrR appears to recognize an extended operator site of about 32–33 bp. This probably accounts for the observed long (44 bp) protected region resulting from OhrR binding to P1 in the DNase I footprinting experiment (Fig. 5). At present, we do not know whether the D2 direct repeats contribute to OhrR binding to P1. The importance of the inverted and direct repeats is being investigated.

Overlapping binding sites for OhrR and RNA polymerase suggest that the repressor binding to the operator prevents RNA polymerase from initiating transcription at P1. Generally, a repressor has a higher binding affinity for an operator site than does RNA polymerase for a promoter. In uninduced cells, most of the OhrR binds to the operator, resulting in repression of P1. Exposure to organic peroxide probably inactivates OhrR and prevents it from binding to the operator, which allows RNA polymerase to bind and initiate transcription. This assumption is supported by the primer extension data showing that the tBOOH treatment increased the transcription initiation at P1 (Fig. 2). Moreover, preliminary investigations suggested that, *in vitro*, organic peroxide might directly modify OhrR and prevent it from binding to P1 (data not shown).

The stability of ohrR–ohr *and* ohr *mRNA*

We have shown that the bicistronic *ohrR–ohr* mRNA is processed by cutting in the loop section of the stem–loop structure 3′ of *ohrR* possibly by an RNase III-like enzyme to give monocistronic *ohr* mRNA and rapidly degraded monocistronic *ohrR* mRNA (Sukchawalit *et al.*, 2001). This reduces the functional level of *ohrR* mRNA and ultimately affects the level of OhrR. As transcription of *ohrR* and *ohr* is driven from the P1, the stability of these mRNAs would have important effects on their expression. Here, we determined the stability of *ohrR–ohr* and *ohr* mRNA by measuring the half-lives of these mRNAs in tBOOH-induced cells. The data show that *ohr* mRNA is highly stable and has a half-life of >15 min (Fig. 6). In contrast, the bicistronic *ohrR–ohr* mRNA was highly labile, and the unprocessed bicistronic mRNA could not be detected 6 min after the addition of rifampicin (Fig. 6). Consistent with previous observations, no monocistronic *ohrR* mRNA could be detected (Fig. 6; Sukchawalit *et al.*, 2001). This differential stability of *ohrR* and *ohr* mRNA would result in high concentrations of functional *ohr* mRNA, giving correspondingly high Ohr levels, and would reduce the level of *ohrR* mRNA. As Ohr is responsible for the detoxification of organic peroxide, a high level of the protein would be beneficial to the bacteria during exposure to organic peroxide stress, although low levels of OhrR also prevent excessive repression of the operon by the autoregulatory process. Thus, the differential stability and rapid processing of the mRNA exerts an additional post-transcription step to regulate the *in vivo* concentration of OhrR. This mechanism is not unique to the regulation of *ohrR*. Post-transcriptional regulation at the level of mRNA stability has been observed in diverse bacteria (Takata *et al.*, 1989; Nilsson *et al.*, 1996; Hebermehl and Klug, 1998; Homuth *et al.*, 1999).

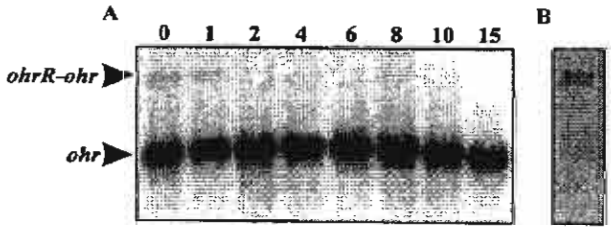

Fig. 6. Analysis of bicistronic *ohrR–ohr* and *ohr* mRNA stability. Total RNA was isolated from culture treated with 100 μM tBOOH for 10 min before the addition of rifampicin. Time zero represents the steady-state level of mRNA before the addition of 150 μg ml^{-1} rifampicin. At the stated time (min) after rifampicin addition, samples were withdrawn and total RNA extracted. RNA (15 μg) from each time point was loaded into each well and separated in a formaldehyde gel. The samples were blotted to a nylon membrane and probed with radioactively labelled *ohr* in (A). The steady-state (0) RNA sample was probed with *ohrR* in (B).

ohrR *is inefficiently translated*

We have observed in both uninduced and tBOOH-induced cells that OhrR is barely detectable by Western analysis, despite the gene having a highly efficient promoter (data not shown). Although we identified a post-transcriptional regulatory step that involves the differential stability of the mRNA, lack of correlation between the promoter strength and the concentration of OhrR suggested further regulation, perhaps at the translational level. *ohrR* produced a leaderless mRNA (Fig. 2). An alternative interpretation that *ohrR* mRNA could be translated from other translation initiation codons located further downstream of the ATG is unlikely, as there are no other commonly used Gram-negative translation initiation codons (ATG to GTG) nearby (Fig. 1B). Furthermore, studies on the translation of leaderless mRNA indicate that the ATG codon is required (Winzeler and Shapiro, 1997; Wu and Janssen, 1997). Here, we investigated the role of the ATG codon in the transcription of the gene and the translation of *ohrR* mRNA. A site-directed mutagenesis of the gene was done to change the 'ATG' codon to a rarely used translation initiation codon, 'CTG' (Fig. 7). Subsequently, the levels of *ohrR* transcription and translation *in vivo* were determined by making transcription (*cat2*; Mongkolsuk *et al.*, 1993) and translation (*cat3*; Mongkolsuk *et al.*, 1993) fusions of *cat* reporter genes to the mutated CTG-*ohrR* at the *Pst*I site. Cat levels specified by *Xanthomonas* harbouring pOPcat2 and pOPcat3 were determined and compared with the levels attained by the strains harbouring non-mutated *ohrR–cat* fusion plasmids (Fig. 7). The results show that changing the translation initiation codon from ATG to CTG did not affect the level of transcription (pOPcat2). In contrast, the translation fusion of CTG-*ohrR* to *cat3* (pOPcat3) was abolished, and no Cat fusion protein was detected (Fig. 7). The evidence confirmed that the translation of *ohrR* mRNA occurred at the proposed ATG and that the codon was required. However, the ATG codon was not important to the transcription of the gene.

We also investigated further the level of transcription and translation of *ohrR* using the *cat* reporter gene fusions. Initially, the transcription and translation fusions were made at the *Kpn*I site located in the middle of *ohrR* (Fig. 7). The results of densitometer analysis show that the Cat levels specified by transcriptionally fused pOKcat2 were at least fivefold higher than the levels specified by the translationally fused pOKcat3 (Fig. 7). To confirm these observations, additional transcriptional and translational *cat* fusions were made at the *Pst*I site located closer to the translation initiation of *ohrR* (Fig. 7). Cat levels specified by pOPcat2 were at least fivefold higher than the levels attained by pOPcat3. These fusions gave similar patterns regardless of the locations of the gene fusions and indicated that *ohrR* mRNA is inefficiently translated and that the translational level of the *ohrR* mRNA occurs in only about 20% of the total *ohrR* mRNA.

High levels of OhrR reduce organic peroxide resistance

The existence of multiple regulatory mechanisms to ensure that OhrR is not produced at high levels implies that the tight regulation of the gene must have important physiological consequences on the cells' ability to respond to oxidative stress. The assumption was tested by measuring the effects of killing concentrations of H_2O_2, a superoxide generator (menadione) and organic peroxides on *X. campestris* pv. *phaseoli* harbouring pBBRohrR.

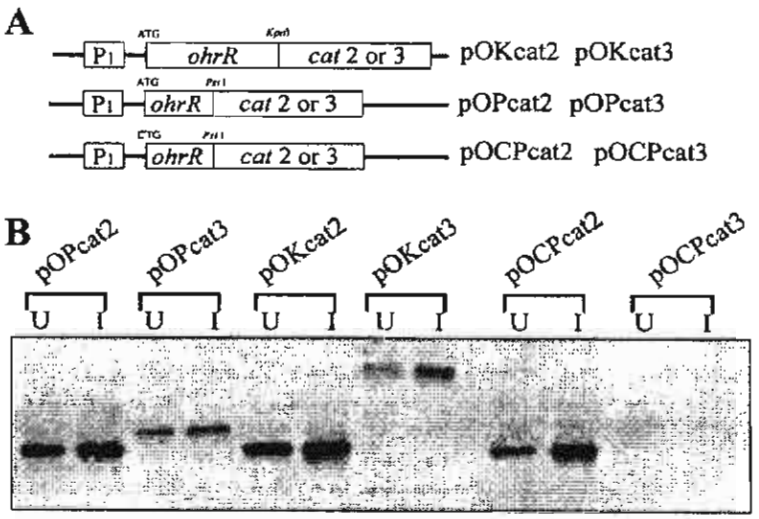

Fig. 7. *ohrR* mRNA is inefficiently translated.
A. Diagrammatic representation of various plasmids showing the site of either transcriptional or translational fusions between *ohrR*, mutated CTG-*ohrR* and *cat*. *cat2* and *cat3* fusions represent transcriptional and translational fusions respectively.
B. Total protein (10 μg) prepared from cultures of *Xanthomonas* harbouring various plasmids was loaded into each lane. Separated protein samples were transferred to membranes after gel electrophoresis. Cat was detected by Western blot. U and I are uninduced and 100 μM tBOOH-induced cultures respectively.

In the bacteria harbouring pBBRohrR, tBOOH and cumene hydroperoxide gave zones of growth inhibition of 32 mm and 31 mm, respectively, compared with 26 mm for the bacteria harbouring pBBRMCS-1. H_2O_2 and menadione produced similar sizes of zone of growth inhibition in both strains. The data indicate that high levels of OhrR have detrimental effects on oxidative stress response by decreasing the organic peroxide resistance level, most probably by repression of the *ohr* expression. Thus, it is not surprising that *Xanthomonas* has evolved multiple mechanisms to ensure proper regulation of *ohrR*. At present, we do not know other genes in the OhrR regulon; thus, it is possible that other stress responses could be affected by high levels of OhrR.

A model for transcriptional and post-transcriptional regulation of ohrR

The transcriptional regulation of *ohrR* involves the autoregulation of the gene at the P1 promoter by OhrR. The binding target for OhrR overlaps with the –35 and –10 sites, enabling repressor to block RNA polymerase from binding to the promoter. In uninduced cells, *OhrR* represses the expression of its own operon. The post-transcriptional regulation of *ohrR* occurs in two steps. First, the bicistronic *ohrR–ohr* mRNA is rapidly processed giving high levels of *ohr* mRNA and rapid degradation of *ohrR* mRNA. *ohr* mRNA is highly stable, in contrast to highly labile bicistronic *ohrR–ohr*. This greatly reduces the functional concentration of *ohrR* mRNA. Secondly, *ohrR* mRNA is inefficiently translated, and translation of the mRNA occurred at only 20% of the transcription level. Multiple regulatory mechanisms at transcriptional and post-transcriptional levels ensure that the intracellular level of OhrR remains low. When cells are exposed to organic peroxides, they presumably inactivate OhrR and prevent the repressor from binding to the operator, resulting in high levels of expression of *ohrR* and *ohr*. Once Ohr removes organic peroxide, OhrR then autoregulates itself and represses the expression of the operon (Fig. 8).

Experimental procedures

Bacterial culture conditions and transformation

All *Xanthomonas* strains were grown aerobically in SB (Silva–Buddenhagen medium: 0.5% peptone, 0.5% yeast extract, 0.5% sucrose and 0.1% glutamic acid, pH 7.0) at 28°C (Mongkolsuk *et al.*, 1997). The oxidant induction experiments were performed on exponential phase cells by the addition of 100 μM tBOOH to cultures followed by incubation for an additional 15 min for Northern analysis and 30 min for Western analysis before cells were harvested for lysate preparation. Antibiotics were used at the following concentrations: for the selection of chromosomal integrated mutants, 15 μg ml^{-1} kanamycin; and for selection of plasmids, 15 μg ml^{-1} gentamicin and 30 μg ml^{-1} kanamycin. All plasmids were transformed into *X. campestris* pv. *phaseoli* by electroporation using previously described conditions (Mongkolsuk *et al.*, 1997).

Western blots

Crude protein (10 μg) was loaded into each lane of a 10% SDS–PAGE gel. The separated proteins were electrophoretically transferred to a sheet of nylon membrane. Membrane blocking, primary antibody reaction, washing and subsequent detection of the immune reaction by alkaline phosphatase-conjugated secondary antibody were performed as described previously (Loprasert *et al.*, 2000).

Nucleic acid purification and ohrR *primer extension*

Total RNA was isolated using the modified hot phenol method from uninduced and tBOOH-induced *X. campestris* pv. *phaseoli* cultures (Mongkolsuk *et al.*, 1997). Primer extension experiments were carried out using ^{32}P-labelled OR1 primer (5′-ATACAACGCAAAGCACAGCTG-3′), 5 μg of total RNA and 200 U of SuperScript II MMLV reverse transcriptase. The extension products were analysed on sequencing gels next to sequence ladders. The sequence ladders were done using a polymerase chain reaction (PCR) sequencing kit with labelled OR1 primer and pBBRohrR plasmid as the template.

Construction of ohrR *fusions*

The intein–OhrR fusion was made by cloning of 480 bp *NcoI–XhoI*-digested PCR products from pBBRohrR template and ohrRI1 (5′-GGCTCGAGTCCCGCGCCAAGG-3′) and ohrRI2 (5′-GGAAACAGCTATGACCATG-3′) into similarly digested pCYB4 (New England BioLabs) giving pINTohrR. The nucleotide sequences of fused genes were determined to confirm fusion in the correct reading frame and that no other mutations had occurred.

Purification of OhrR

The intein fusion system was used by making the fusion at the carboxy-terminus of OhrR. *Escherichia coli* harbouring pINTohrR was grown to mid-log phase before 1 mM IPTG was added and incubation continued for 3 h. The culture was harvested, and cell pellets were resuspended in the column buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100) and sonicated. The lysate was spun at 10 000 g for 15 min, and lysate was loaded on to a chitin bead column, which was subsequently washed extensively with column buffer. The on-column cleavage of fusion protein was done by the addition of buffer C [20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM EDTA and 30 mM dithiothreitol (DTT)] at 4°C overnight. The eluted fractions containing OhrR were pooled and dialysed against Z buffer (10 mM HEPES, pH 8.0, 1 mM EDTA, 20 mM $MgCl_2$, 60 mM KCl and 20% glycerol).

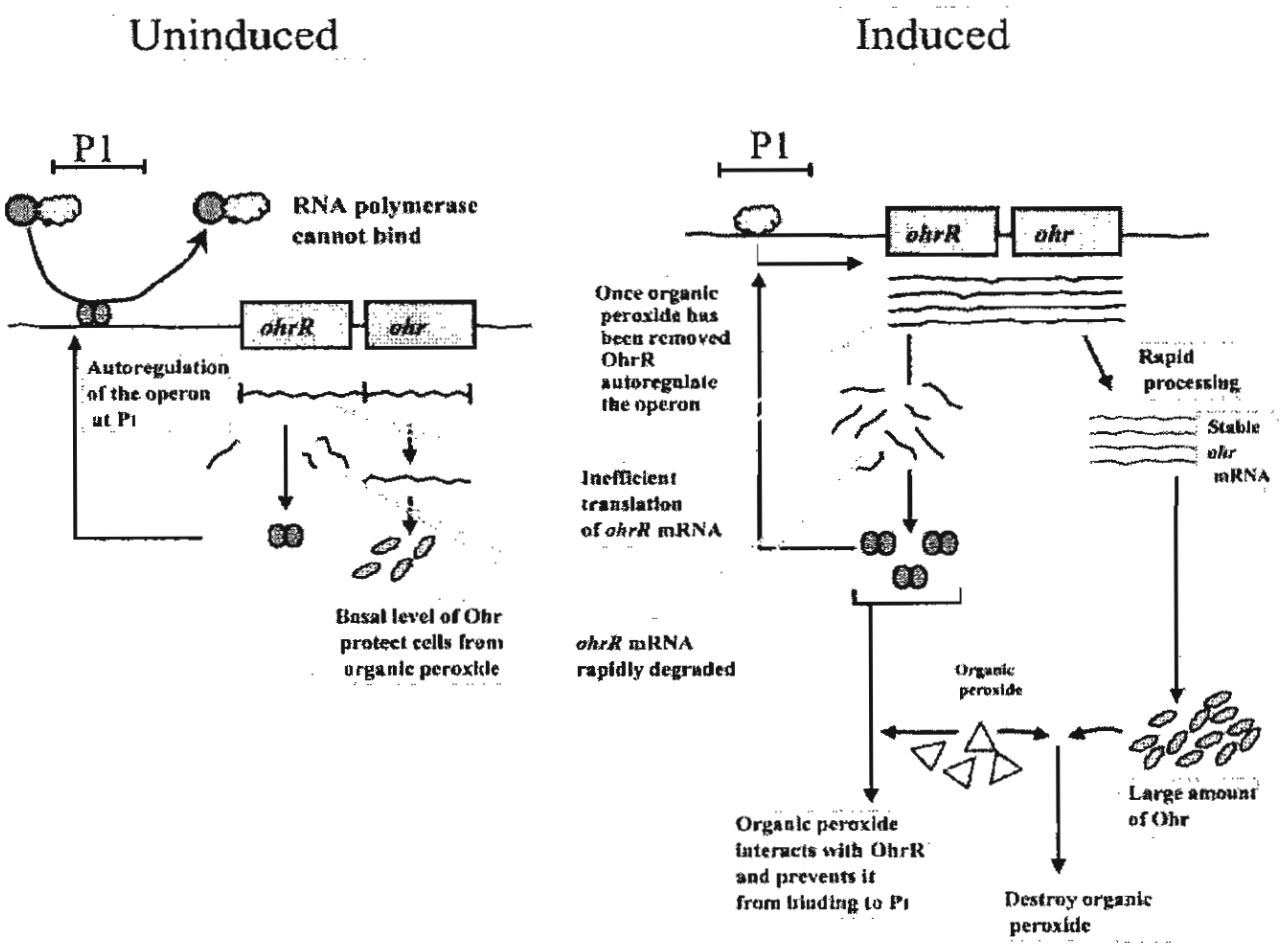

Fig. 8. A model for *ohrR* regulation at transcriptional and post-transcriptional levels.

DNA bandshift assay and DNase I footprinting

Strand-specific, radioactively labelled DNA fragments were prepared by PCR using labelled M13 reverse primer for the coding strand and BT18 for the non-coding strand with pohrRsfi as templates. The PCR products generated 293 bp fragments, which were used in footprinting the coding strand. For the non-coding strand, the PCR product was digested with *Eco*RI to give 180 bp that was used in the footprinting experiment. The DNA bandshift reactions were performed by adding 3 fmol of labelled probe to the buffer [20 mM Tris, pH 7.0, 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μg ml^{-1} BSA, 5 μg ml^{-1} calf thymus DNA, 0.5 mg ml^{-1} poly-(dI–dC)]. Purified OhrR (400 ng) was added, and the reaction was incubated at 25°C for 15 min. For DNase I footprinting, 25 μl of 0.2 mM Mg^{2+} and 0.1 mM Ca^{2+} and 0.5 U of DNase I were added to the binding reaction, and incubation was continued for 1 min before 200 μl of stop solution (20 mM EDTA, pH 8.0, 1.0% SDS and 0.2 M NaCl) was added. The mixture was extracted with phenol–chloroform and ethanol precipitated. The pellets were resuspended in sequencing buffer and loaded onto a sequencing gel. The DNA sequence ladder for the non-coding strand was performed with fmol sequencing kits (Promega) using labelled M13 primer and pahpC plasmid (Mongkolsuk *et al.*, 1997). Sequence reactions were loaded on to a denatured DNA sequencing gel.

Determination of mRNA stability

Xanthomonas campestris pv. *phaseoli* strain 182 cultures were used in the determination of mRNA stability. Exponential phase cultures of the bacteria (OD_{600} of 0.7) were treated with 150 μg ml^{-1} rifampicin to inhibit new RNA synthesis. Then, at 1 min intervals, 10 ml of culture was withdrawn, pelleted rapidly and extracted for total RNA (Mongkolsuk *et al.*, 1997). Subsequent steps in the RNA extraction, formaldehyde gel electrophoresis and construction of *ohr* and *ohrR* probes were performed as described previously (Sukchawalit *et al.*, 2001).

Site-directed mutagenesis and gene fusions

To investigate the role of the *ohrR* translation initiation codon

in transcription and translation of the gene, the codon was mutated from ATG to CTG using primers CT (5′-ATTGCAACCCTGGACACCACC-3′), CTR (5′-GGTGGT GTCCAGGGTTGCAAT-3′) and pBBRohrR as the template in a PCR mutagenesis reaction. The mutated gene CTG-*ohrR* was digested with *Sfi*I–*Pst*I, and the 139 bp fragment was cloned into similarly digested pUFRcat2-Km, resulting in a transcription fusion of the gene to the *cat2* in pOPcat2. The translation fusion of the gene was constructed using a similar strategy to that described for the non-mutated *ohrR*, except that CTG-*ohrR* was used as the starting material. This gave pOPcat3. pOPcat2 and pOPcat3 were transformed into *X. campestris* pv. *phaseoli*, and Cat levels were determined.

Construction of ohrR *transcriptional and translational fusions*

The transcriptional fusions of *ohrR* to *cat* were made by cloning either the 610 bp *Bam*HI–*Kpn*I or the 139 bp *Sfi*I–*Pst*I fragments in front of a promoterless *cat* in the promoter probe vector pUFR027cat2-Km, giving pOKcat1 and pOPcat1 respectively. These recombinant plasmids were confirmed by restriction mapping. The translational fusions were made by cloning these fragments into the polylinker region of the plasmid pSM-cat3 (containing *cat* coding sequence minus the ribosome binding site; Mongkolsuk *et al.*, 1993) giving pKcat3 and pPcat3. The cloning sites were chosen so that the *ohrR* fused in frame with the *cat* coding sequence. All fusion joints were sequenced. Moreover, these OhrR–Cat fusions should give proteins with a higher molecular weight than Cat. Western analysis was used to confirm the size of the recombinant translation fusions. pKcat3 and pPcat3 were digested with *Hin*dIII and *Eco*RI, and the DNA fragments containing the *ohrR–cat* fusion were cloned into pUFR027cat2-Km giving pOKcat3 and pOPcat3 respectively. All recombinant transcriptional and translational fusion plasmids on the broad-host-range replicons were electroporated into *X. campestris* pv. *phaseoli*.

Qualitative determination of oxidant resistance levels

The zone of growth inhibition method was used to measure the resistance level to various oxidants. Exponential phase cells (10^8) were mixed with semi-soft SB agar and poured on top of an SB plate. After the semi-soft agar had solidified, 6 mm 3M paper disks individually impregnated with 6 µl of 1 M tBOOH, cumene hydroperoxide, menadione and H_2O_2 were placed on top of the cell lawn. The zone of growth inhibition was measured after 24 h incubation at 28°C.

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OhrR, a transcription repressor that senses and responds to changes in organic peroxide levels in *Xanthomonas campestris* pv. *phaseoli*

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Summary

We report the physiological role of OhrR as an organic peroxide sensor and transcription repressor in *Xanthomonas campestris* pv. *phaseoli*. *In vivo* exposure of *X. campestris* pv. *phaseoli* to either *tert*-butyl or cumene hydroperoxides efficiently neutralized OhrR repression of expression from the OhrR-regulated P1 promoter. H_2O_2 was a weak and non-physiological inducer of the system while other oxidants and metabolites of organic peroxide metabolism did not induce the expression from the P1. Northern blotting results indicated a correlation between concentrations of *tert*-butyl hydroperoxide used in the treatment and the induction of *ohr* (an OhrR-regulated gene) expression. In addition, the levels of *ohr* mRNA in cultures induced by various concentrations of *tert*-butyl hydroperoxide were reduced in cells with high levels of an organic peroxide metabolising enzyme (AhpC-AhpF) but not in cells with high catalase levels suggesting that organic peroxide interacts with OhrR. DNA band shift experiments using purified OhrR and the P1 promoter fragment showed that organic peroxide treatment prevented binding of the protein to the P1 promoter by oxidation of OhrR, as the inhibition of binding to the P1 promoter was reversed by addition of a reducing agent, DTT. The highly conserved cysteine residue C22 of OhrR is required for organic peroxide inducible gene expression. A mutant protein, OhrRC22S can repress the P1 promoter activity but is insensitive to organic peroxide treatment. Thus, OhrR is the first transcription repressor characterized that appeared to evolve to physiologically sense organic peroxides.

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Introduction

Organic peroxides are highly toxic compounds to biological systems because of their abilities to react with intracellular macromolecules and to generate reactive organic radicals (Halliwell and Gutteridge, 1984). Bacteria have evolved several pathways to ensure efficient removal of organic peroxides. Alkyl hydroperoxide reductase (AhpC) is widely distributed in diverse organisms ranging from bacteria to humans and is the best characterized organic peroxide detoxification system that metabolizes organic peroxides to their corresponding alcohols (Chae *et al.*, 1994; Poole and Ellis, 1996). A second organic peroxide protection system, organic hydroperoxide resistance protein (Ohr), was first identified in *X. campestris* pv. *phaseoli* (Mongkolsuk *et al.*, 1998) and subsequently shown to be present in many Gram-positive and Gram-negative bacteria (Atichartpongkul *et al.*, 2001; Fuangthong *et al.*, 2001; Ochsner *et al.*, 2001; Shea and Mulks, 2002). Inactivation of *ohr* results in increased sensitivity towards organic peroxides (Mongkolsuk *et al.*, 1998; Atichartpongkul *et al.*, 2001). As yet, the way in which the Ohr system works is unknown.

The abilities to sense changes and respond to oxidative stress are crucial for aerobic organisms. Bacteria have complex sensing mechanisms and response regulators which alter patterns of gene expression as to prevent oxidative damages to cells. Two of the most well-characterized bacterial sensors of oxidative stress and transcription regulators are OxyR, a global peroxide sensor (Toledano *et al.*, 1994; Zheng and Storz, 2000) and SoxR, a global superoxide sensor (Demple *et al.*, 2002). AhpC and Ohr appear to have overlapping physiological functions, but their patterns of expression and regulation differ. *ahpC* is regulated by OxyR (Loprasert *et al.*, 2000), whereas *ohr* is regulated by a novel, organic hydroperoxide-inducible, transcription repressor, OhrR, that belongs to the MarR family (Fuangthong *et al.*, 2001; Sukchawalit *et al.*, 2001). *ohrR* is found in both Gram-negative and Gram-positive bacteria (Fuangthong *et al.*,

2001; Sukchawalit *et al.*, 2001). Interestingly, genes in the OxyR regulon are highly induced by treatment of cells with H_2O_2 and organic peroxide whereas genes in the OhrR regulon are highly induced only by organic peroxide (Loprasert *et al.*, 2000; Sukchawalit *et al.*, 2001). In *X. campestris* pv. *phaseoli*, *ohrR* is uniquely regulated at both the transcriptional and post-transcriptional levels. OhrR autoregulates expression from its own promoter (P1), and RNA processing and production of a highly labile *ohrR* mRNA, coupled with inefficient translation of the mRNA limits the intracellular level of OhrR (Mongkolsuk *et al.*, 2002). Here, we report that OhrR has evolved to sense and respond to changes in organic peroxide levels. The sensing mechanism involved oxidation of the highly conserved C residue that prevents the protein from binding to its target site in the P1 promoter region.

Results

Organic peroxide is the in vivo *inducer of OhrR*

High level expression of *ohrR* from an expression vector results in strong repression of expression from P1 promoter. Transcription from P1 can be lifted by treatment with an organic peroxide (Sukchawalit *et al.*, 2001). This is a sensitive system with which to test the ability of various compounds to induce transcription from P1. *In vivo*, an organic peroxide is metabolized to the corresponding organic alcohol by alkyl hydroperoxide reductase (Poole and Ellis, 1996). However, the *in vivo* system cannot differentiate whether an organic peroxide moiety or its metabolite is acting as an inducer of the system. Thus, we tested the ability of organic alcohols to induce transcription from P1 promoter. Chloramphenicol acetyltransferase (Cat) levels in *X. campestris* pv. *phaseoli* pP1/pBBRohrR (Mongkolsuk *et al.*, 2002) treated with 100 μM *tert*-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CuOOH) and corresponding alcohols were determined and compared to untreated cells. As expected, tBOOH and CuOOH strongly induced *cat* expression from P1 as shown by high Cat levels while *tert*-butyl and cumic alcohols did not (Fig. 1A). The *in vivo* role of OhrR as an organic peroxide sensor was further investigated. We have previously shown that induction of *ohr* requires a functional *ohrR* (Mongkolsuk *et al.*, 2002). We hypothesized therefore that if an organic peroxide is responsible for the inactivation of OhrR and subsequent derepression of *ohr* expression, then high levels of an organic peroxide metabolizing enzyme, such as alkyl hydroperoxide reductase, would be expected to reduce the magnitude of tBOOH induced expression of *ohr*. In contrast, high level of enzymes not involved in organic peroxide metabolism, such as catalase, should not affect *ohr* induction. The levels of *ohr* mRNA in uninduced and tBOOH-induced *X. campestris* pv. *phaseoli* cultures having normal AhpC-AhpF, high AhpC-AhpF and high catalase levels were determined. The results show that a reduction in the level of the tBOOH induced *ohr* mRNA was detected only in cells having high levels of AhpC and AhpF (Fig. 1B). No changes were detected in the other two strains (Fig. 1B). In addition, there was a correlation between the level of *ohr* mRNA and the inducing concentration of tBOOH,

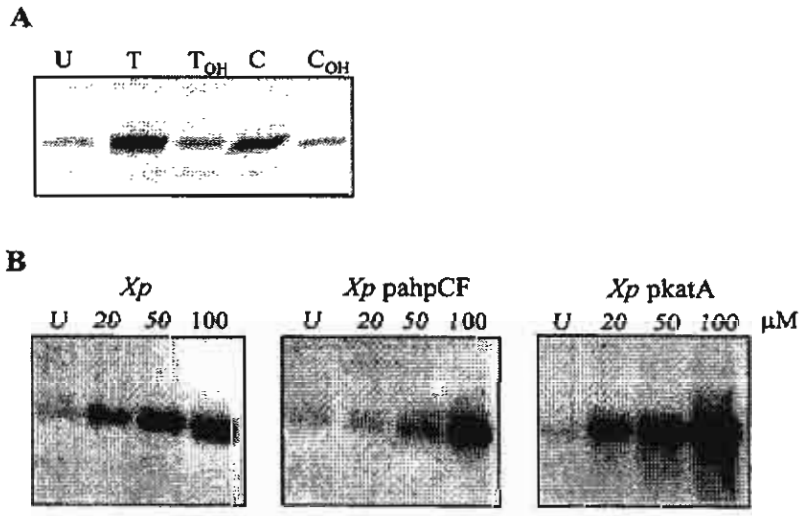

Fig. 1. Organic peroxide is the *in vivo* inducer of OhrR.
A. Western analysis of Cat production by *X. campestris* pv. *phaseoli* harbouring pP1 and pBBRohrR in response to various inducers. *Xanthomonas campestris* pv. *phaseoli* pP1/pBBRohrR (Sukchawalit *et al.*, 2001) was treated with 1 mM *tert*-butyl alcohol (T_{OH}), 1 mM cumic alcohol(C_{OH}), 100 μM tBOOH (T) and 100 μM CuOOH (C). Cell collection, lysate preparation, gel electrophoresis and Western analysis of Cat were done as previously described (Loprasert *et al.*, 2000). Total protein (30 μg) was loaded into each lane. U represents an uninduced culture.
B. Northern analysis of *ohr* expression in response to tBOOH treatments in *X. campestris* pv. *phaseoli* and strains having high AhpC-AhcF or catalase levels. *Xanthomonas campestris* pv. *phaseoli* (*Xp*) strains with high catalase level (*Xp* pkatA) or high AhpC-AhpF levels (*Xp* pahpCF) were treated with 20, 50, 100 μM tBOOH for 10 min. Total RNA extraction and Northern blotting analysis of *ohr* mRNA were done as previously described (Mongkolsuk *et al.*, 1998). Total RNA (10 μg) was loaded into each well of a 2% agarose formaldehyde gel. U represents uninduced culture.

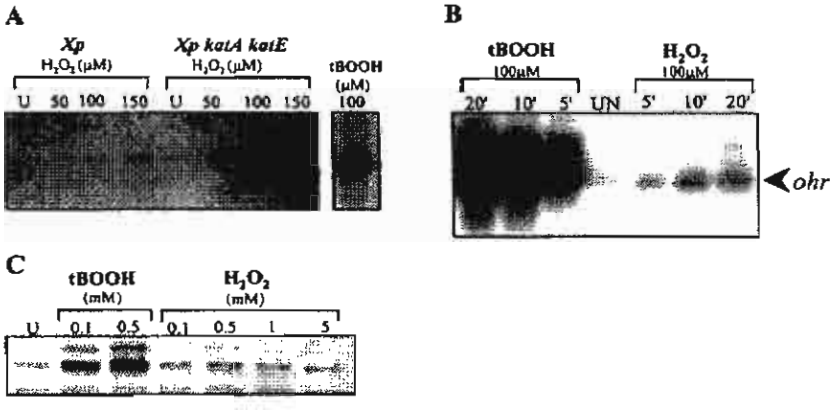

Fig. 2. H_2O_2 is not a physiological inducer of OhrR.
A. Northern blot analysis of *ohr* expression in response to H_2O_2 in a *katA katE* mutant and the kinetic of induction. Cultures of *X. campestris* pv. *phaseoli* (*Xp*) and a *katA katE* mutant (*Xp katA katE*) were treated with increasing concentrations of H_2O_2. In addition, a *X. campestris* pv. *phaseoli* culture was induced with 100 μM tBOOH.
B. The kinetic of *ohr* induction in *X. campestris* pv. *phaseoli* in response to 100 μM tBOOH or H_2O_2 at 5, 10 and 20 min. The levels of *ohr* mRNA were determined using Northern blotting analysis as described in Fig. 1B legend except in tBOOH-treated cells, 5 μg of total RNA was loaded. U represents uninduced culture.
C. Western analysis of dose–response of H_2O_2 treatment and derepression of P1 promoter. Log-phase cultures of *X. campestris* pv. *phaseoli*pP1/pBBRohrR were induced with 0.1, 0.5 mM tBOOH or 0.1, 0.5, 1, 5 mM H_2O_2 for 15 min. U represents uninduced culture. Total protein (10 μg) was loaded into each lane and detection of Cat performed as described in the *Experimental procedures*.

suggesting that OhrR senses changes in the intracellular concentration of the organic peroxide.

Surprisingly, H_2O_2 weakly induced *cat* expression from P1 at 20-fold lower levels than those obtained on an organic peroxide induction (data not shown). Weak induction of gene expression from P1 by H_2O_2 raises the question: does H_2O_2 acts as a physiological inducer of the OhrR regulon? Thus, we examined the expression *ohr*, a gene controlled by OhrR in response to peroxide treatment. *In vivo*, the low level of induction of *ohr* by H_2O_2 could be caused by high catalase activity. Thus, *ohr* induction by H_2O_2 was examined in a *katA katE* [KatA is the major catalase in *X. campestris* pv. *phaseoli* log-phase cells and KatE is a growth-phase-regulated catalase (Vattanaviboon and Mongkolsuk, 2000)] double mutant and the parental strain. Northern blotting results showed that the levels of *ohr* mRNA induced with 100 and 150 μM H_2O_2 were higher in the *katA katE* mutant than in the parental strain (Fig. 2A). Nonetheless, these levels were more than 10-fold lower than the level attained after induction with organic peroxide. In the control experiment, the expression of an OxyR-regulated gene, *ahpC* was strongly induced by H_2O_2 in both strains (data not shown). The data suggest that low level H_2O_2 induction of *ohr* expression was partially due to elimination of H_2O_2 by catalase.

A possibility for lower induced levels of *ohr* by H_2O_2 is that OhrR is oxidized at different rate by H_2O_2 and tBOOH. The idea was tested by examining the kinetic of H_2O_2 and tBOOH induction of *ohr* expression. The results in Fig. 2B show that tBOOH treatment rapidly induced *ohr* expression and full induction was achieved after exposure to tBOOH for 10 min. Similarly, the peak of *ohr* mRNA level induced by H_2O_2 was also detected after the treatment for 10 min, however, this level was 20-fold less than the level attained by tBOOH treatment. These findings ruled out the idea that low level of *ohr* induction by H_2O_2 was due to the slow rate of OhrR oxidation.

Because H_2O_2 was a much less efficient inducer than tBOOH, experiments were done to test the effects of treating cells with increasing doses of H_2O_2 on the induction of *cat* expression from the P1 promoter. Western analysis of Cat level clearly demonstrated that treatment of cells with H_2O_2 at concentrations between 100 and 500 μM resulted in corresponding increase in the amount of Cat specified by the P1 promoter (Fig. 2C). However, further increase in the concentration of H_2O_2 upto 5 mM did not result in further increase in the Cat level (Fig. 2C). Moreover, the H_2O_2 fully induced levels were 20-fold lowered than the Cat level in cells treated with 100 μM tBOOH. This clearly showed that the low level of OhrR oxidation by H_2O_2 was due to its inability to efficiently oxidize the protein. These data lead us to conclude that OhrR is not involved in sensing changes in H_2O_2 levels. The physiological importance of the H_2O_2 induction of the OhrR regulon remains unclear. Nonetheless, low level induction of the OhrR regulon might serve as an additional defence against low concentrations of organic peroxides, such as nucleic acid and lipid peroxides produced as a result of exposure to high concentrations of H_2O_2 (Kappus, 1987; Turton *et al.*, 1997)

The ability of other oxidants to induce *cat* expression from P1 was also investigated. A superoxide generator (menadione) and *N*-ethylmaleimide (NEM) did not cause

induction, although these substances strongly induced expression of genes in the OxyR regulon (data not shown and Mongkolsuk *et al.*, 1997; Loprasert *et al.*, 2000). We also tested other compounds (at a concentration of 1 mM), such as salicylate, benzoate, butylate and hydroxytoluene, that commonly induce expression of genes under the MarR family of repressors (Alekshun and Levy, 1999). None of these compounds induced *cat* expression from P1 (data not shown). These findings suggest that both the peroxide and the organic moieties of organic peroxides are required for efficient interaction with, and inactivation of, OhrR.

Organic peroxide and H_2O_2 treatments inhibited OhrR binding to P1 in vitro

The putative mechanism of inactivation of OhrR and induction of gene expression is thought to involve organic peroxide, either directly or indirectly, resulting in modified OhrR by oxidation, rendering it inactive. An *in vivo* promoter assay cannot differentiate between direct and indirect modification of OhrR by an organic peroxide. We have used a DNA mobility shift assay to show that OhrR interacts specifically with the P1 promoter region and the location of the OhrR binding site was mapped by DNase I footprinting experiment (Mongkolsuk *et al.*, 2002). The DNA mobility shift assay allowed us to test the *in vitro* effects of oxidants, such as CuOOH, tBOOH, H_2O_2 and other chemicals on the ability of OhrR to bind to P1. CuOOH, tBOOH or H_2O_2 treatment prevented OhrR from binding to the P1 fragment (Fig. 3A–C). The inactivation of OhrR by organic peroxides (tBOOH and CuOOH) occurred at concentrations more than 10-fold lower than the concentration of H_2O_2 needed for OhrR inactivation. Addition of other oxidants, such as menadione, NEM or related compounds such as *tert*-butyl and cumic alcohols to the binding reaction mixture did not interfere with OhrR binding to the P1 promoter region (data not shown). The *in vitro* experiment provides crucial data to support the hypothesis that organic peroxide and H_2O_2 interact directly with and probably oxidize OhrR, so rendering the protein inactive as a repressor. Next, we tested whether oxidation of OhrR by peroxide is a reversible process. We determined if a reducing agent, such as dithiothreitol (DTT), could reverse the inhibitory effect of an organic peroxide on OhrR binding to the P1 promoter. The results show that 10 mM DTT reversed the inhibitory effects of tBOOH on OhrR binding to the P1 promoter (Fig. 3D). DTT was also able to reverse the inhibitory effect of H_2O_2 (data not shown). These data supported the idea that tBOOH oxidizes OhrR to render it inactive and the oxidized protein could be reduced to give back its biological activity. It remains to be seen *in vivo* if oxidized OhrR can be reactivated by any of the intrinsic cellular reducing systems. These data, coupled with the *in vivo* results favour the hypothesis that an organic peroxide is responsible for efficient oxidation of OhrR and, as a consequence, inactivation of the repressor OhrR.

C22 residue of OhrR is required for peroxide oxidation

A cysteine residue in proteins is often a target for oxidation as in the case of OxyR where oxidation of highly conserved C residues lead to formation of a disulphide bond

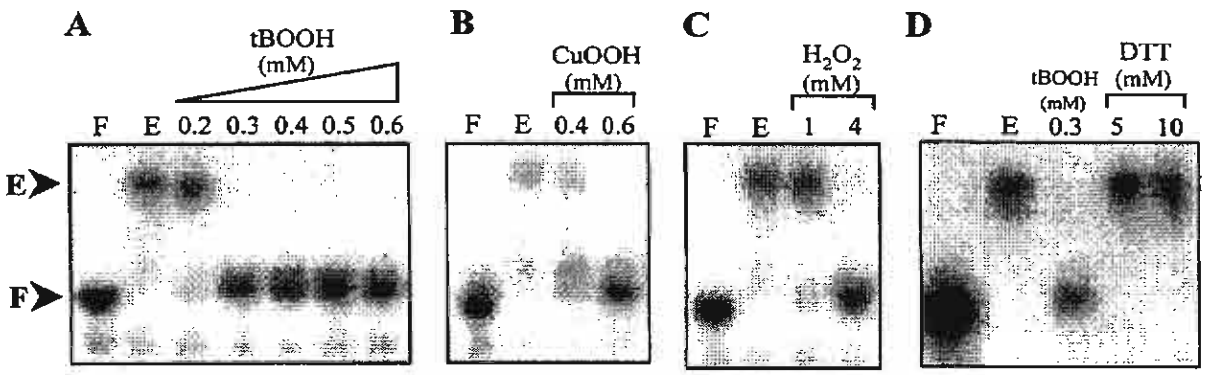

Fig. 3. The effect of organic peroxide and DTT on OhrR binding to the P1 promoter. DNA band shift experiments using purified OhrR and radioactively labelled P1 promoter fragment were done as described in Mongkolsuk *et al.* (2002). The binding reaction consisted of 15 ng OhrR and labelled P1 probe.
A. Effect of adding increasing concentrations of tBOOH to the binding reaction.
B. Effect of adding CuOOH to the binding reaction.
C. Effect of adding H_2O_2 to the binding reaction.
D. Effect of addition of DTT to the binding reaction containing 0.3 mM tBOOH, DTT was added 1 min after addition of tBOOH to the binding reaction. F and E represent free and OhrR-bound probe respectively.

A

X. campestris pv. phaseoli (AAK62673)	LLQLDNQLCFALYSANLAMH
Acinetobacter sp (CAA70318)	I-E-------LI-T----L-
Pseudomonas aeruginosa (AAG06237)	Q-K----------TS-Q-T
Caulobacter vibrioides (AAK22899)	I-R----------G-ANR-T
Agrobacterium tumefaciens (AAL41860)	--R--Q-I-----G-AH-FT
Vibrio cholerae (AAF96901)	P-L-E-----P----SN-VI
Sinorhizobium meliloti (CAC45533)	A-A-GR-----V---AH-FN
Brucella melitensis (AAL53650)	N-D-ADM----V--TAN-LS
Streptomyces coelicolor (CAB87337)	W-R--Q-I--S-SA-SR-FG
Bacillus subtilis (CAA06594)	HMK-E-----L--ASSRE-T

B

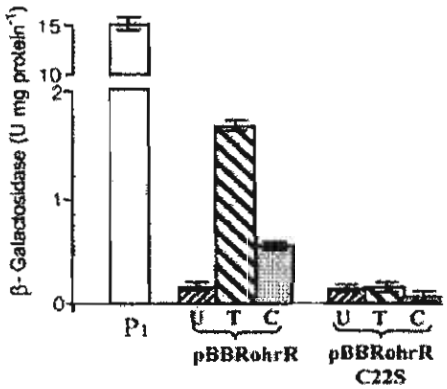

Fig. 4. The conservation of C22 in OhrR and the effect of OhrRC22S mutation on expression of *lacZ* from the P1 promoter.
A. Conserved regions around C22 (▼) in OhrR from various Gram-negative and Gram-positive bacteria. (–) represents the same amino acid as in *X. campestris* pv. *phaseoli* OhrR.
B. pBBRohrR and pBBRohrRC22S were transformed into *Xp* P1lacZ. P1 represented the *Xp* P1lacZ with a plasmid vector. tBOOH (T) or CuOOH (C) at final concentration of 100 μM was added to the culture of *Xp* P1lacZ harbouring pBBRohrR or pBBRohrRC22S and incubated for additional 15 min prior to cell harvest and enzyme assay. U represents uninduced culture. β-galactosidase was assayed as described in the *Experimental procedures*.

(Zheng *et al.*, 1998). This converts OxyR to an activator (oxidized) form. Comparison of OhrR amino acid sequences from various organisms showed a highly conserved C residue (C22) located close to the amino terminus. In addition, amino acid sequences surrounding the C residue are also highly conserved suggesting that the region is important for OhrR function (Fig. 4A). Whereas other C residues in the protein are not in highly conserved positions (data not shown). A site-directed mutagenesis that changed C22S was performed to examine the regulatory role of the conserved C residue. The mutated *ohrR*C22S was cloned in pBBR1MCS-5 (Kovach *et al.*, 1994) resulting in pBRRohrRC22S. The plasmid vector, pBBRohrR and pBBRohrRC22S were transformed into *Xp* P1lacZ (*X. campestris* pv. *phaseoli* mini-Tn5 P1lacZ, Km^R), a strain that has P1 promoter fused to *lacZ* in a mini-Tn5 vector and subsequently transposed into the chromosome. Analysis of β-galactosidase activity showed that both wild-type OhrR and mutated OhrRC22S repressed the P1 promoter activity (Fig. 4B). This indicated that the C22 residue has no role in the binding of OhrR to its target site within the P1 promoter. In contrast, treatment with 100 μM tBOOH or CuOOH highly induced *lacZ* expression driving from the P1 promoter In cells harbouring pBBRohrR but not in cells harbouring pBBRohrRC22S (Fig. 4B). These data indicated that C22 of OhrR is important to the protein ability to sense organic peroxide and this residue is the target for oxidation by organic peroxide that probably changes the protein conformation and renders it inactive. Recent findings indicate that the conserved C residue in *Bacillus subtilis* OhrR Is being oxidized by organic peroxide to a sulphenic acid intermediate that cause inactivation of the protein (Fuangthong and Helmann, 2002).

The data presented in this report imply that the physiological role of OhrR is to sense and respond to changes in organic peroxide levels. Addition of organic peroxide causes inactivation by oxidation of OhrR leading to derepression of *ohr* expression. We have shown that high level of OhrR conferred increased resistance to organic peroxide killing (Mongkolsuk *et al.*, 2002). Oxidation of OhrR by an organic peroxide results in loss of the protein ability to bind to its target site. A recent finding shows that OhrR from *B. subtilis* behave similarly (Fuangthong and

Helmann, 2002). Efficient interaction with the inducer and subsequent oxidation of OhrR requires that the inducer has both peroxide and organic moieties. Replacing either of these entities renders the inducer ineffective or reduces its efficiency. It remains to be seen how OhrR differentiates between organic peroxide and H_2O_2. One explanation is that the OhrR peroxide sensing site is buried in a hydrophobic region which favours interaction with the organic moiety of an organic peroxide. Full oxidation of the protein might require that the ligand remains at the active site for a prolonged period of time. Alternatively, protein–ligand interaction with the organic moiety of the inducer may facilitate the oxidation reaction, e.g. by aligning the peroxide group and its target. In contrast, because H_2O_2 can diffuse into the cell, the low level induction detected may be a result of H_2O_2 reacting in a non-directed way with the sensing site of OhrR. These possibilities will be investigated.

Experimental procedures

Bacterial culture conditions

All *Xanthomonas* strains were grown aerobically in SB (Silva–Buddenhagen medium, 0.5% peptone, 0.5% yeast extract, 0.5% sucrose and 0.1% glutamic acid, pH 7.0, Mongkolsuk *et al.*, 1997) at 28°C. The oxidant induction experiments were performed on exponential phase cells by addition of stated concentrations of oxidants to cultures followed by incubation for additional 15 min for Western analysis and 10 min for Northern analysis before cells were harvested for lysate preparation and RNA extraction. Antibiotics were used at the following concentrations: for selection of chromosomal integrated mutants 15 μg ml^{-1} kanamycin; and for selection of plasmids, 15 μg ml^{-1} gentamicin and 30 μg ml^{-1} kanamycin. All plasmids were transferred into *X. campestris* pv. *phaseoli* by electroporation using previously described conditions (Mongkolsuk *et al.*, 1998).

Western immunodetection of Cat

Cell lysates were prepared by resuspended cell pellets from 10 ml log-phase cultures in 0.5 ml of 50 mM sodium phosphate buffer pH 7.0. The cell suspension was sonicated intermittently for 2 min and spun at 10 000 *g* for 20 min. Protein concentration in clear lysate was measured using the Bradford assay (Bradford, 1976). Subsequently, 20 μg total protein was loaded into each lane of an 10% SDS–PAGE gel. The separated proteins were electrophoretically transferred to a sheet of PVDF membrane. The blocking of membrane, primary antibody reaction, washing and subsequent detection of immune reaction by alkaline phosphatase conjugated second antibody were done as previously described (Loprasert *et al.*, 2000). Densitometer analysis was performed as previously described (Sukchawalit *et al.*, 2001).

Northern analysis of ohr *expression*

Xanthomonas campestris pv. *phaseoli* strains were treated with different concentrations of tBOOH or H_2O_2 for 10 min. Cells were pelleted and total RNA was extracted using the modified hot phenol method (Mongkolsuk *et al.*, 1997). In the induction kinetic experiment, aliquots of cells were removed at the indicated time and cells were pelleted by centrifugation before total RNA was extracted. RNA samples were loaded into 1.5% formaldehyde agarose gel and after electrophoresis the separated RNA samples were transferred to a nylon membrane. Pre-hybridization, hybridization, stringent washing conditions and *ohr* probe preparation were done as previously described (Mongkolsuk *et al.*, 1998).

Purification of OhrR

The poly His-OhrR fusion protein was made using BT377 (5′-ATTCTCGAGTCCCGCGCCAAGGCT-3′) and BT 378 (5′-CGAATTCGCCGATGGTCCC-3′) primers and pBBRohrR as DNA template in a PCR reaction using previously described conditions except 1 U of *pfu* polymerase was used instead of *Taq* polymerase. The 560 bp PCR products were digested with *Nco*I and *Xho*I and cloned into similarly digested pET Blue-2 (Invitrogen). This gave pET-OhrR3His that has OhrR fused to poly His at the carboxyl terminus. The gene sequence was confirmed by DNA sequencing. The fusion protein was purified from *Escherichia coli* BL21(DE3)/pLysS harbouring pET-OhrR3His. Essentially, cell pellet was resuspend in resuspension buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, 1 mM PMSF and 10% glycerol) and lysed by intermittent sonication. The cell debris was removed by centrifugation at 10 000 *g* at 4°C for 30 min before loading into a heparin agarose column equilibrated with binding buffer without 100 mM NaCl. Bound protein was eluted with NaCl gradient (0.05–1.0 M). Fractions containing OhrR was pooled and concentrated before loading onto Superdex 75 column and protein eluted at the rate of 0.5 ml min^{-1} in binding buffer with 100 mM NaCl. Fractions containing OhrR was pooled and protein concentration was determined by a dye-binding method (Bradford, 1976). The purity of OhrR is greater than 90%.

DNA band shift assay

The DNA band shift reactions were performed by adding 3 fmol of labelled 170 bp P1 promoter fragment probe to the binding buffer (20 mM Tris pH 7.0, 50 mM KCl, 1 mM EDTA, 5% glycerol, 50 μg ml^{-1} BSA, 5 μg ml^{-1} calf thymus DNA, 0.8 μg ml^{-1} polydI/dC). Then 15 ng of purified OhrR was added and the reaction incubated at 37°C for 30 min (Loprasert *et al.*, 2000).

Site-directed mutagensis of ohrR

Polymerase chain reaction (PCR) based site-directed mutagenesis was used to change the OhrR C22S. Essentially, mutagenic primers BT17 (5′-GAGCTGTCCTTTGCGT TGT-3′) and BT18 (5′-ACAACGCAAAGGACAGCTC-3′) were used and pBBRohrR as DNA template were mixed with PCR reaction and performed using previously described conditions (Mongkolsuk *et al.*, 2000). Two PCR products were re-

annealed and re-PCR with M13-Forward and M13-Reverse primers giving 710 bp PCR products which were digested with *EcoR*I and *Sac*I and cloned into pBBR1 MCS-5 (Kovach *et al.*, 1994) giving pBBRohrRC22S. The sequence of the mutated DNA was verified using an automated DNA sequencer.

Construction of Xp P1lacZ

A 170-bp fragment contalning P1 was cloned into a mini-Tn5 vector pUTPlacZ1 (de Lorenzo and Timmis, 1994) in front of a promoterless *lacZ* giving pP1lacZ. The promoter probe vector was transferred into *X. campestris* pv. *phaseoli* and selected for Km^R and Ap^S phenotypes for transposition of the mini-Tn. The intergration of the mini-Tn containing P1lacZ was confirmed by PCR using primers located in P1 and *lacZ*. Beta-galactosidase assays were done according to Ochsner *et al.* (2001).

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