รายงานฉบับสมบูรณ์

โครงการ: การศึกษากลไกในระดับโมเลกุลของ organic peroxide sensor/transcription regulators และการตอบสนองของขบวนการนี้ต่อการเปลี่ยนแปลงของสภาพแวดล้อม

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

สิงหาคม 2548

สัญญาเลขที่ RTA4580010

รายงานฉบับสมบูรณ์

โครงการ

การศึกษากลไกในระดับโมเลกุลของ organic peroxide sensor/transcription regulators และการตอบสนองของขบวนการนี้ต่อการเปลี่ยนแปลงของสภาพแวดล้อม

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป

ABSTRACT

Organic hydroperoxide and peroxide stress responses in soil and pathogenic bacteria such as *Agrobacterium tumefaciens, Xamthomonas campestris, Vibrio harveyi, Pseudomonas aeruginosa* and *Burkhoderia pseudomallei*, are investigated. Several global sensor and transcriptional regulators including *ohrR, oxyR* and *soxR* have been isolated and molecularly characterized. Their physiological functions under normal growth as well as under stresses are investigated. The enzymes involved in peroxide scarvenger i.e. catalase-peroxidase, monofunctional catalase, organic hydroperoxide thiol peroxidase (Ohr), glutathione peroxidase, glutathione reductase, alkyl hydroperoxide reducatase and superoxide dismutase are functionally studied. Taken together, the results give a new insight into the understanding of bacterial peroxide stress responses. The research provides new knowledges that could be applied to find out novel drug targets in the future.

บทคัดย่อ

คณะผู้วิจัยได้ทำการศึกษาการตอบสนองต่อออร์แกนิด ไฮโดรเปอร็อกไซด์ และสารเปอร็อกไซด์ ในแบคทีเรียก่อโรคหลายชนิด ได้แก่ *Agrobacterium tumefaciens, Xamthomonas campestris, Vibrio harveyi, Pseudomonas aeruginosa* และ *Burkhoderia pseudomallei* โดยได้ทำการแยกและศึกษายีนที่ทำหน้าที่เป็นตัว sensor และตัวควบคุมการ transcription เช่น *ohrR, oxyR* และ *soxR* ทั้งการทำงานภายใต้สถาวะปกติ และภายใต้สภาวะ stresses นอกจากนี้คณะผู้วิจัยยังได้ทำการศึกษาเอนไซม์ที่เกี่ยวข้องกับการทำลายพิษจากสารเปอร็อกไซด์ เช่น catalase-peroxidase, monofunctional catalase, organic hydroperoxide thiol peroxidase (Ohr), glutathione peroxidase, glutathione reductase, alkyl hydroperoxide reducatase และ superoxide dismutase ว่ามีบทบาทและหน้าที่อย่างไร จากผลการศึกษาทำให้สามารถทราบและเข้าใจหน้าที่และบทบาทของยีนต่างๆ ในแบคทีเรียเหล่านี้ในการตอบสนองต่อสารเปอร็อกไซด์ ซึ่งความรู้เหล่านี้สามารถนำไปประยุกต์ใช้ในการพัฒนาหาเป้าหมายใหม่ของยาที่ใช้รักษาโรคจากแบคทีเรียเหล่านี้ได้ในอนาคต

CONTENT

	Pages
Abstract	3
Executive summary	5
CHAPTER I	11
Pseudomonas aeruginosa	
Glutathione peroxidase-like in *Pseudomonas aeruginosa*: a novel family of enzymes playing physiological role against oxidative stress	11
CHAPTER II	25
Vibrio harveyi	
Induction of peroxide and superoxide protective enzymes and physiological cross protection against peroxide killing by a superoxide generator in *Vibrio harveyi*	25
CHAPTER III	37
Agrobacterium tumefaciens	
3.1. *ohrR-ohr* are the primary sensor/regulator and protective genes against organic hydroperoxide stress in *Agrobacterium tumefaciens*	37
3.2. Atypical gene regulation and evaluation of the physiological role of *soxR* in *Agrobacterium tumefaciens*	63
CHAPTER IV	76
Xanthomonas campestris	
4.1. Novel organic hydroperoxide sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress	76
4.2. Chemical modulation of physiological adaptation and cross protective responses against oxidative stress in soil bacterium and phytopathogen, *Xanthomonas*.	97
4.3. The unique glutathione reductase from *Xanthomonas campestris* : gene expression and enzyme characterization.	105
CHAPTER V	121
Burkholderia pseudomallei	
DpsA protects the human pathogen *Burkholderia pseudomallei* against organic hydroperoxide	121
สรุปผลงานตลอดโครงการ	136
Appendix (Reprints)	152

Executive Summary

โครงการ:

การศึกษากลไกในระดับโมเลกุลของ organic peroxide sensor/transcription regulators และการตอบสนองของขบวนการนี้ต่อการเปลี่ยนแปลงของสภาพแวดล้อม

Molecular and Physiological Characterization of Family of Organic Peroxide Sensor and Transcription Regulators and How The System Response to Changes in The Environment

1. ความสำคัญและที่มาของปัญหา

The ability to sense changes in the stress level is absolutely essential for bacterial survival. Soil and pathogenic (both plant and animal) bacteria are exposed to reactive oxygen species (ROS) including H_2O_2 , organic peroxide and superoxide as results of environmental degradation and host defense response. One of the ROS, organic peroxide is highly toxic to biological molecules since it can participate in the generation of more organic radicals which could react with cellular macromolecules. To-date there have been only two sensor /transcription regulators of oxidative stress response, OxyR and SoxR have been characterized in all bacteria. These two proteins could not be responsible for all highly complex processes of oxidative stress response. Recently, we have identified OhrR, the first transcription repressor from *Xanthomonas* which senses only organic peroxide. Currently not much is known about the biochemical and structure of the protein. Although, search of the bacterial genomes show multiple copies of closely related OhrR homologues suggesting the family of OhrR like proteins may serve other sensing functions. *Agrobacterium tumefaciens, Pseudomonas aeruginosa, Vibrio harveyi* will be used as model organisms. The characterization of these regulators and their target genes will form basis for understanding of how other bacteria senses changes in organic peroxide level and response to it in other microorganisms.

2. วัตถุประสงค์

The major goal of the research is to understanding the function of these OhrR homologues and the genes regulated. Most of the genes regulated by OhrR such as *ohr*, glutathione S- transferase and chloroperoxidase have not been characterized and their biochemical and physiological functions are unknown. The knowledge will help elucidate the physiological function of these proteins and more importantly in general how bacteria can sense changes in the environment and send appropriate signals to activate or repress expression of genes in response to the stress. This is the central issue on how living organisms recognize changes in the environment and the signal transduction processes.

3. ระเบียบวิธีวิจัย

Our laboratories will use biochemistry, microbiology and molecular genetics techniques to elucidate the functions of these regulators and the genes they regulated. For biochemical and molecular biology characterization of OhrR and their target genes, large quantity of purified proteins are needed. These proteins will be produced using tightly regulated T-7 expression and poly-His tag fusion systems for high level expression and easy purification. The insertion inactivation technique will be use to construct knock out mutants and subsequent physiological and determination of resistance level to oxidants in these mutants will help define physiological roles of these genes.

4. แผนการดำเนินการวิจัยในแต่ละช่วง 6 เดือน

วัตถุประสงค์	แผนงานวิจัย	ปีที่ 1		ปีที่ 2		ปีที่ 3	
		1 - 6	7 - 12	1 - 6	7 - 12	1 - 6	7 - 12
P.aeruginosa							
1. *ohrR1 ohrR2*	1.1 Transcription organization and localization of promoters 1.2 In vivo promoter analysis 1.3 Construction and characterization of *ohrR1* and *ohrR2* mutant	←	→				
2. *P. aeruginosa* Glutathione peroxidases (Gpx)	2.1 Purification and biochemical characterization of three Gpx 2.2 Mutant construction and characterization of 3Gpx	←	→				
***Agrobacterium* oxidative stress response**							
1. In silico analysis of oxidative stress protective genes	1. Analysis of oxidative stress response genes	←	→				
2.*Agrobacterium*: Adaptive and cross protection response.	2.1 Oxidants induce protection against exposure of lethal concentrations of oxidants 2.2 Constructions of *oxyR*, *soxR* mutants	←	→	←	→		
3. *Agrobacterium ohrR1* to *ohrR5*	3.1 Transcription organization of *ohrR1* to *ohrR5* 3.2 Construction of mutants in *ohrR1* to *ohrR5* 3.3 Targets genes for *ohrR1* to *ohrR5* regulation 3.4 Cross regulation between different regulators	←	→				
4. *Agrobacterium* glutathione transferase	4. Biochemical and genetic and physiological characterization			←	→		
5. *Agrobacterium* chloroperoxidase	5. Biochemical and genetic and physiological characterization				←	→	
6. *Agrobacterium*	6. Biochemical and genetic and physiological					←	→

unknown oxidoreductase	characterization						
Vibrio harveyi							
1. Oxidant induction of oxidative stress protective enzyme	1. Oxidants induction of catalase, AhpC	◄	►				
2. Adaptive and cross protection against oxidative stress killing	2. Oxidants induce protection against lethal concentration of oxidants	◄	─	►			
3. The effect of heavy metals on oxidative stress response	3. Cd and Ni induce oxidative stress			◄	─	─	►

5. ประโยชน์ที่คาดว่าจะได้รับ

The proposal address basic research in the area of microbial gene regulation. The research will determine how bacteria in the environment senses changes in the chemicals in the surrounding environment. This could be important since increasing environmental degradation due to deliberate or accidental released of pollutants. These pollutants some of which are strong oxidants and they could modulate the oxidative stress response of microbes. Altered oxidative stress response could ultimately changes the disease development and progression since oxidative stress is often the mechanism hosts uses to defense against microbial infection. In addition, elucidation of sensing peroxide mechanism could be use to develop a biosensor system for detection of strong oxidant pollutants released into the environment. The understanding of microbe defense against organic peroxide can also be use to our benefit since inhibition of this process will make microbes more susceptible to host defense. Thus, this system is a potential new target for drug development.

6. Outputs

ผลงาน	ปีที่ 1	ปีที่ 2	ปีที่ 3
1. จำนวนผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ	4	5	5
2. จำนวนหนังสือ			
3. จำนวนผลงานการจดสิทธิบัตร			
4. จำนวนนักวิจัยรุ่นใหม่ที่สร้างจากโครงการ 4.1 สถาบันเดียวกัน 4.2 ต่างสถาบัน	2 4		
5. จำนวนนักศึกษาระดับปริญญาเอก	1	1	1
6. จำนวนนักศึกษาระดับปริญญาโท	1	1	1
7. อื่น ๆ			

7. ชื่อสารสารวิชาการระดับนานาชาติที่คาดว่าจะลงตีพิมพ์ (ระบุค่า impact factor)

	Impact factor (1999)
Molecular Microbiology	6.361
Journal of Bacteriology	3.712
Applied and Environmental Microbiology	3.541
Microbiology	2.700
Arch Microbiology	2.209
FEMS Microbiology Letters	1.673
Current Microbiology	1.165

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Expected Output ของทีมวิจัยแต่ละคน (First author or Corresponding author)

ผลงาน	ปีที่ 1	ปีที่ 2	ปีที่ 3	รวม
จำนวนผลงานตีพิมพ์ในวารสารวิชาการนานาชาติรวมทั้งโครงการ	**> 4**	**>5**	**> 5**	**>14**
หัวหน้าโครงการ				
รศ. ดร. ศกรณ์ มงคลสุข	2	3	3	8
ผู้ร่วมวิจัย				
ดร. สุวิทย์ ล้อประเสริฐ	1	1	1	3
ดร.ไพบูลย์ วัฒนวิบูลย์	1	1	1	3
Young Scientists				
ดร. สุนีย์ กอปรศรีเศรษฐ์		1		1
ดร. ยุวดี มหาคุณกิจเจริญ		1		1
ดร. ผดุงศรี วิชัยวาณิชย์		1		1
ดร. กอบชัย ภัทรกุลวณิชย์		1		1
ดร. กรประภา เครือวัลย์		1		1
นักศึกษาปริญญาเอก				
นางสาววราวรรณ เอี่ยมพึ่งพร		(1)		
นางสาวทัศนี ชูเชื้อ		(1)		
นางเบญจภรณ์ ประภักดี		(1)		

CHAPTER I

Glutathione peroxidase-like in *Pseudomonas aeruginosa*: a novel family of enzymes playing physiological role against oxidative stress

Abstract

Glutathione peroxidase is an antioxidant enzyme existed in diverse eukaryotic organisms. Here we reported isolation and characterization of three glutathione peroxidase-like genes (AAG06214, AAG04227 and AAG04676) designated *gpxA, gpxB* and *gpxC*, respectively from *Pseudomonas aeruginosa*, an important hospital acquired, human pathogen. Analysis of the putative amino acid sequences against the classical bovine GPx suggests lacking of glutathione binding domain in all *P. aeruginosa* GPxA, GPxB and GPxC. However, amino acid residues important for catalytic activity are quite conserved except in GPxC. Interestingly, the expression of *gpxA* could be highly induced by exposure of bacteria to sub-lethal concentration of various oxidant especially organic hydroperoxides and was co-transcribed with *ohrR2*, the gene homologous to organic hydroperoxide regulator (*ohrR*). Similar to *gpxC*, the expression of *gpxB* could not be induced by oxidants. Expression of cloned *gpxA* and *gpxB* from the plasmid vector in the mutant lacking functional organic hydroperoxide resistance enzyme (*P. aeruginosa ohr*) rendered cells more resistant to organic peroxides (both cumene and t-butyl hydroperoxides) killing treatments indicating that these glutathione peroxidase-like proteins could function complementarily to Ohr. Heterologous expression of *gpxA* or *gpxB* in *Escherichia coli* TA4315 (alkyl hydroperoxide reductase null mutant) increased degradation of organic peroxides but not H_2O_2. Our data elucidate an essential role of *gpx*-like family in protection of bacterial cells from oxidative stress.

Introduction

Pseudomonas aeruginosa is one of the most common Gram-negative bacteria found in nosocromial infections. This opportunistic pathogen causes significant mortality and morbidity in cystic fibrosis patients and in immunocompromised patients including those who suffered from burns, cancer and AIDS (6, 13). *P. aeruginosa* is a strictly aerobic microorganism which generally encounters oxidative stress generated by aerobic metabolisms as well as from phagocytic cells during infection. The ability to withstand oxidative attack is necessary to bacterial survival. The known enzymatic defense systems *P. aeruginosa* used to cope with such stress including two types of superoxide dismutases (SOD, namely [Fe]-SOD]

and [Mn]-SOD), two isozymes of catalases (KatA and KatB), three alkyl hydroperoxide reductases (AhpA, AhpB and AhpCF) and an organic hydroperoxide resistance enzyme (Ohr) (7, 8, 19).

Glutathione peroxidases (GPx) are diverse enzymes catalyzing hydrogen peroxide (H_2O_2), organic hydroperoxide and lipid hydroperoxides using a reduced glutathione as a reducing agent thereby having the protective activity against oxidative stress (27). Mammal GPxs usually contain a rare amino acid selenocysteine encoded by UGA stop codon in the active sites (9). Moreover, several *gpx* from plants, nematode and included the *gpx*-like from bacteria do not possess the UGA stop codon implying that these enzymes contain a cysteine instead of selenocysteine. In animal, where extensively studied have been described, various forms of GPx are known to be existed; the cytosolic GPx, the extracellular plasma GPx, the cytosolic gastrointestinal GPx, the phospholipids hydroperoxide GPx (PHGPx) and the selenium-independent epididymis GPx. These isozymes differ in their amino acid sequences, substrate specificity, and cellular locations (9, 27).

In prokaryotic cells, the *gpx*-like gene has been initially been identified in *Neisseria meningitidis* (17). Disruption of this *gpx*-like gene makes bacteria more sensitive to a redox cycling paraquat and H_2O_2 (16). *Synechocystis sp* possesses two *gpx*-like genes whose purified gene products capable of reducing organic hydroperoxides and lipid hydroperoxides in a GSH-independent manner (5). Moreover, these enzymes require NADPH as the electron donor. In this report, we isolated a family of *gpx*-like genes designated *gpxA, gpxB* and *gpxC* from *P. aeruginosa*. This is the first report clearly showed the *in vivo* function of prokaryotic *gpx*-like in detoxification of and protection of cells from organic hydroperoxides in pathogenic bacteria.

Analysis of glutathione peroxidase-like genes

A bioinformatic tool available at http//www.ncbi.nlm.nih.gov allowed us to perform BLASTP search using bovine cellular GPx amino acid sequence (4) against *P. aeruginosa* PAO1 genome sequence (23). The searched results showed 3 open reading frames namely AAG06214, AAG04227 and AAG04676 giving high score of identity to bovine GPx. The genes encoded these proteins were later designated *gpxA, gpxB* and *gpxC*, respectively. Alignment of the putative GPxA, GPxB and GPxC with bovine GPx were performed with CLUSTAL W program (26). The percent identities between bovine GPx and GPxA, GPxB or GPxC were 39, 34, and 20, respectively. It is noteworthy that the GSH binding residues corresponding to R57, K91, R103, R184 and R185 of the bovine GPx (4, 27) were absent in

all PAO1 GPx-like proteins suggesting that these enzymes probably do not use GSH as a physiological reducing substrate. *In vitro* functional analysis of purified GPx-like enzymes, namely GPx1 and GPx2 from *Synechocystis* whose primary structure homologous to GPxA, GPxB and GPxC (56, 43 and 33% for GPx1, respectively; and 46, 53 and 36% for GPx2, respectively) revealed that these *gpx*-like enzymes use NADPH instead of GSH as reducing molecules. The three distant protein loops namely N.A..C (position 47-52 in bovine GPx), L.FPCNQF..Q (position 77-87) and WNF (position 165-167), that are important for catalytic activity, are quite conserved in all GPx-like enzymes except for GPxC whose amino acid CNQ in the position 72-82 region are changed to SDD (Fig. 1) (4). However, all amino acid residues at the catalytic triad including C52, Q87 and W165 are conserved. Similar to other bacterial GPx-like enzymes, these *P. aeruginosa* GPxs are likely contain cysteine instead of selenocysteine.

Gene arrangement and expression patterns

The physical maps and gene arrangement of *gpxA, gpxB* and *gpxC* are shown in Fig. 2. *gpxA* located immediately upstream to *ohrR2* (this reading frame was named *ohrR2* because PAO1 possesses two *ohrR* genes). *gpxB* was surrounded by *slyD* and *tetR* while *gpxC* localized between genes encoding transporter and outer membrane proteins. Northern blot analyses were performed to characterize the expression patterns of *gpxA, gpxB and gpxC* in response to oxidative stress producing substances including organic hydroperoxide, H_2O_2, and superoxide generating agent menadione and paraquat. Under normal physiological condition, *gpxA* was transcribed in relatively high level judged by the band of mRNA hybridized with the radioactively labeled probe. Surprisingly, the expression of *gpxA* was inducible as cells were exposed to organic hydroperoxides while treating cells with other oxidants such as menadione, paraquat and H_2O_2 failed to significantly increase its expression. At the same concentration, it was clearly shown that CuOOH was a stronger inducer than tBOOH. We can notice a small hybridized band at the size of 700 bp only in the CuOOH-induced sample after probing with *gpxA*. The size is good agreement with the monocistronic mRNA of *gpxA*. This may be due to a posttranscription modification of this mRNA in a similar manner as the *ohrR-ohr* operon in *Xanthomonas campestris* (14, 24). Alternatively, there is another promoter for *gpxA*.

The size of positively hybridized band was approximately 1.2 kb indicating that *gpxA* was not transcribed monocistronically. This size of RNA was corresponded to that of *gpxA* plus ohrR2. Northern experiment was then repeated by changing the DNA probe to *ohrR2*.

Similar size and pattern of the hybridized bands were observed (data not shown). The fact that *gpxA* expression could specifically be induced by organic hydroperoxides implied that this gene could play an important role on protection of PAO1 from organic hydroperoxides and the finding that *gpxA* was co-transcribed with *ohrR2*, the gene homolog to organic peroxide sensor and transcription repressor *ohrR* of *X. campestris* (46 % identity) (24), suggested the possibility that the expression of *gpxA* is under the regulation of *ohrR2*. This hypothesis is investigating.

The result in Fig. 2 showed two hybridized bands of 1.3 and 0.7 kb after probing with *gpxB* suggesting that the gene was probably co-transcribed with other gene. When we repeated probing with *slyD*, similar patterns of two positive bands of 1.3 and 0.6 kb were obtained. Therefore, *gpxB* was transcribed in an operon with *slyD* encoding peptidyl-prolyl cis-trans isomerase (10). However, the expression of *gpxB* as well as *gpxC* could not be induced by pretreatments with oxidants (Fig. 2). Unlike *gpxA* and *gpxB*, *gpxC* was transcribed monocistronically only.

gpxA* and *gpxB* could function complementarily *ohr

Eukaryotic glutathione peroxidase can catalyze a variety of peroxides including H_2O_2, phospholipids hydroperoxides, and fatty acid hydroperoxids. The prokaryotic GPx-like enzymes have been demonstrated *in vivo* to have the ability to reduce organic hydroperoxides. In bacteria, two well-characterized mechanisms that protect cells from organic hydroperoxide toxicity are alkyl hydroperoxide reductase and organic hydroperoxide resistance enzymes (15, 22). PAO1 possess multicopies of *ahp* and a single copy of *ohr* (19). The *ohr* mutant (2) (POHR) has been constructed and showed significantly increased sensitivity to organic hydroperoxide killing. This strain is quite ideal for testing the ability of the isolated *gpx*-like genes on protection of cells from organic hydroperoxides. The full-length copies of *gpxA, gpxB* and *gpxC* were amplified using specific oligonucleotide primer pairs, designed from putative genes analyzed from *P. aeruginosa* genome sequence (23), and PAO1 genomic DNA as template in PCR reactions. The lists of the primers are BT189 5' CCTCAACAAGGAAGACAA 3'and BT190 5' GGTGCTCATTCAAGCAGC3' for *gpxA*; BT208 5' GGAGTACGTCATGAGTGC 3'and BT209 5' GGAGATCAACCCAGGGCC 3' for *gpxB*; and BT210 5' GGAGCCATTCCATGAAAC 3' and BT211 5' GACCCTCAGGGCTGGCTG 3' for *gpxC*. After checking the nucleotide sequences, the genes were subcloned into broad-host-range plasmid vector, pBBR1MSC-4 (12). The recombinant plasmids, pgpxA, pgpxB and pgpxC were finally transferred into the POHR

strain by electroporation. The resistance level against organic hydroperoxide killings in the POHR harboring pgpxA, pgpxB or pgpxC were determined by plating exponential phase cultures on medium containing appropriate concentrations of tBOOH or CuOOH. The ability of each strain to survive and form colonies was expressed in term of percent survival comparing with the number of the colony forming units of the same strain on plates without organic hydroperoxides. The result showed that the presence of CuOOH and tBOOH at concentration of 450 and 350 μM, respectively did not cause any adverse effects on the survival of the PAO1 strain but significantly reduced the ability to form colony of the POHR strain to the level of approximately 5% survival. High expression of *gpxA* in the POHR (POHR/pgpxA) could partially make the mutant more resistant to CuOOH and tBOOH to the level of 60 and 70% survival, respectively. Similarly, POHR harboring pgpxB plasmid conferred resistance to CuOOH and tBOOH to the level of 80 and 60% survival, respectively. The results suggested that both gpxA and gpxB could partially function complementarily to *ohr* to protect bacterial cells from CuOOH and tBOOH toxicity and implied the essential role of *ohr* required for full protection. We can notice that high expression of gpxA was slightly better than that of gpxB for protecting POHR from tBOOH toxicity and vice versa for protection against CuOOH.

However, elevated level of gpxC in the POHR bearing pgpxC plasmid failed to render cells increased resistance to both CuOOH and tBOOH. The inability of gpxC to function complementarily *ohr* in POHR is not unexpected because its putative amino acid sequence is quite different from GPxA (34% identity), GPxB (38% identity) and other known gpx-like proteins including *Synechocystis* GPx1 (33% identity), GPx2 (36% identity) and *Neisseria meningitidis* GPxA (23%). We have shown earlier that one of some amino acid residues at the active domain surround Q87 in GPxC (PSDDF..Q) were differed from the conserved sequence (PCNQF..Q) suggesting that this gpxC may be the mutated and non-functional copy gene. Alternatively, both CuOOH and tBOOH are not the suitable substrates for this enzyme. These hypotheses need further investigations.

This is the first *in vivo* observation that high expression of prokaryotic *gpx*-like gene could protect bacterial cells from organic hydroperoxides and suggesting the crucial and physiological role of the *gpx*-like family in the oxidative stress response in this human pathogen.

Heterologous expressions of *gpxA* and *gpxB* in *E. coli* increase *in vivo* degradation of organic hydroperoxides

Since we did not have the purified GPxA and GPxB yet, *in vivo* experiments were set to determine their ability to directly detoxify tBOOH and CuOOH using a modified xylenol orange colorimetric method to measure organic hydroperoxide degradation rates in various bacterial strains. The exponential phase cultures (OD500 = 0.5) of PAO1 and POHR harboring pgpxA, pgpxB and pBBR1MSC-4 vector were incubated with 100 μM of tBOOH and CuOOH. The rate of tBOOH and CuOOH degradation was measured at 10 min interval. Unfortunately, all strains could totally degrade tBOOH and CuOOH within the first ten minutes (data not shown) and we could not increased the organic peroxide concentrations to the higher level because it will cause serious effect on the bacterial cells. This may be due to the existence of several copy of *ahp* in the POHR strain. We then change the host cells to an *Escherichia coli*, the other well-characterized bacteria. The major mechanism *E. coil* used to defend themselves from organic hydroperxide is involved the function of AhpCF. Inactivation of *ahpCF* in *E. coli* (TA4315) (22) makes cells highly susceptible to organic hydroperoxides. The ability of GPxA, GPxB and GPxC to degrade organic hydroperoxides was assayed in TA4315 harboring pgpxA, pgpxB and pgxpC. The log-phase cultures (OD_{600} of 0.5) of TA4315 bearing plasmid for high expression of each *gpx*-like gene and the vector control were incubated with 100 μM tBOOH or CuOOH. The rate of tBOOH and CuOOH degradation were measured. The TA4315/pBBR1MSC-4 control still degrade about 40% and 30% of tBOOH and CuOOH, respectively after 30 min incubation suggesting the involvement of the mechanisms other than AhpCF capable of effectively degrading organic hydroperoxides. Several kinds of non-heme peroxidase including bacterioferritin-comigratoty protein (11) and thiol peroxidase (3) have been shown to have peroxidatic activity against organic hydroperoxides. The strains with high expression of *gpxA* and *gpxB* (TA4315/pgpxA and TA4315/pgpxB) degraded both tBOOH and CuOOH at higher rate than the control while the degradation rate of TA4315/pgpxC was similar to that of the control. The results suggest that both GPxA and GPxB possess *in vivo* activity to degrade organic hydroperoxides. However, when we considered the rate of degradation, it was clearly showed that the rate to degrade tBOOH in the strain with high expression of *gpxA* was significantly higher than that with *gpxB*. Contradictorily, the strain having pgpxB degrade CuOOH at more rapid rate than that having pgpxA. The results suggested that although GPxA and GPxB could directly degrade organic hydroperoxides, they favor distinct type of organic hydroproxides. The former prefers small and water soluble peroxide i.e. tBOOH while the latter likes more

complex and hydrophobic peroxide i.e. CuOOH. These data clearly showed the physiological function of these *gpx*-like enzymes in degradation of organic hydroperoxides.

The ability to degrade H_2O_2 was also determined by repeating the experiment in catalase deficient *E. coli* strain UM255 (18) harboring pgpxA, pgpxB, pgpxC or pBBR1MSC-4 and used 100 μM H_2O_2 instead of organic hydroperoxide. The results showed no differences in the rate of degradation in all tested strains suggesting that these *gpx*-like genes are likely not involved in H_2O_2 degradation. Since, the reduction rate in the UM255 containing pBBRMSC control was comparatively high, it was indicated that not only KatG (catalase HPI) and KatE (catalase HPII) are important for H_2O_2 degradation (18), other enzymes also play a role in degradation of H_2O_2. AhpCF are one of the enzymes has been shown to have the ability to reduce H_2O_2 (21).

Our evidences suggest a novel and essential role of the *gpx*-like genes especially *gpxA* and *gpxB* in prevention of *P. aeruginosa* from organic hydroperoxide toxicity. The enzymatic and kinetic properties of these GPx are actually unknown and need further investigations. Lacking of the putative GSH binding sites suggests the enzymes are unlikely used this thiol molecule as a substrate. Recently, it has been shown that a putative GPx from *Plasmodium falciparum* reacts with thioredoxin much more effective that the GSH (25). Hence thioredoxin is probably the physiological reducing substrate for these peroxidases. The idea is consistent with the preliminary observation in *E. coli* that purified GPx-like protein (formerly BtuE) possesses *in vitro* thioredoxin peroxidase activity (3).

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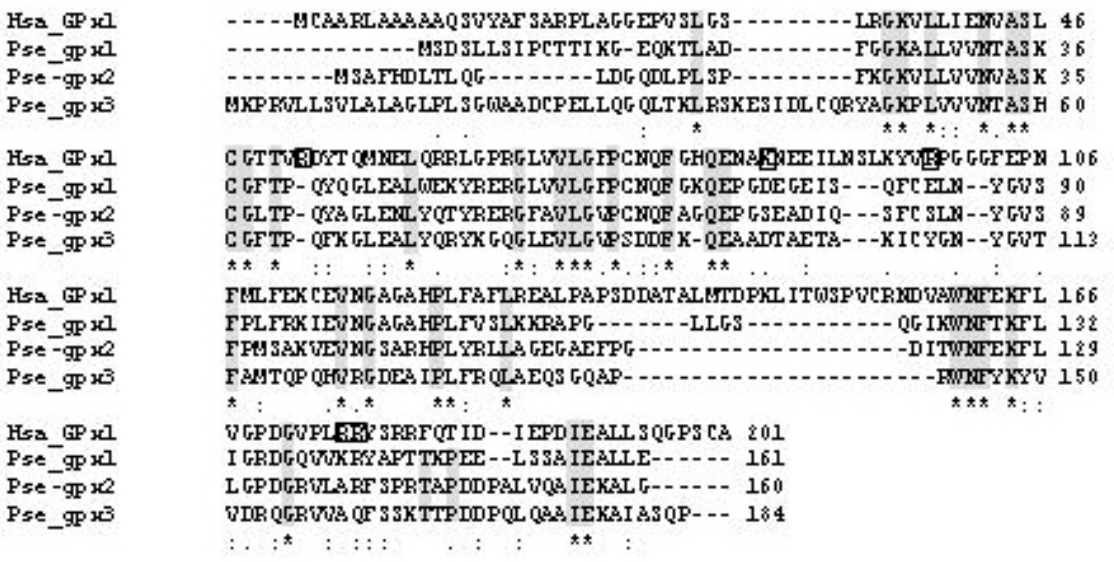

Fig. 1. Multiple alignments of *gpxA, gpxB* and *gpxC* with human *gpx*.

Amino acid sequences of GPX proteins were retrieved from public sequence databases using the BLAST program (1). The alignments were performed by using the multiple alignment feature of CLUSTAL W version 1.7 (26) with maximal fixed-gap and gap extension penalties.

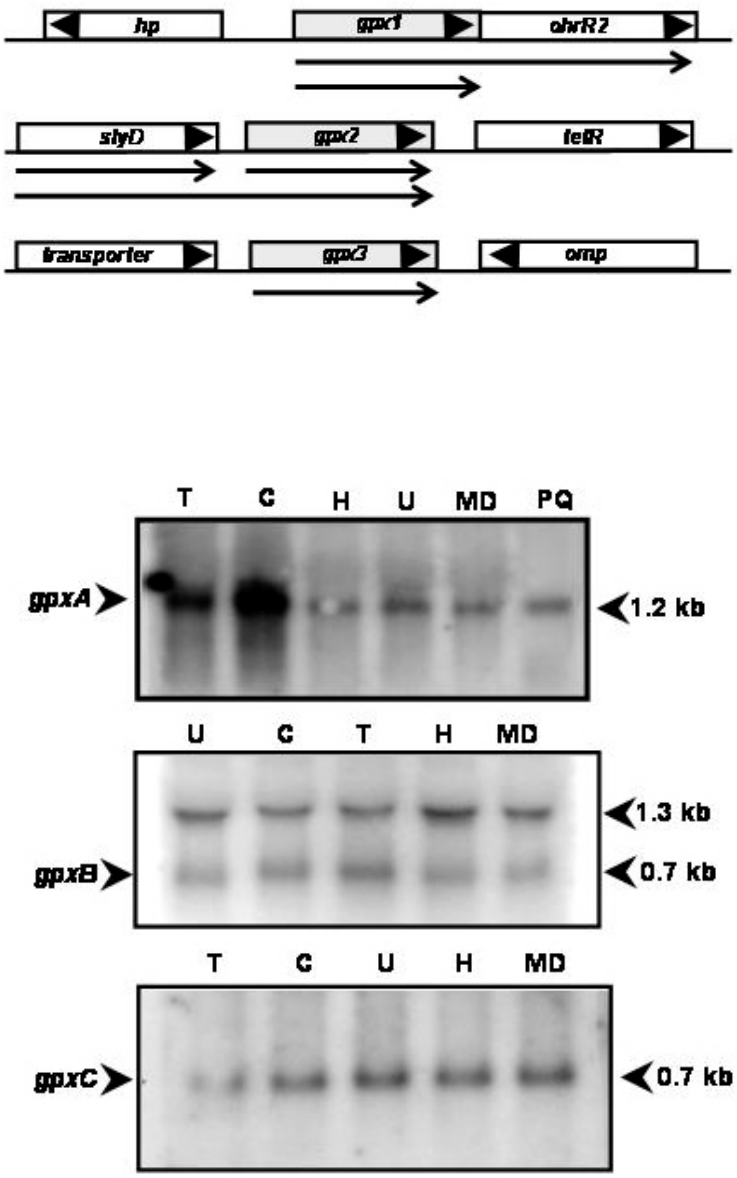

Fig. 2. Physical maps and the expression patterns of *gpx*-like genes from *P. aeruginosa* Northern blot analysis was performed under standard protocols (20). Total RNA (10 mg) extracted from PAO1 log-phase cells cultured under uninduced condition (U) and induced with 200 μM of H_2O_2 (H), CuOOH (C), tBOOH (T), H_2O_2, menadione (MD) or paraquat (PQ) for 15 min, were separated in 2% formaldehyde agarose gel electrophoresis, transferred to nylon membrane and hybridized with radioactively labeled DNA probe.

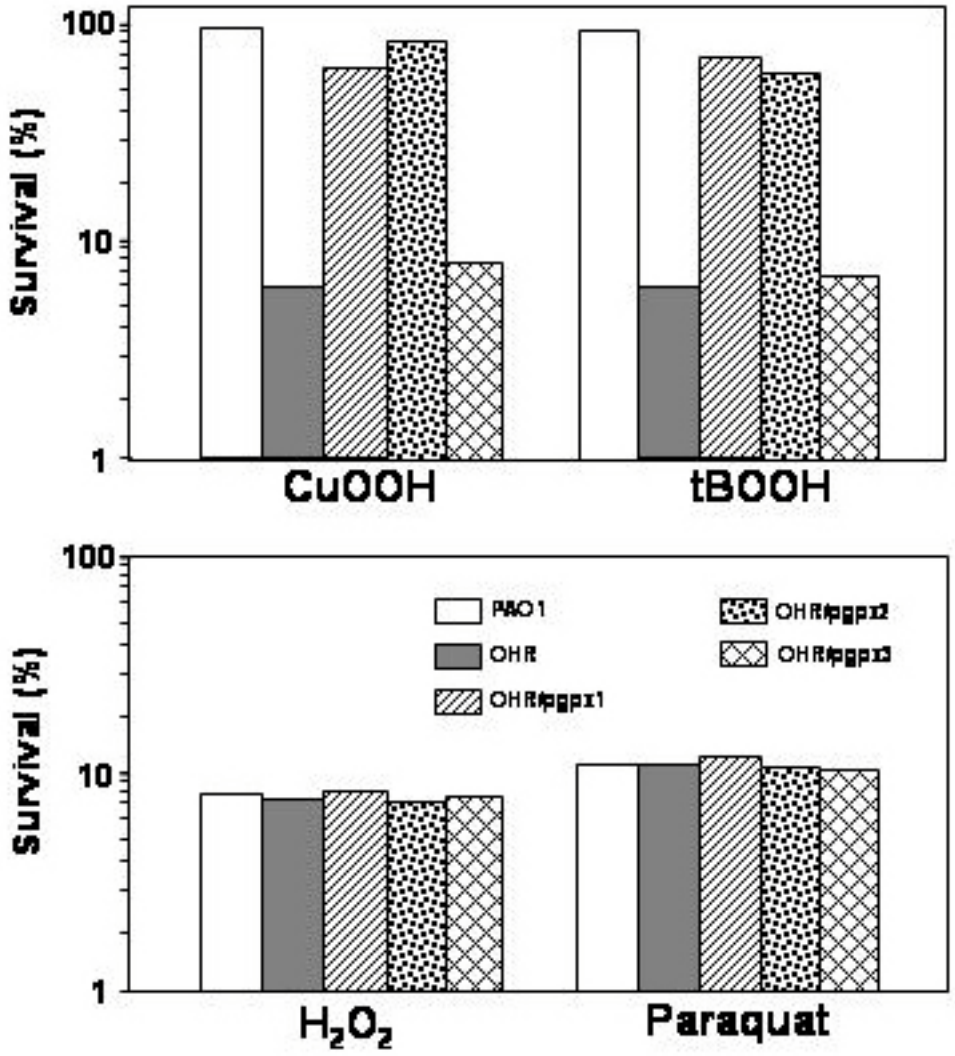

Fig. 3. The effect of high expression of *gpx*-like genes in *P. aeruginosa ohr* mutant Overnight cultures of PAO1, and POHR harboring pgpxA (POHR/pgpxA), pgpxB (POHR/pgpxB), pgpxC (POHR/pgpxC) and pBBRMSCx (POHR) were inoculated into fresh LB medium and incubated until $OD_{600} \approx 0.5$. Appropriate dilutions of bacterial cells were plated onto LB agar containing 450 μM CuOOH, 350 μM tBOOH, 500 μM H_2O_2 or 500 μM paraquat. The survived colonies were counted after 24 h incubation at 37°C and percent survival comparing to the viable count on LB agar were determined.

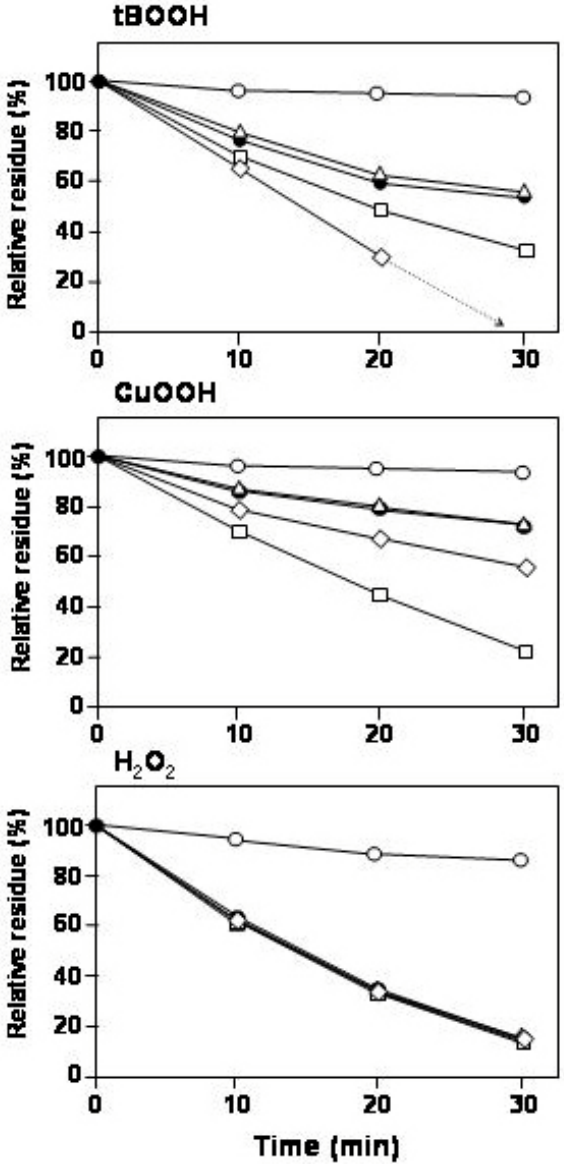

Fig. 4. Functional analysis of *gpx*-like genes in *E. coli*

The degradation of organic hydroperoxides were measured as described by Ochsner *et al*. (19) with some modifications (28). The log phase cultures of *E. coli* strains (TA4315 for CuOOH and tBOOH; UM255 for H_2O_2) harboring pgpxA (◊), pgpxB (□), pgpxC (Δ), and pBBRMSCx (●) were adjusted to OD_{600} of 0.5 with fresh LB medium prior to an addition of CuOOH or tBOOH to a concentration of 100 μM. Residual organic hydroperoxide concentrations were determined at 10-min intervals by a xylenol orange-iron reaction. At indicated time, 1 ml of the culture was removed and cell pelleted. Then, 100 μl of the clear supernatant was added to 400 μl of 25 mM sulphuric acid in a 1 ml cuvett. Subsequently, 500 μl of freshly prepared reaction buffer (200 μM ammonium ferrous sulfate, 200 μM xylenol orange and 25 mM sulphuric acid) was added to the mixture. After exactly 10 min incubation at room temperature, the absorbance at 540 nm was monitored. The concentration of residual organic peroxide in the culture was calculated using standard organic hydroperoxide in LB medium. ○ represents the degradation rate in LB broth without bacterial cultures.

CHAPTER II

Induction of peroxide and superoxide protective enzymes and physiological cross protection against peroxide killing by a superoxide generator in *Vibrio harveyi*

Summary

Vibrio harveyi is a causative agent of destructive luminous vibriosis in farmed black tiger prawn (*Penaeus monodon*). *V. harveyi* peroxide and superoxide stress responses toward elevated levels of a superoxide generated by menadione were investigated. Exposure of *V. harveyi* to sub-lethal concentrations of menadione induced high expression of genes in both the OxyR-regulon (e.g., a monofunctional catalase or KatA and an alkyl hydroperoxide reductase subunit C or AhpC), and the SoxRS regulon (e.g., a superoxide dismutase and a glucose-6-phosphate dehydrogenase). *V. harveyi* expressed two detectable, differentially regulated SOD isozymes, [Mn]-SOD and [Fe]-SOD. [Fe]-SOD constitutively throughout the growth phase while [Mn]-SOD was expressed at the stationary phase and could be induced by a superoxide generator. Physiologically, pretreatment of *V. harveyi* with menadione induced cross-protection against subsequent exposure to killing concentrations of H_2O_2. This induced cross-protection required newly synthesized proteins. However, the treatment did not induce significant protection against exposures to killing concentrations of menadione itself or cross protect against an organic hydroperoxide (t-butyl hydroperoxide). Unexpectedly, growing *V. harveyi* in high salinity media induced protection against menadione killing. This protection was independent of superoxide dismutase induction. Stationary phase cells were more resistant to menadione killing than exponential phase cells. The induction of oxidative stress protective enzymes and stress altered physiological responses could play a role in the survival of this bacterium in the host marine crustaceans.

1. Introduction

Vibrio harveyi is a luminous bacterium commonly found as part of the normal flora in light organs of certain marine cephalopods and in the intestine of tropical marine animals. It is also recognized throughout Asia and Australia as a destructive pathogen of cultured marine animals, and particularly in farmed black tiger prawns, *Penaeus monodon* [1-3].

Reactive oxygen species (ROS) are important components of the defense response generated by crustacean haemocytes against invading microorganisms [4,5]. At the same time, environmental degradation and contamination of natural waters with herbicides and chemical residues that are strong oxidants could modulate the bacterial oxidative stress response making bacteria more resistant to such stress. Thus, it is possible that environmental exposure to strong oxidants could alter disease progression and outcomes in crustaceans. Most ROS including superoxide anions, peroxides and hydroxyl radicals are highly toxic and can cause damage to macromolecules. In order to colonize and proliferate in a host, bacteria must protect themselves from such ROS by detoxification and they have multiple, appropriate defense responses that can be adjusted according to nature and amount of ROS. For example, *Escherichia coli* has independent multigene responses to at least two types of oxidative stress. Excess H_2O_2 triggers the expression of genes in the OxyR regulon, including those for catalase and alkyl hydroperoxide reductase. Excess superoxide radicals trigger the SoxRS regulon that coordinates the transcriptional induction of many promoters including those for a manganese containing superoxide dismutase ([Mn]-SOD) gene and a glucose-6-phosphate dehydrogenase (G6PD) gene [6,7]. SODs are essential enzymes for aerobic organisms. They are important for the maintenance of cells and DNA integrity during aerobic metabolism since they eliminate superoxide radicals that induce lipid damage and oxidative inactivation of essential enzymes. More importantly, SOD prevents the formation of the highly toxic and mutagenic hydroxyl radicals, the product of superoxide radicals, reduced iron and H_2O_2 [8].

Defense against ROS is important mechanisms for bacteria to survive in stressful environment. We have previously shown that prior exposure of *V. harveyi* to sub-lethal concentrations of peroxide resulted in a several fold increased in expression of OxyR regulated catalase and alkyl hydroperoxide reductase genes [9]. Here, we describe the effects of a superoxide generator menadione on induction of peroxide scavenging and superoxide protective enzymes and on menadione induced cross-protection to peroxide killing.

2. Materials and Methods

2.1. Bacterial growth conditions

A *V. harveyi* strain pathogenic to shrimp [3] was grown aerobically in LBS medium (LB supplemented with 2%, w/v NaCl) at 30 ^{o}C. To ensure uniform growth, overnight cultures were inoculated into fresh LBS medium to give an OD_{600} of 0.1. By subsequent monitoring of bacterial growth spectrophotometrically at OD_{600}, exponential phase cells (OD_{600} 0.8, were obtained at approximately 3 h) and stationary phase cells (OD_{600} 5.5, at approximately 18 h cultivation) were used in experiments.

2.2. Quantitative determination of resistance levels to oxidants

Induced adaptive and cross protection response experiments were performed by adding either 400μM menadione, 250 μM H_2O_2 or 200 μM tBOOH to exponential phase cultures. The cultures were then allowed to grow for an additional 30 min before aliquots of cells were treated with killing concentrations of menadione (200, 300 and 400mM), H_2O_2 (100, 200, and 300 mM) or tBOOH (100, 150, and 200 mM). After 30 min treatment, samples were pelleted and washed once with fresh medium before appropriate dilutions were plated on LBS agar plates. Cells that survived various treatments were counted after 24 h incubation at 30 ^{o}C. Surviving fractions are defined as the number of colony forming units (c.f.u.) obtained after the treatment divided by c.f.u. prior to treatment. All experiments were repeated at least three times and representative data are shown.

2.3. Catalase activity gels and assays

Crude bacterial lysates were prepared by resuspending *V. harveyi* cell pellets in 50 mM sodium phosphate buffer, pH 7.0 containing 1 mM phenylmethylsulfonyl fluoride and exposing them to intermittent sonication on ice until the suspensions become clear. The lysates were then centrifuged at 10 000Xg for 10 min before being used for enzyme assays and activity gel staining. Total catalase activity was spectrophotometrically measured by the disappearance of H_2O_2 at OD_{240} [10]. Catalase activity was visualized on 7.5% non-denaturing polyacrylamide gels prepared as previously described [9].

2.4. Superoxide dismutase activity gels and assays

Xanthine-xanthine oxidase coupled reduction of cytochrom *c* was used to monitor total SOD activity [11]. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. To visualize SOD activity, non-denaturing electrophoresis was performed using a 10% polyacrylamide gel (pH 8.7) with a

5% stacking gel (pH 8.0) followed by staining with nitroblue tetrazolium/ riboflavin photochemical stain as described by Beauchamp and Fridovich [12]. In the enzyme inhibition tests H_2O_2 or potassium cyanide were added to a riboflavin-TEMED solution at final concentrations of 5 and 2 mM, respectively. [Cu, Zn]-SOD is inactivated by cyanide and [Fe]-SOD by H_2O_2 while [Mn]-SOD is resistant to both [13].

2.5. Western immunoblot analysis

For Western immunoblot analysis of AhpC levels, conditions of SDS-PAGE, blotting to PVDF membranes and immunodetection against anti-*E.coli* AhpC polyclonal antibody were performed as previously described [9]. Antibody reactions were developed using an alkaline phosphatase antibody detection kit from Promega (USA).

3. Results and discussion

3.1. Analysis of SOD isozymes during different phases of growth

Analysis of genomic DNA sequences from the GenBank database of a closely related bacterium, *V. cholerae* showed three possible open reading frames with homology to [Mn]-SOD (AAF95835), [Fe]-SOD (AAF95193) and [Cu-Zn]-SOD (AAF94737). This suggested that *V. harveyi* might also possess more than one SOD isozymes. Thus, experiments were done to test for different SOD isozymes using cell lysates prepared at various growth phases of *V. harveyi.* Lysate proteins were separated on native-PAGE and stained for SOD activity as described in the experimental procedures. The results showed that exponential phase cells produced only one detectable SOD isozyme and that stationary phase cells produced an additional SOD (Fig. 1A). However, the additional isozyme in stationary phase cells was present in minor quantity as indicated by staining intensity. It was possible to distinguish amongst the SOD isozymes using specific enzyme inhibitors. Results revealed that the major SOD band was abolished by H_2O_2 treatment, identifying it as an [Fe]-SOD (data not shown). By contrast, activity of the minor band present at stationary phase could be detected in the presence of either H_2O_2 or cyanide treatments, identifying it as an [Mn]-SOD (data not shown). Induction of [Mn]-SOD by cells entering the stationary phase has been previously reported for bacteria [14].

3.2. Induction of [Mn]-SOD and glucose-6-phosphate dehydrogenase by menadione pretreatment

First, we examined the effects of menadione and peroxide pre-treatments on levels of two enzymes, SOD and G6PD that are known to be regulated by superoxide anion in other bacteria. Total G6PD and SOD activity was measured in lysates prepared from cells untreated and pretreated with 400 μM menadione, 250 μM H_2O_2 and 200 μM *tert*-butyl hydroperoxide (tBOOH). G6PD levels increased 1.8 fold in response to menadione pre-treatment, while pre-treatments with peroxides (both H_2O_2 and tBOOH) had no effect (Fig.1B). Similarly, SOD activities increased 1.4 fold with menadione pre-treatment (Fig. 1C). H_2O_2 and tBOOH pre-treatment have no effect on total SOD activity (Fig.1C).

Additional experiments were done to determine which SOD isozyme was induced by the menadione treatment. By activity staining of a native gel (Fig. 1A), the intensity of the [Fe]-SOD band from menadione-induced and uninduced cultures was not significantly different. However, the [Mn]-SOD band was detected only in menadione-induced cultured and this was correlated with the increase in total SOD activity (Fig. 1C). The results suggested that menadione induce expression of [Mn]-SOD most likely occurred via the production of superoxide anion.

Thus, induction of G6PD and SOD activities seem to be specific to the menadione pretreatment as has been observed in other bacteria. For example, superoxide induction in *E. coli* is regulated by a superoxide sensor and transcription repressor called SoxR. SoxR is, in turn, regulated by expression of an AraC family transcription regulator called SoxS [7]. Genetic analysis has shown that SoxR/S regulate both [Mn]-SOD and *zwf* (coding for G6PD) genes. Analysis of the genome sequences of *V. cholerae* revealed an open reading frame with coding potential for a SoxR homologue (AAF95998) suggesting that such a regulator might also be mediate the superoxide stress response in *V. harveyi*.

3.3. Induction of peroxide scavenging enzymes in V. harveyi pretreated with menadione

Different bacteria have evolved different responses to oxidative stress. Pretreatment of *V. harveyi* with a sub-lethal concentration of H_2O_2 or organic hydroperoxide could induce the expression of catalase (*katA*) and alkyl hydroperoxide reductase catalytic subunit C (*ahpC*) genes in an OxyR-regulon [9]. In vivo, menadione gives rise to production of superoxide anion that can be dismutated or chemically broken down to H_2O_2 [15]. The effect of menadione pretreatment on levels of AhpC and catalase was investigated in *V. harveyi* cultures pre-treated with 400 μM menadione for 30 min before harvesting. The levels of AhpC and catalase were measured by enzyme assay and by Western immunoblot analysis coupled with activity staining. The results (Fig. 2A, B) showed that pre-exposure of *V.*

harveyi to menadione resulted in a several fold increase in the level of both AhpC and KatA. Densitometer analysis of AhpC in Western blots indicated a 5-fold increase in the protein level. Total catalase activity increased three fold from 39.5 ± 5.6 U to 126.9 ± 10.1 U (mg protein)$^{-1}$. These results suggested that superoxide anions activated the expression of *ahpC* and *katA* genes in an OxyR regulon. Activation of OxyR-regulated genes by superoxide anions would most likely occur via the production of peroxide as the result of superoxide anion breakdown. Elevated H_2O_2 would then oxidize OxyR and convert it from an inactive reduced state to an active oxidized state that is known to positively regulated genes in the regulon [6,16]. Consistent with the idea, we have already shown that H_2O_2 is a potent inducer of catalase and AhpC in *V. harveyi* [9]. Similar findings have been reported in other bacteria [17].

3.4. The effects of menadione induced adaptive and cross protective responses against peroxide killing

Pre-exposure to a low concentration of an agent often confers resistance to sub-sequent challenge with a lethal concentration of the same agent (i.e. an adaptive response). Thus, we measured the physiological effects of menadione pre-treatment on superoxide and peroxide killing. *V. harveyi* cultures pre-treated with menadione and subsequently challenged with a lethal concentration (Fig. 3A) showed no adaptive protection even though menadione induced high levels of G6PD, [Mn]-SOD and the peroxide scavenging enzymes, catalase and AhpC.

In addition to adaptive response, ROS can sometimes induce protection to an unrelated agent. This cross-protection response has been observed in many bacteria including *V. harveyi*. Since menadione induced many peroxide scavenging enzymes, its ability to induce protection against H_2O_2 and an organic peroxide was also investigated. *V. harveyi* cultures pre-treated with 400 μM menadione prior to being challenged with lethal doses of H_2O_2 or tBOOH showed over 100-fold more resistance to H_2O_2 killing than uninduced cells (Fig. 3B). By contrast, there was no cross-protection against tBOOH killing (Fig. 3C). Addition of the protein synthesis inhibitor, chloramphenicol, completely abolished cross protective response to H_2O_2 kiliing, indicating that it required newly synthesized protein(s) (Fig. 3B).

Superoxide anion-induced cross-protection to H_2O_2 or tBOOH killing has been observed in other bacteria such as *Rhizobium leguminosarum* [18] and *Xanthomonas campestris* [17]. Although menadione was a potent inducer of AhpC, the catalytic subunit of alkyl hydroperoxide reductase, this induction did not result in protection against tBOOH for *V. harveyi*. Similarly, we have observed that *V. harveyi* pre-treated with H_2O_2 induced high

levels of AhpC but do not confer protection against tBOOH killing [9]. These results suggest that other protective enzymes are required for protection against tBOOH toxicity in *V. harveyi.*

3.5. High salinity protects V. harveyi from menadione killing

The absence of an adaptive response to menadione killing would seem to be a disadvantage to bacteria. However, in warm marine water, the physiological habitat of *V. harveyi*, bacteria are expose to a high salinity environment. We discovered that growing *V. harveyi* in LBS (LB broth supplemented with 2% NaCl) resulted more resistance to menadione killing than with growth in normal LB broth (data not shown). The effect of high salinity in growth media on menadione killing of *V. harveyi* was investigated in LB broth without NaCl and with 1, 2, 3, 4, and 5% sea salt (Sigma, USA) supplementation. Full strength marine water corresponds to approximately 3.5%. Exponential phase cultures in LBS (2% NaCl) were subsequently treated with 200 mM menadione for 30 min. The results showed that high salinity made *V. harveyi* more resistant to menadione killing (Fig. 4A). Similar findings have been reported in other bacteria treated with the redox cycling agent paraquat [19]. It is known that *V. harveyi* causes the most serious outbreaks in farmed tiger prawns during summer months in Thailand when the salt concentration in coastal shrimp farms is relatively higher than in other seasons [3]. Since shrimps are euryharic and adjust internal osmotic balance to match that of the environment, it is possible that high salt concentration protects *V. harveyi* from superoxide anions generated by host prawn hemocytes as a part of their defense mechanism.

3.6. Stationary phase cells were more resistant to menadione killing

Stationary phase *V. harveyi* are more resistant to lethal doses of H_2O_2 and the thiol depleting agent (iodoacetamide) than exponential phase cells [9]. By contrast, stationary phase cells are highly susceptible to *t*BOOH killing [9]. Thus, the pattern of resistance or sensitivity to oxidative stress of stationary cells is likely dependent on the type of stress. Resistance to lethal concentrations of menadione was compared for stationary phase cells and exponential phase cells. The survival curves (Fig. 4B) showed that cells from stationary phase cultures were 100-fold more resistant to menadione killing than exponential phase cells. However, the intracellular level of SOD did not account for this stationary phase resistance (data not shown). We have already shown that [Mn]-SOD induced by menadione pre-treatment (Fig. 1A) could not protect cells from lethal concentrations of menadione (Fig. 2A). On the other hand, multi-stresses resistance of stationary phase cells is common in many bacteria [20-23]

and expression of genes controlled by a stationary phase-specific sigma factor RpoS (σ^{38}) is responsible for this increased resistance [21,22,24,25]. Indeed, stationary cells of a *V. cholerae rpoS* mutant are more susceptible to stringent environments and particularly oxidative stress [25]. This increased menadione resistance of *V. harveyi* stationary phase cells may arise by similar mechanism.

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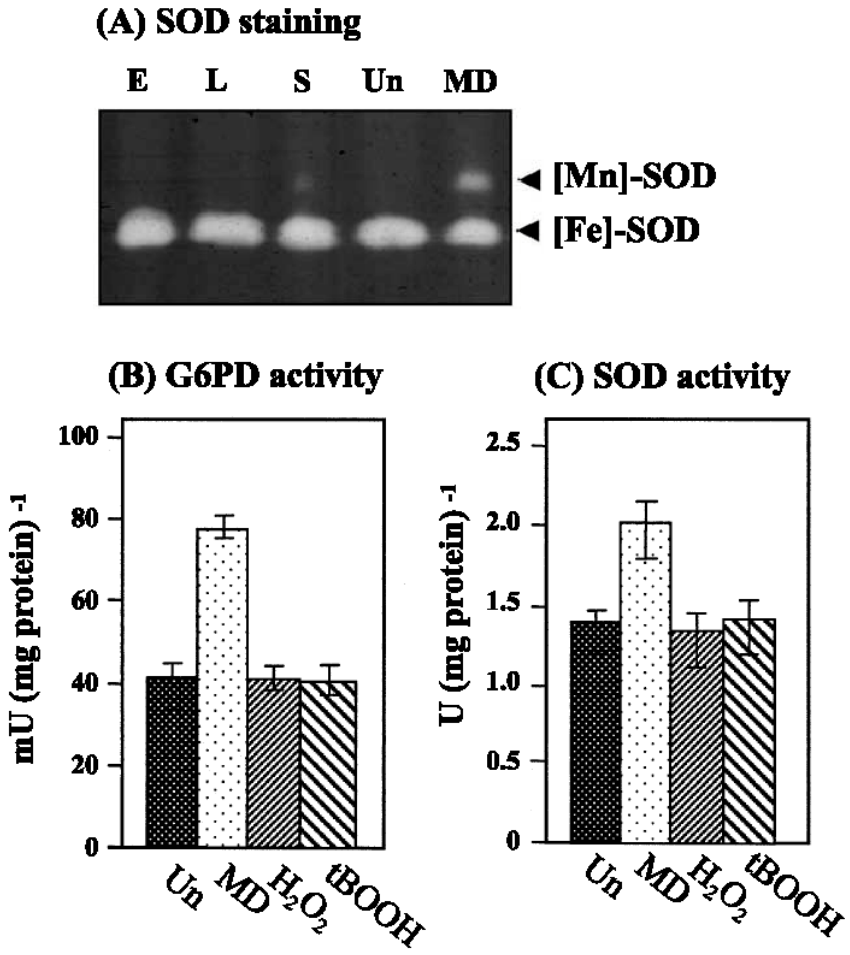

Fig. 1. Visualization of SOD isozymes and induction of SOD and glucose-6-phosphate dehydrogenase in *V. harveyi* by pretreatment with menadione

SOD activity staining (A) was performed as described in the experimental procedures to visualize SOD isozymes in various growth phases; early exponential (E), late-exponential (L) and stationary phases (S), and in uninduced (Un) and menadione-induced (MD) cultures. Glucose-6-phosphate dehydrogenase activity (B) and total SOD activity (C) in lysates prepared from *V. harveyi* culture induced with menadione (MD), H_2O_2, tBOOH and uninduced control (Un).

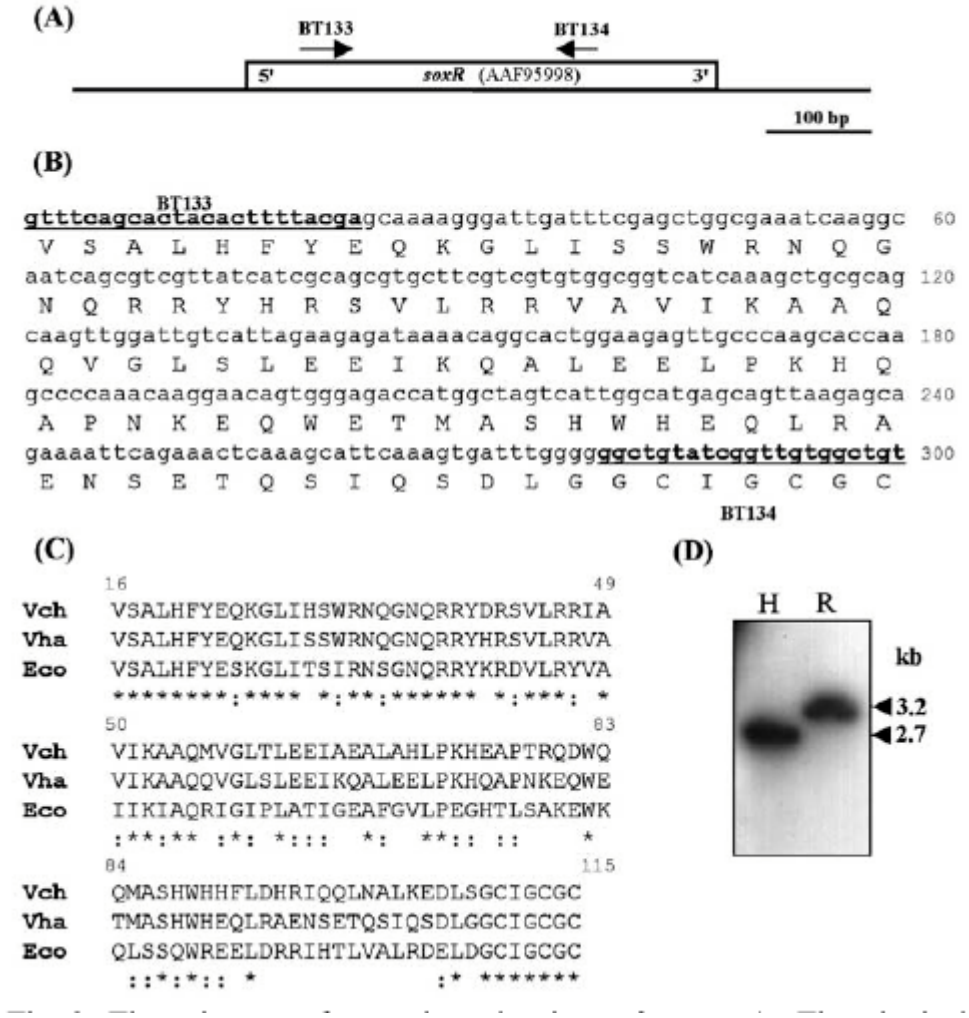

Fig. 2. Induced peroxide scavenging enzymes in *V. harveyi* by pretreatment with menadione

Crude lysate (80 μg protein) prepared from the exponential phase of uninduced (Un) and menadione-induced (MD) cultures were separated by PAGE and stained for catalase activity (A) or processed Western immunoblot to detection of AhpC (B) as described in the materials and methods.

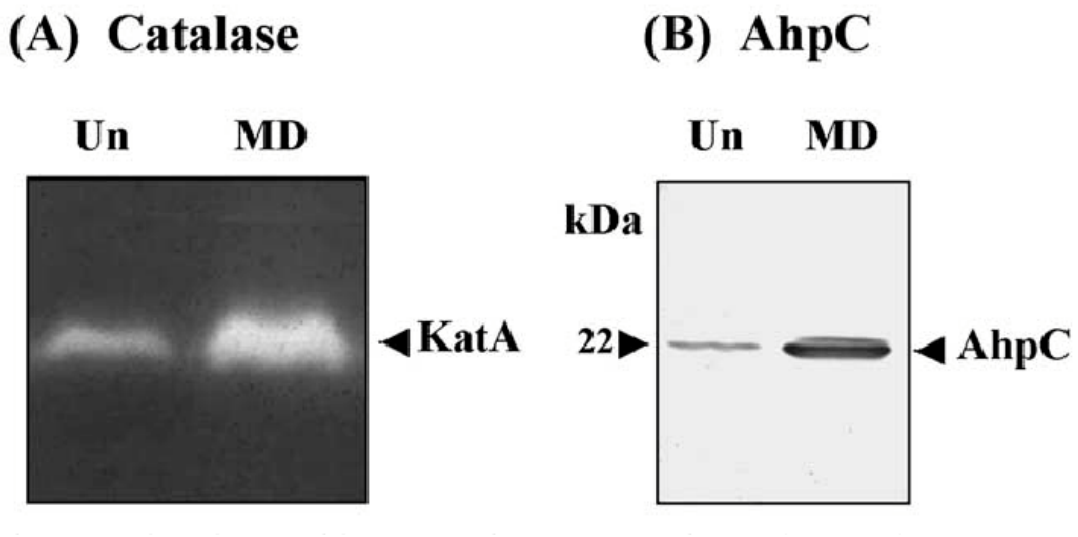

Fig. 3. Induced protective responses in *V. harveyi*

Cultivation conditions, induction and killing were as described in the materials and methods. (A) menadione survival curves for uninduced (●) and menadione-induced cultures (△); (B), H_2O_2 survival curves for cultures uninduced (●), menadione-induced (△) and menadione induced in the presence of a protein synthesis inhibitor (▲) at various concentrations of H_2O_2. (○) represents the survival of untreated cultures in the present of protein synthesis inhibitor alone. (C) *t*BOOH survival curves for uninduced (●) and menadione-induced (△) cultures. Values presented are means and standard deviations of three replicates.

CHAPTER III

3.1. *ohrR-ohr* are the primary sensor/regulator and protective genes against organic hydroperoxide stress in *Agrobacterium tumefaciens*

Abstract

The genes involved in organic hydroperoxide protection in *Agrobacterium tumefaciens* were functionally evaluated. Gene inactivation studies and functional analyses have identified *ohr,* encoding a thiol peroxidase, as the gene primarily responsible for organic hydroperoxide protection in *A. tumefaciens*. An *ohr* mutant was sensitive to organic hydroperoxide killing and had a reduced capacity to metabolize organic hydroperoxides. *ohr* is located next to, and is divergently transcribed from, *ohrR,* encoding a sensor and transcription regulator of organic hydroperoxide stress. Transcription of both *ohr* and *ohrR* was induced by exposure of cells to organic hydroperoxides, but not to other oxidants. This induction required functional *ohrR*. Results of gel mobility shift and DNaseI foot printing assays using purified OhrR, combined with in vivo promoter deletion analyses, confirmed that OhrR regulated both *ohrR* and *ohr* by binding to a single OhrR binding box that overlapped the *ohrR* and *ohr* promoters. *ohrR* and *ohr* are both required for the establishment of a novel cumene hydroperoxide-induced adaptive response. Taken together, the results of biochemical, gene regulation and physiological studies support the role of *ohrR-ohr* as the primary system in sensing and protecting *A. tumefaciens* from organic hydroperoxide stress.

Introduction

Agrobacterium tumefaciens is a soil bacterium that causes crown gall disease in a wide range of plants by transferring some of its DNA (T-DNA) into the plant host. The T-DNA is then stably integrated into the plant genome, where its expression leads to the synthesis of plant hormones that stimulate tumor growth (1). In general, soil bacteria are exposed to hydroperoxides from various sources, such as their own aerobic metabolism and exposure to other soil bacteria and fungi that produce hydroperoxides. In addition, during plant microbe interactions, bacterial phytopathogens are exposed to reactive oxygen species (ROS) including; H_2O_2, superoxide anions and lipid hydroperoxides that are generated as part of active plant defence responses. While the levels of plant lipoxygenases, that catalyze the formation of fatty acid hydroperoxides from fatty acid precursors, have been shown to

increase in response to microbial invasion (23), the role of ROS during *Agrobacterium*-plant interactions is not clear.

In order to grow and proliferate, bacterial phytopathogens and soil bacteria must overcome these ROS. In regard to the protection against organic hydroperoxide toxicity, there are two major families of enzymes, peroxiredoxins (Prx) and Ohr, that have been shown to be important in many bacteria (Poole, 2005 #65}(12, 24). AhpC (alkyl hydroperoxide reductase), an enzyme of the Prx family that catalyzes the reduction of organic hydroperoxides to their corresponding alcohols, has been well characterized biochemically and genetically (32). The enzyme not only detoxifies organic hydroperoxides, but is also involved in the degradation of low concentrations of intracellular H_2O_2 (16). The physiological functions and biochemical properties of other members of the Prx family such as; Tpx (Thiol peroxidase), BCP (bactoferritin co-migratory protein), and 2-Cys Prx are less clear partly due to their limited distribution in only a few bacterial species (10, 31, 32). Nonetheless, they have been shown to be capable of metabolizing organic hydroperoxide. Ohr (organic hydroperoxide resistance protein), a thiol peroxidase, was initially discovered in *Xanthomonas campestris* due to its ability to complement organic hydroperoxide sensitive phenotypes in an *Escherichia coli ahpC* mutant (6). Ohr is uniquely regulated and its expression is highly induced only by organic hydroperoxides. Purified Ohr has hydroperoxide peroxidase activity and catalyzes the reduction of organic hydroperoxides to their corresponding alcohols (24, 25). Both *ahpC* and *ohr* are found in diverse species of bacteria (6, 12-14, 22). They have similar biochemical properties but differ in both their physiological function and pattern of gene expression in response to stresses. The expression of *ahpC* is regulated by OxyR, a peroxide sensor and transcription regulator (8, 9) while *ohr* is controlled by OhrR, an organic peroxide-inducible transcription repressor (11, 12, 19, 20).

The aims of this study were to functionally evaluate the roles of various genes, formerly known to be involved in organic hydroperoxide detoxification, and determine which gene(s) plays the primary role in the process. The analysis of the biochemical properties of *ohrR* and *ohr* mutants and the expression patterns of *ohrR* and *ohr* indicate that this system plays a primary role in sensing and protecting *A. tumefaciens* from organic hydroperoxides.

Materials and Methods

Bacterial growth conditions

Agrobacterium tumefaciens NTL4 (17) and mutant strains were grown aerobically in Luria Bertani (LB) medium at 30 ^{o}C with continuous shaking at 150 rpm. To ensure synchronous

growth, overnight cultures were inoculated into fresh LB medium to give an OD_{600} of about 0.1. Exponential phase (OD_{600} about 0.6, after 4 h of growth) and stationary phase (OD_{600} of about 5.0, after 30 h of growth) cells were used in all experiments. The peroxide induction experiments were executed with exponential treated with varying concentrations of peroxides for 15 and 30 min for Northern analysis and enzymatic assays, respectively.

Molecular biology techniques

General molecular genetics techniques including, genomic DNA preparation, plasmid preparation, restriction endonuclease digestions, ligation, transformation in *Escherichia coli*, agarose gel electrophoresis, Southern and Northern Blot analyses were performed using standard protocols (2). Plasmid purification for DNA sequencing was performed using the Qiagen Miniprep kit (Qiagen, Germany). DNA was sequenced using a BigDye terminator cycle sequencing kit (PE Biosystems) and run on an ABI 310 automated DNA sequencer. Routinely, *A. tumefaciens* was transformed by electroporation as previously described (17).

Purification of OhrR

The fusion system was used by making the fusion at the carboxy-terminus of ohrR The OhrR-His tag was produced by cloning of 473bp *Nco*I digested PCR products pOhrR template and specific oligonucleotide primers, BT992 and BT486 into *Nco*I-*Hinc*II digested pETBlue-2 (Novagen) giving pETohrR.

E. coli harboring pETohrR was grown to mid-log phase before 1 mM IPTG was added followed by incubation for 3 h. The cultures were harvested by centrifugation and cell pellets were resuspended in 50 mM PB, sonicated, and then spun at 10,000 X g for 15 min. The cleared lysate was then loaded onto an Affi-Gel heparin column (Bio-Rad), followed by extensive washing with column buffer (25 mM Tris-HCl, pH 8, 25 mM NaCl, 2 mM EDTA). The His-tagged protein was eluted by the addition of elution buffer (25 mM Tris-HCl, pH 8, 500 mM NaCl, 2 mM EDTA, 1 mM DTT). The eluted fraction was dialysed against (25 mM Tris-HCl, pH 8, 100 mM NaCl, 2 mM EDTA, 1 mM DTT). The purity of the protein was evaluated on SDS/PAGE gels.

Gel mobility shift and DNase I protection assays

^{32}P-labelled DNA fragments were prepared by PCR using oligonucleotide primers, BT536 and BT537 (see Table 1) and *A. tumefaciens* NTL4 genomic DNA as the template to generate a 363-bp fragment spanning the *ohr* and *ohrR* promoter region. Gel mobility shift assays were performed as previously described (20). Gel mobility shift reactions contained 3 fmol of labelled probe in 25 µl of reaction 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, 0.5 mg ml^{-1} polydI/dC, 400 ng of

purified OhrR). DNaseI footprinting assays using the 336 bp PCR generated DNA fragment spanning the *ohr-ohrR* intergenic region and purified OhrR were performed as described previously (20).

Construction of *A. tumefaciens bcp, prx, ohr* and *ohrR* mutants

The specific primers used for PCR amplification of gene internal fragments of *A. tumefaciens bcp1, bcp2, prx1, prx2, prx3, ohr* and *ohrR1* were designed based on the nucleotide sequences corresponding to putative open reading frames Atu1830 (BT907 and BT908), Atu3655 (BT911 and BT912), Atu1480 (BT532 and BT533), Atu0779 (BT1173 and BT1174)), Atu2399 (BT1319 and BT1320), Atu0847 (BT538 and BT539), and Atu0846 (BT546 and BT547), respectively, in the *A. tumefaciens* genome sequence ((15), see Table 1). The PCR products were ligated into pDrive prior to the subcloning of the *Eco*RI fragments into pKNOCK-Gm or pKNOCK-Km and insertion-mutants were constructed using a protocol previously described (7). Mutants were confirmed by PCR using two primers flanking the insertion site and by Southern blot analysis.

Construction of pBcp1, pBcp2, pPrx1, pPrx2, pPrx3, pOhr and pOhrR

The full-length genes were PCR amplified from *A. tumefaciens* genomic DNA using specific pairs of primers (BT909 and BT910 for *bcp1*; BT913 and BT914 for *bcp2*; BT574 and BT575 for *prx1*;BT1046 and BT1047 for *prx2*; BT1317 and BT1318 for *prx3*; BT 487 and BT488 for *ohr* and BT485 and BT486 for *ohrR1*) (see Table 1) and *Pfu* polymerase. The PCR products were cloned into pCR® Blunt (Invitrogen, USA), sequenced and subcloned into the broad-host-range plasmid, pBBR1MSC-4 (4) to generate the high expression plasmids pBcp1, pBcp2, pPrx1, pPrx2, pPrx3, pOhr and pOhrR.

Organic hydroperoxide degradation assay

The degradation of organic hydroperoxides was measured as previously described (14) with some modifications. Overnight cultures of various *A. tumefaciens* strains were inoculated into 20 ml LB medium at a final OD_{600} of 0.1. Log phase cultures (after 4 h growth) were adjusted to an OD_{600} of 0.5 with fresh medium prior to addition of organic hydroperoxide at a concentration of 200 μM. Residual organic hydroperoxide concentrations were determined at 10-min intervals using a xylenol orange-iron reaction. At varying time intervals, 1 ml of the culture was removed and the cells pelleted. 100 μl of the cleared supernatant was then added to 400 μl of 25 mM sulphuric acid in a 1-ml cuvett. 500 μl of freshly prepared reaction buffer (200 μM ammonium ferrous sulfate, 200μM xylenol orange and 25 mM sulphuric acid) was then added to the mixture. After 10 min of incubation at room temperature, the absorbance at 540 nm was determined. The concentration of residual organic

peroxide in the culture was calculated from a standard curve generated using LB medium containing known organic hydroperoxide concentrations.

Determination of oxidant resistance by plate sensitivity assay

The resistance levels of *A. tumefaciens* strains to oxidants were determined using a plate sensitivity assay as previously described (28). Serial dilutions of exponential phase cells were made in LB medium and 10 µl of each dilution was spotted onto a LB agar plate containing 0.2 mM CuOOH. The plates were incubated at 30^{o}C for 24 h before bacterial colonies were scored.

Determination of adaptive protection to CuOOH

Induced adaptive resistance to CuOOH killing was measured by adding 50 µM CuOOH to exponential phase cultures of *A. tumefaciens* strains prior to treatment with lethal concentrations of H_2O_2 (1, 5, 10 mM) for 30 min. After treatment, the cells were washed with fresh LB medium and the number of viable cells was determined as described previously (27). The surviving fraction was defined as the number of colony forming units (cfu) recovered after treatment divided by the cfu prior to treatment. Three independent experiments were performed in each case.

β-galactosidase assay

Crude bacterial lysates were prepared and protein assays were performed as previously described (6). In brief, 20 ml exponential phase cultures were harvested and washed once with 50 mM sodium phosphate buffer pH 7.0 (PB). Bacterial suspensions in 0.5 ml PB containing 1 mM PMSF, a protease inhibitor, were lysed by intermittent sonication followed by centrifugation at 10,000x*g* for 20 min. The total protein concentration was determined for each of the cleared lysates prior to their use in enzyme assays. β-galactosidase was assayed as described earlier (18).

Results and Discussion

Evaluation of the physiological and biochemical roles of putative genes encoding organic hydroperoxide scavenging enzymes

The objective of the investigation was to evaluate the roles of various genes encoding putative organic hydroperoxide metabolizing enzymes in protecting *A. tumefaciens* from organic hydroperoxide exposure. BLAST algorithm (5) searches of the annotated genome of *A. tumefaciens* (15) identified at least nine predicted open reading frames (ORF) that had a high degree of sequence similarity to enzymes that have been shown to be involved in organic hydroperoxide metabolism in other organisms. These ORFs could be grouped into either the

peroxiredoxin (TSA/AhpC) or Ohr families (6, 10, 12, 24). The ORFs belonging to the peroxiredoxin family were *prx1* (peroxiredoxin, Atu1480), *prx2* (Atu0779), *prx3* (Atu2399), *bcp1* (bacterioferritin co-migratory protein, Atu1830), and *bcp2* (Atu3655), while *ohr* (Atu0847) was homologous to the Ohr family of thiol peroxidases. As an initial step toward understanding the physiological function of these genes in protecting *A. tumefaciens* from lethal doses of organic hydroperoxides, mutants lacking a functional copy of either; *prx1, prx2, prx3, bcp1, bcp2*, or *ohr* were constructed by insertion inactivation using the pKNOCK system (7). The resistance levels of these mutants and the wild type strain NTL4 to organic hydroperoxides were determined using plate sensitivity assays. Surprisingly, only the *ohr* mutant showed increased sensitivity (100-fold relative to wild type NTL4) toward organic hydroperoxides (0.2 mM CuOOH and 0.8 mM tBOOH, data not shown). It was possible that *prx1, prx2, prx3, bcp1 and bcp2* played minor roles in organic hydroperoxide resistance such that expression of the Ohr system masked the effects of mutations in these genes. Thus, double mutants were constructed in which *ohr* was inactivated along with either, *prx1, prx2, prx3, bcp1 or bcp2*. Each of the double mutants showed resistance levels to tBOOH and CuOOH that were similar to those of the *ohr* mutant (data not shown). Another approach used to evaluate the *in vivo* function of the putative organic hydroperoxide protective genes was to test whether high-level expression of plasmid-borne *prx1, prx2, prx3, bcp1 or bcp2* affected the organic hydroperoxide sensitive phenotype of an *ohr* mutant. Each of the genes was cloned into pBBR1MSC-4 to create pPrx1, pPrx2, pPrx3, pBcp1 pBcp2 and pOhr (See Materials and Methods). Each plasmid was introduced into an *A. tumefaciens ohr* mutant and the organic hydroperoxide resistance levels were determined. As expected, pOhr restored the CuOOH resistance level of an *ohr* mutant to that of wild type (data not shown). By contrast, expression of plasmid-borne *prx1, prx2, prx3, bcp1 or bcp2* did not alter the CuOOH resistance level of the *ohr* mutant strain (data not shown). Hence, *prx1, prx2, prx3, bcp1 or bcp2* are unlikely to play important roles in the protection of *A. tumefaciens* from organic hydroperoxide toxicity under the conditions tested.

The genetic and physiological data clearly indicate that *ohr* is the major protective system against organic hydroperoixde stress. The finding that *prx1, prx2, prx3, bcp1 and bcp2* did not participate in organic hydroperoxide resistance was surprising. This was especially true for *A. tumefaciens prx1* that encodes an *AhpC* (alkyl hydroperoxide reductase) homologue. AhpC is a structurally and functionally conserved hydroperoxide-metabolizing enzyme that has been shown to be involved in organic hydroperoxide resistance in other

bacteria (32). It remains to be seen whether some of these genes might have functions under specific conditions.

Analysis of organic hydroperoxide degradation in various mutants

Out of the 6 genes tested only *ohr* played a role in protecting the bacteria from organic hydroperoxide toxicity. It is possible that some of these gene products could contribute to organic hydroperoxide degradation; however, their contributions might not be sufficient to confer significant resistance to the lethal concentrations of organic hydroperoxide used in the study. In order to detect more subtle changes in the capacity to detoxify organic peroxides, the effects of either gene inactivation or over-expression, on a strains ability to degrade organic hydroperoxide, were determined. The *ohr* single and *ohr prx1* and *ohr bcp1* double mutants were incubated with CuOOH and the rate of hydroperoxide degradation was determined. These genes were initially chosen for further analysis due to the fact that homologs of both *prx1* and *bcp1* had been shown to be involved in organic hydroperoxide resistance in other bacteria (10, 26). The results, shown in Fig. 1, indicated that the wild type strain NTL4 rapidly metabolized CuOOH (Fig. 1) while only the *ohr* mutant showed a significant reduction in the ability to metabolize CuOOH. After 15 min incubation in media containing CuOOH, the amount of CuOOH remaining was 60% for the *ohr* mutant and 40% for wild type strain NTL4 (Fig. 1). The reduced capacity to metabolize CuOOH in the *ohr* mutant could be complemented by the introduction of plasmid-borne *ohr* in pOhr, resulting in a rate of CuOOH degradation that was similar to that in NTL4 (Fig. 1). CuOOH degradation assays were also performed on the double mutants, *ohr prx1* and *ohr bcp1*, to determine whether inactivation of these genes in an *ohr* mutant background had any additive effect in CuOOH degradation. The results clearly indicated that both of the double mutants tested had rates of CuOOH degradation that were similar to that of the *ohr* single mutant (Fig. 1). The CuOOH degradation assay was also used to assess the effects of high-level expression of genes putatively involved in organic hydroperoxide metabolism on *ohr* mutant ability to degrade CuOOH. The expression plasmids were transformed into *ohr* mutant and transformants ability to degrade CuOOH determined. High-level expression of *prx1, prx2, prx3, bcp1 or bcp2* in an *ohr* mutant did not significantly alter the rate of CuOOH degradation (data not shown).

The fact that inactivation of *ohr* leads to an over 50% reduction in the ability of *A. tumefaciens* to metabolize CuOOH further supports the contention that *ohr* is the primary enzyme involved in organic hydroperoxide protection. It should be noted that the *ohr* mutant still retained a significant capacity to degrade CuOOH suggesting that other enzymes are also

involved in the process. Obvious candidates for this role were the peroxyredoxin homologs encoded by *prx1*, *prx2*, *prx3*, *bcp1* and *bcp2*. However, inactivation of each of these genes had no effect on the ability of the bacterium to either resist lethal exposure to CuOOH or to degrade CuOOH present in the culture medium. While participation of these genes in organic peroxide metabolism cannot be ruled out, it is likely that other, as yet unidentified, enzymes are responsible for the residual CuOOH degradation observed in the *ohr* mutant

Regulation of ohrR-ohr expression in response to stresses

The expression patterns of genes involved in stress protection should correlate with their physiological roles. Thus, regulators of these genes must have mechanisms to sense and respond to changes in levels of the appropriate stresses. In general, genes involved in stress protection are tightly regulated and their expression is highly induced by stresses. The levels of *ohr* mRNA were determined under uninduced and oxidant induced growth conditions using Northern analysis. Compared to uninduced cultures, the levels of *ohr* mRNA markedly increased during growth in the presence of 200 μM tBOOH, 50 μM CuOOH or 50 μM LOOH by 20-, 30- and 15-fold, respectively, as determined by densitometry (Fig. 2A). The observed pattern of oxidant-induced *ohr* expression was similar to the pattern observed in several other microorganisms, where *ohr* expression was highly induced only by organic hydroperoxides. (6, 14, 29) and is consistent with *ohr*'s proposed physiological role as the major protective system against organic hydroperoxide toxicity. An increase in *ohr* expression upon exposure to an organic hydroperoxide (an Ohr substrate) would certainly contribute to bacterial survival under this stress condition. It should be noted that treatment of *A. tumefaciens* cultures with inorganic oxidants including a superoxide generator (200 μM menadione, MD), and 200 μM H_2O_2 failed to induce *ohr* expression (Fig. 2A). The lack of H_2O_2 induced expression of *ohr* is at odds with results in Pseudomonas and other bacteria showing that treatment with high concentrations of H_2O_2 resulted in low-level induction of *ohr* expression (30) (29) leading to the suggestion that *ohr* may also play some role in H_2O_2 protection (22). In these cases it is unclear whether the inducer is H_2O_2 or some byproduct of H_2O_2 treatment such as organic hydroperoxides that are produced during exposure to high concentrations of H_2O_2. If *ohr* induction is due to the accumulation of byproducts resulting from H_2O_2 exposure then lack of induction in *A. tumefaciens* could be a reflection of the organism's ability to rapidly detoxify H_2O_2.

In order to assess the role of the peroxide sensing repressor *ohrR* in regulating *ohr* expression, an *ohrR* insertion mutant was constructed and the mutation's effects on *ohr* transcription during uninduced and oxidant induced conditions were investigated. The results

in Fig. 2A clearly demonstrated that *A. tumefaciens ohrR* is a repressor of *ohr* expression since its inactivation resulted in constitutively high expression of *ohr* that was unaffected by oxidant exposure. The expression analysis was extended to determine the pattern of oxidative stress induced expression of *ohrR*. *ohrR* expression was highly induced (10-15 fold) by treatments with the organic hydroperoxides, tBOOH and CuOOH but not the superoxide generator menadione or H_2O_2 (Fig.2B). Thus, *ohrR s*hares a similar organic hydroperoxide inducible expression profile with *ohr*. As would be expected for a regulatory protein, under uninduced or induced conditions the levels of *ohrR* mRNA was much lower than the levels of *ohr* mRNA as judged by densitometry (Data not shown). A 363 bp fragment containing the *ohr* promoter was transcriptionally fused to a promoterless *lacZ* in the promoter probe vector pUFR027*lacz*, a derivative of pUFR027 (3) to yield pPohr. pPohr was then used to monitor *ohr* promoter activity in response to inducing concentrations of hydroperoxides and the superoxide generator, menadione in wild-type strain NTL4 and an *ohrR* mutant. The results shown in Fig. 4A mirrored those of the Northern analyses and indicated that the organic hydroperoxides, CuOOH, tBOOH and LOOH were potent inducers of *ohr* promoter activity with magnitudes of induction ranging from 2.5- to 3-fold. The increases in promoter activity appeared to be dose dependent in the low dosage range (i.e. 0.2 mM and below). However, as the inducing concentrations of the various organic hydroperoxides increased, significant reductions in *ohr* promoter activity were observed (Fig. 4A). This was most likely due to organic hydroperoxide toxicity resulting in growth arrest and cell death. It has recently been reported that expression of the *X. campestris* ohrR-ohr system is more responsive toward low concentrations of the complex organic hydroperoxide, LOOH, as compared to the simple organic hydroperoxide, tBOOH (34). In *A. tumefaciens*, the *ohr* promoter was more responsive to the moderately complex hydroperoxide, CuOOH, than to either LOOH or tBOOH suggesting that the relative sensitivity of regulatory system to respond to different organic hydroperoxides is organism specific. The differences in the sensitivity to the various organic hydroperoxide inducers between the two bacteria are probably due to differences in the structure of the OhrRs. *A. tumefaciens* and *X.campestris* pv. phaseoli OhrRs each contain a Cys residue at position 21 that is absolutely conserved among all OhrRs and has been shown to be required for sensing organic hydroperoxide (18, 21). *X. campestris ohrR* also contains additional cysteine residues at positions 127 and 131 and there is evidence that C^{127} interacts with C^{22} during peroxide sensing (Panmanee *et al*., manuscript in preparation). *A. tumefaciens* OhrR lacks cysteine 127 suggesting that the hydroperoxide sensing mechanisms of the *A. tumefaciens* and X. campestris proteins may be different. Minor differences in the efficiency

of different types of organic hydroperoxides in inducing *ohr* could be advantageous to *A. tumefaciens* when it encounters mixtures of organic hydroperoxides of varying toxicity. In any case, organic hydroperoxides were still much more efficient inducers of the system than either the inorganic oxidant H_2O_2 or the superoxide generator, menadione, regardless of concentration (Fig. 4A). As expected, inactivation of *ohrR* resulted in *ohr* promoter activity was constitutively high and unaffected by organic hydroperoxide treatments (Fig. 4B). Furthermore, the *ohr* promoter activity in the *ohrR* mutant was 2-fold higher than the fully induced level observed in the wild type strain (Fig. 4B) suggesting that, even under fully induced conditions, some OhrR probably still bound to the *ohr* promoter. This could provide additional fine-tuning of the expression of OhrR regulated genes. Finally, high-level expression of *ohrR* from an expression vector lead to the repression of *ohr* promoter and this effect could be negated by CuOOH treatment (Fig. 4B). The observation is consistent with the idea that OhrR acts as the transcription repressor of *ohr* promoter.

The *in vivo* promoter analyses were extended to the *ohrR* promoter. The *ohrR* promoter activity was induced by organic hydroperoxide treatments, but was unaffected by either H_2O_2 or menadione treatment (data not shown). The pattern of sensitivity of the *ohrR* promoter to induction by organic hydroperoxides was similar to the pattern for the *ohr* promoter. CuOOH was the most potent inducer followed by LOOH and tBOOH. The organic hydroperoxide inducibility of the *ohrR* promoter was lost in an *ohrR* mutant background with absolute levels of *ohrR* promoter activity that were higher than those in wild type strain NTL4 (Fig. 4C). Moreover, complementation with plasmid borne *ohrR* in pOhrR restored the normal pattern of hydroperoxide inducibility (Fig. 4C). These observations indicated that OhrR negatively autoregulated its expression. Consistent with the results of the Northern blotting experiments (Fig. 2B), comparative analysis of induced *ohr* and *ohrR* promoter activities showed that the *ohr* promoter was the stronger of the two with up to 9-fold higher promoter activity under a given condition.

Mapping of regulatory elements within the ohr and ohrR promoters

As a first step in the characterization of both the *ohrR* and *ohr* promoters, primer extension experiments were performed to determine the transcription start sites of both genes. The results in Fig. 3A, show that *ohr* transcription initiates at a C residue, 21 bases upstream from the translation initiation codon. Immediately, upstream of the *ohr* transcription start site were found E. coli RNA polymerase ^{70}-like -10 (TATAAG) and -35 (TTGCGT) sequence elements that were separated by 17 bases (Fig. 3A). The transcriptional start site of *ohrR* was mapped to a G residue 81 bases upstream of the ATG codon. Examination of the region

upstream of the transcription start also revealed the presence of E. coli RNA polymerase ^{70}-like –10 and –35 sequence motifs TTGAAT and GATAAT, respectively, separated by 17 bases (Fig. 3A). Quantitative analysis of *ohr* and *ohrR* primer extension products indicated that transcription initiation from these promoters was highly inducibed by CuOOH (Fig. 3A). Thus, the increase in *ohr* and *ohrR* transcripts in response to CuOOH treatment detected in Northern experiments was due to increases in transcription initiation.

Genetic evidence indicated that *ohrR* regulates its own expression in addition to that of *ohr*. This fact, combined with the close proximity of the divergently transcribed *ohr* and *ohrR* promoters suggested that they might share regulatory sites. Examination of the *ohr-ohrR* intergenic region revealed the presence of the AT-rich inverted repeat sequence, 5'-gcgTACAATTnAATTGTAcgc-3', that was similar to the putative OhrR box sequence thought to be involved in the binding of OhrR to target promoters in *B. subtilis* and *X. campestris* (11, 13) (Fig. 3B). The inverted repeat was situated 19 bps upstream of the *ohrR* –35 promoter element and over-lapped the region between the *ohr* –35 and –10 promoter elements (Fig. 3A), suggesting that a single OhrR box could be involved in the regulation of both the *ohr* and *ohrR* promoters.

In order to probe the function of the putative OhrR binding site a number of promoter-*lacZ* transcriptional fusion plasmids were constructed that contained varying amounts of sequence upstream of the *ohr* and *ohrR* promoters (Fig. 5A). The ability of each fusion to be induced by organic hydroperoxide treatments was tested in vivo. The results shown in figure 5B and C indicate that the OhrR box is necessary for normal organic hydroperoxide inducible regulation of both promoters. Deletion of the sequence upstream of position –55 (pP_{921}) in the *ohr* promoter had no appreciable effect on promoter function relative the full-length control promoter (pP_{ohr}) (Fig. 5B). Deletion of the sequence upstream of –22, in pP_{1236}, that removed the upstream half of the putative OhrR binding box along with the -35 promoter element, resulted in inactivation of the promoter (Fig. 5B). Thus, the *ohrR* promoter resides in the region within 55 bp of the *ohr* transcription start containing the OhrR box and the –10 and –35 promoter elements.

A similar analysis of the *ohrR* promoter showed that a fusion (pP_{974}) containing 80 bp upstream of the *ohrR* transcription start and spanning the OhrR-box, as well as the –10 and –35 promoter elements, was regulated normally. Deletion of all or part of the Ohr box, in fusion plasmids pP_{920} (deleted to -36), and pP_{975} (deleted to -61), respectively, yielded expression patterns that were similar to that of the full-length *ohrR* promoter in an *ohrR* mutant i.e. high-level constitutive expression that was unaffected by organic hydroperoxide

(Figs. 4C and 5C). Taken together the data indicated that the *ohrR* promoter was located within 80 bps of the *ohrR* transcription start and that the OhrR box was required for organic hydroperoxide dependent regulation. One interesting finding was the fact that over-expression of OhrR from plasmid pOhrR restored organic hydroperoxide-dependent regulation to the *lacZ* fusion plasmid pP_{975} (Fig. 5C). Since this fusion contained only the proximal half of the OhrR box, the result suggested that OhrR could still bind to this site, albeit with a lower affinity than to the full OhrR box.

Binding of OhrR to the *ohr-ohrR* intergenic region

The direct interaction of OhrR with the *ohr* and *ohrR* promoters was tested using purified *A. tumefaciens* OhrR and a 363 bp DNA fragment spanning the *ohr-ohrR* intergenic region, that contained the putative OhrR binding box, using gel mobility shift assays. OhrR specifically bound to the intergenic region since binding was blocked by the addition of excess unlabelled probe fragment (UP), but not by non-specific competitor DNA, pBBR1MSC-4 (UD) (Fig. 6). The genetic and physiological analyses reported in this work indicated that the likely role of OhrR is as a sensor of organic hydroperoxide. More direct evidence of this was obtained when the organic hydroperoxide CuOOH was added to the gel mobility shift reactions containing purified OhrR and the 363 bp intergenic region probe (Fig. 6). Addition of CuOOH to the binding reaction led to the loss of OhrR binding to its target site (Fig. 6). This is consistent with the proposed mechanism of OhrR sensing of organic hydroperoxide in which oxidation of a sensing cysteine residue(s) lead to inactivation of the repressor that, in turn, allows RNA polymerase to bind to the promoter and activate transcription (18, 19, 21). In light of both the in vivo and in vitro data, it is clear that *A.tumefaciens* OhrR has evolved to sense and respond to organic hydroperoxide.

Similar gel mobility shift experiments were performed using deleted OhrR promoter fragments spanning either all (P_{920}) or part (P_{975}) of the OhrR box. Consistent with the *lacZ* fusion results, no binding of OhrR to fragment P_{920} was detected. However, fragment P_{975}, containing half of the OhrR box, was still bound by OhrR, but with an affinity that was at least 10-fold lower than OhrR binding to the 363 bp intergenic region probe (compare Fig. 6 Lane X with Fig. 6? Lane ?). This was in good agreement with the *lacZ*-fusion results using pP_{975} where hydroperoxide inducibility of this promoter deletion was restored when OhrR was expressed at high levels from plasmid pOhrR (Fig. 5C). This implies that OhrR binds to the target half-site in the proper configuration and retains its function. Finally, precise localization of the OhrR binding site within the *ohr-ohrR* intergenic region was accomplished by DNaseI footprinting (Fig. 7). OhrR protected a region a 49 bp region from –6 to –54

relative to the ohr transcription start. The extent of protection was typical of previously mapped OhrR binding sites in *B. subtilis* and *X. campestris* (13, 20) and indicates that OhrR binding represses expression of both genes by covering the –10 and –35 elements of the *ohr* promoter as well as the –35 region of the *ohrR* promoter. Given the data presented here it seems reasonable to assume that multiple OhrRs may bind within this region with varying affinities. The physiological significance of OhrR's ability to bind, at high concentrations, to sites that are very different in sequence from the highly conserved OhrR boxes that have been identified to date is not known. Nonetheless, it raises the possibility the expression of genes that do not contain a concensus OhrR box in their promoters could still be regulated by OhrR under conditions where reduced OhrR concentrations are significantly increased. Such conditions might occur during brief periods when most organic hydroperoxide has been removed and *ohrR* expression is not yet to be fully repressed. This could be important when transcription of some genes needs to be temporarily turned off after exposure to organic hydroperoxide stress. Transient inactivation of gene expression during oxidative stress has previously been observed in the yeast *Schizosaccharomyces pompe* (33). Pap1, an activator of oxidative stress response genes during exposure to low peroxide concentrations, is inactivated at high peroxide concentrations due to the oxidation of the Pap1 activator Tpx1. Reduction of the Tpx1 pool leading to Pap1 reactivation only occurs with the high-level peroxide dependent induction of Srx1. Thus, changes in the peroxide levels from low to high results in the transient inactivation of genes involved in the low-level oxidant stress response. The possible occurrence of a similar phenomenon involving OhrR in *A. tumefaciens* is currently being investigated.

Physiological analysis of *ohr* and *ohrR* mutants

Inactivation of either *ohrR* or *ohr* had no effects on aerobic growth rate or colony formation on a complex medium (data not shown) and an *ohr* mutant was less resistant to organic hydroperoxides, (CuOOH and tBOOH) than the wild type strain NTL4 (data not shown). However, the sensitivity of the *ohrR* mutant to CuOOH was more pronounced than that to tBOOH suggesting that the *A. tumefaciens ohrR-ohr* system has evolved to be more efficient at sensing and protecting the bacteria from moderately complex organic hydroperoxides such as CuOOH than to other simple (tBOOH) or to more complex organic hydroperoxides (LOOH). In a few bacteria, *ohr* has been implicated in H_2O_2 protection and metabolism (22, 25). In *A. tumefaciens*, it is unlikely that *ohr* plays any protective role against H_2O_2 since high-level expression of *ohr* on an expression vector had no effect on resistance to either H_2O_2 or the superoxide generator, menadione (data not shown). These observations are

consistent with *ohr* being the major detoxification enzyme involved in organic hydroperoixde degradation in *A. tumefaciens*. Inactivation of *ohrR* lead to small increases in resistance levels to CuOOH that were probably due to increased expression of *ohr*. No changes in resistance levels to inorganic oxidants were detected in an *ohrR* mutant (data not shown).

The ability to adapt to stress is crucial for bacterial survival under stressful conditions. It has often been observed that low-level exposure to a particular stress can elicit an adaptive response that results in an increased resistance to a subsequent high-level exposure to the same stress. An adaptive response to H_2O_2 exposure has been observed in many bacteria including *A. tumefaciens* (27). While adaptive responses to organic hydroperoxide are rare, an adaptive response to lipid hydroperoxide involving the *ohrR/ohr* system has been reported in *X. campestris* pv. phaseoli (34). In *A. tumefaciens*, we have reported lack of adaptive response to an organic hydroperoxide, tBOOH (27). In light of data on the transcription regulation of *ohr* and the ability of OhrR to sense various organic hydroperoxides, the adaptive response of *A. tumefaciens* to CuOOH was investigated. The results indicated that pre-exposure to a low concentration (200 μM) of CuOOH conferred 10-fold resistance to subsequent exposure to a killing concentration (1, 5 and 10 mM) of CuOOH relative to uninduced cells (Fig. 8). Moreover, inactivation of either *ohr* or *ohrR* resulted in complete loss of CuOOH adaptive response (Fig. 8). The results indicate that Ohr is the primary protective enzyme against organic hydroperoxide toxicity in *A. tumefaciens* and that the establishment of an adaptive response to CuOOH requires the *ohr-ohrR* system.

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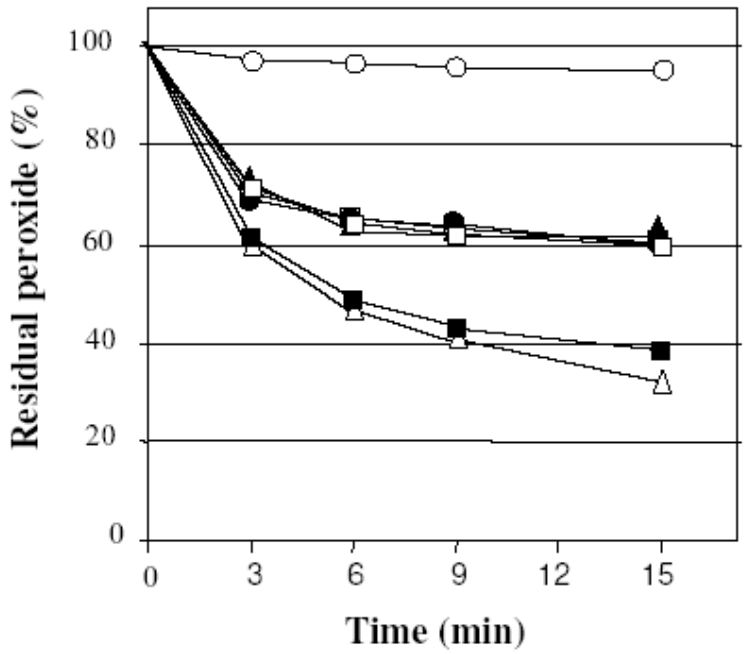

Fig. 1. Degradation of CuOOH by various *A. tumefaciens* strains

The rate of CuOOH degradation in culture medium containing 200 μM CuOOH by *A. tumefaciens* parental NTL4 (■), *ohr* mutant (□), *ohr prx1* mutant (▲), *ohr bcp1* mutant (●), and *ohr* mutant harbouring pOhr (Δ) The levels of CuOOH remaining in the culture media at the various time points are reported along with those of a media control without bacteria (○).

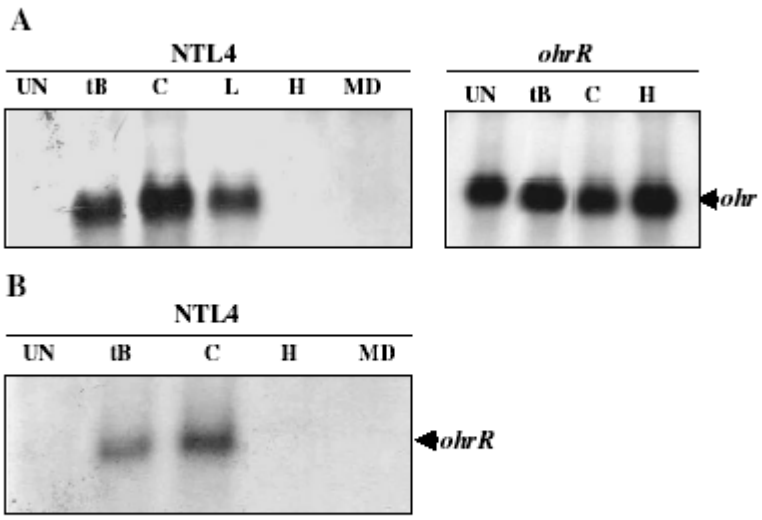

Fig. 2. Organic hydroperoxide induced gene expression of *ohr* and *ohrR*

Northern blots of total RNA extracted from exponential phase cultures of *A. tumefaciens* parental strain NTL4 and an *ohrR* mutant under uninduced conditions (UN) and after exposure to --Include the concentrations used--200 μM tBOOH (tB), 50 μM CuOOH (C), 50μ M LOOH (L), 200 μM H_2O_2 (H) and 200 μM menadione (M) and hybridized with a radioactively labeled *ohr* (A) and *ohrR* (B) specific probe.

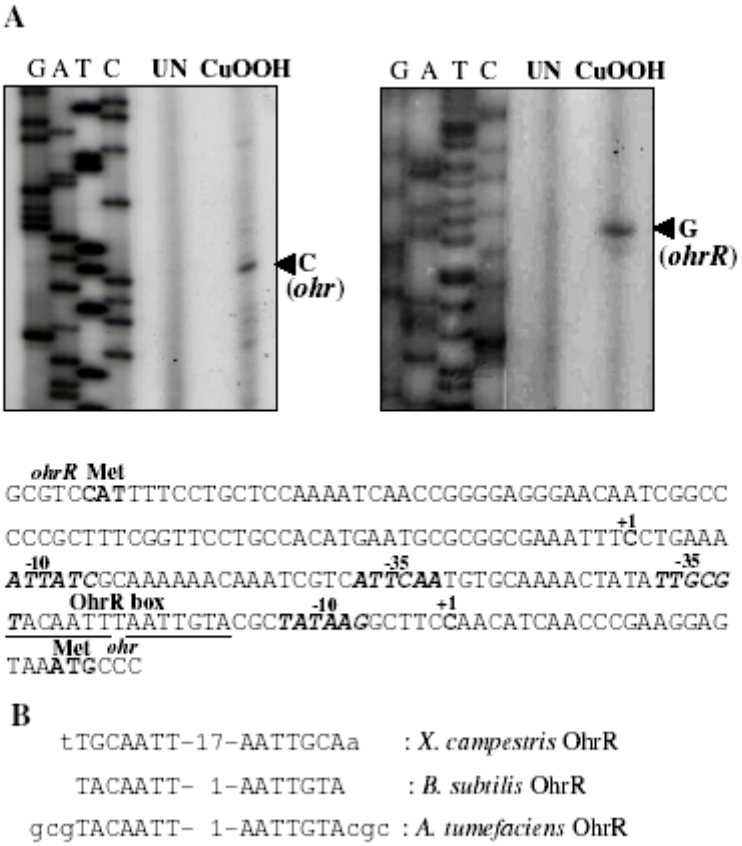

Fig. 3. Localization of *ohr* and *ohrR* promoters and alignment of OhrR binding box

(A) Primer extension of RNA extracted from uninduced (UN) and CuOOH-induced cultures. The experiment was performed using a [^{32}P]-labeled oligonucleotide primer as described in Materials and Methods. The C T A and G lanes of a dideoxy are sequencing ladder using the same primer as that used for the primer extension are shown. The *ohr* and *ohrR* transcription start sites are marked by arrowheads in the primer extension autoradiographs and +1 in the accompanying sequence. Putative -35 and -10 regions are shown in bold italics. A translation initiation codons (ATG) are in bold. The putative OhrR box is underlined.

(B) Alignment of putative OhrR binding sites from *X. campestris* (11), *B. subtilis* (13) and *A. tumefaciens*. Numbers indicate the number of intervening nucleotides.

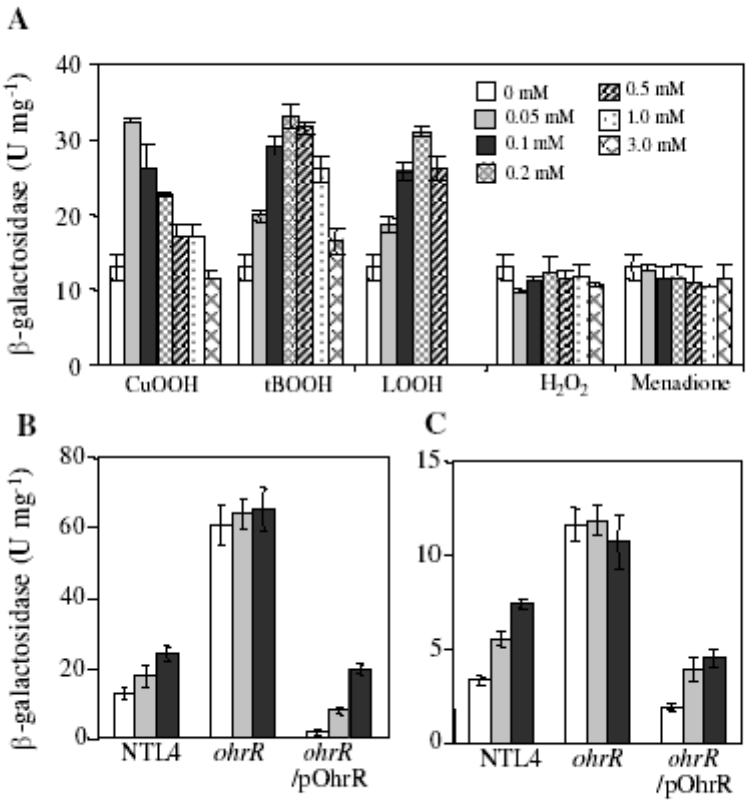

Fig. 4. *In vivo* characterization of the *ohr* and *ohrR* promoters

β-galactosidase activity of exponential phase cultures of *A. tumefaciens* strains, containing either an *ohr* or *ohrR* promoter-*lacZ* transcriptional fusion plasmid, exposed to CuOOH, tBOOH, LOOH, H_2O_2 or menadione at various concentrations. (A), *A. tumefaciens* harboring pP_{ohr} (B), *A. tumefaciens* (NTL4), *A. tumefaciens ohr* mutant (*ohr*) and *A. tumefaciens ohr* mutant containing pOhrR (*ohr*/pOhrR) harboring pP_{ohr} exposed to tBOOH (gray shade), CuOOH (black shade) or unexposed (open bar). (C) Experiments were performed as describe in (B) but using *A. tumefaciens* strains containing the *ohrR* promoter-*lacZ* fusion plasmid, pP_{ohrR}. Values are the mean and SD of four replicate experiments.

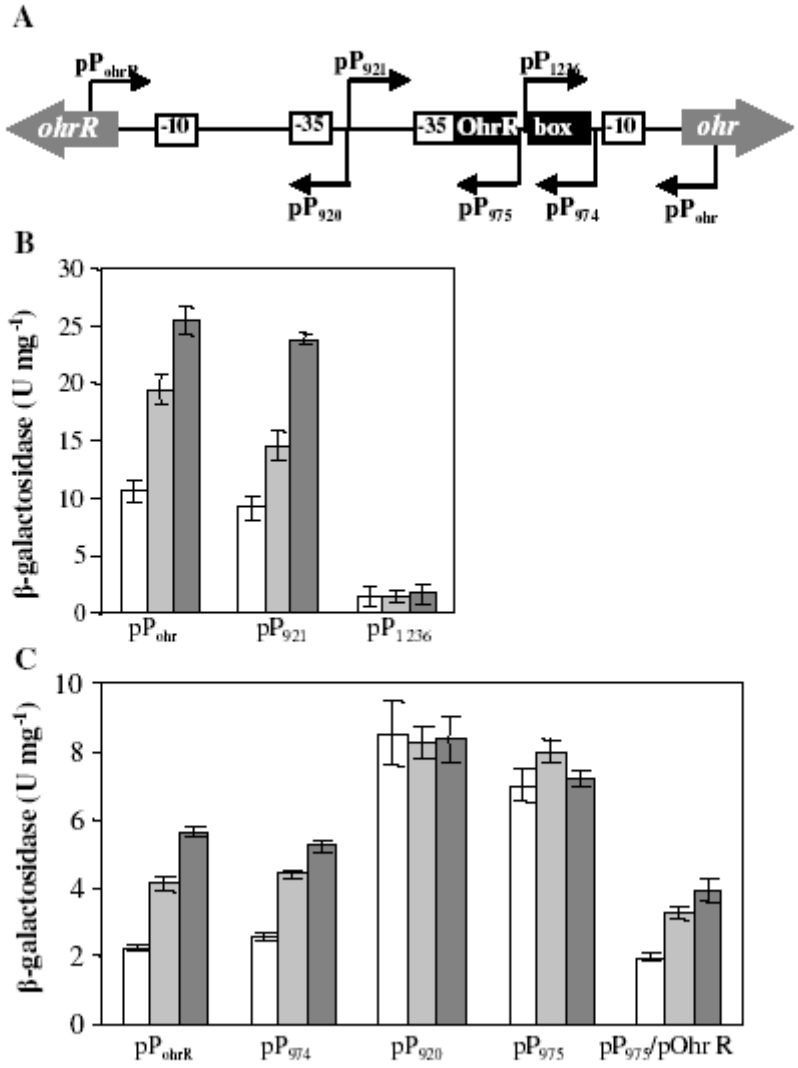

Fig. 5. *ohr* and *ohrR* promoter deletion analyses

In (A) a map of the *ohr-ohrR* intergenic region showing the upstream end points of promoter fragments used to construct the various promoter-*lacZ* fusion plasmids. In (B), β-galactosidase activity of *A. tumefaciens* harboring the *ohr* promoter-*lacZ* fusion pP_{ohr} or its deletions pP921 and pP1236. In (C), β-galactosidase activity of *A. tumefaciens* harboring pP_{ohrR} or its deletions. pP_{975}/pOhrR represents *A. tumefaciens* containing pP_{975} and carrying pOhrR for the expression of *ohrR*. Cells were cultured to exponential phase before induction with tBOOH (gray shade), CuOOH (black shade) or uninduced (open bar). Values are the mean and SD from four replicate experiments.

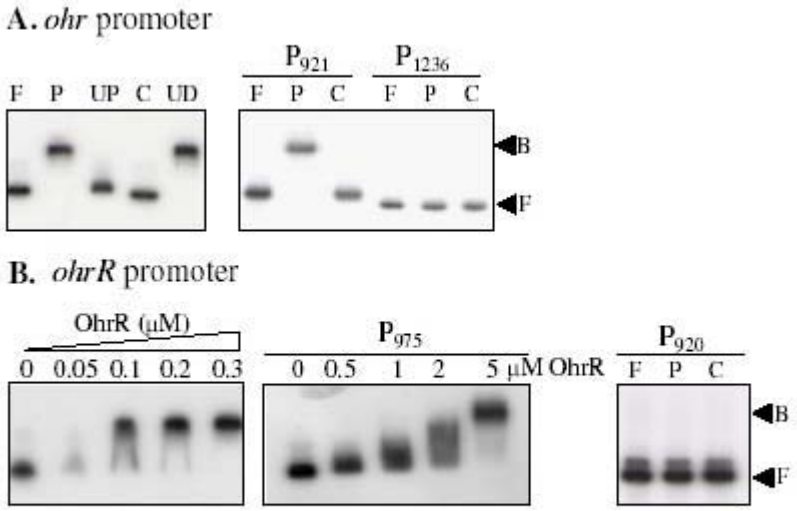

Fig. 6. OhrR binds the *ohr* and *ohrR* promoters

The results of DNA mobility shift assays using ^{32}P-labeled *ohr* (A) and *ohrR* (B) promoter fragments and purified OhrR. F indicates free probe; P, indicates a reaction containing purified OhrR and labeled probe. UD and UP indicate reactions containing 2 μg of unrelated DNA (pBBR1MCS-4 plasmid) and 1 μg unlabeled promoter, respectively. C indicates reactions in which CuOOH (1.0 mM) was added to the binding reaction. If not indicated, the amount of purified OhrR in the binding reaction was 0.3 μM. B represents bound probe.

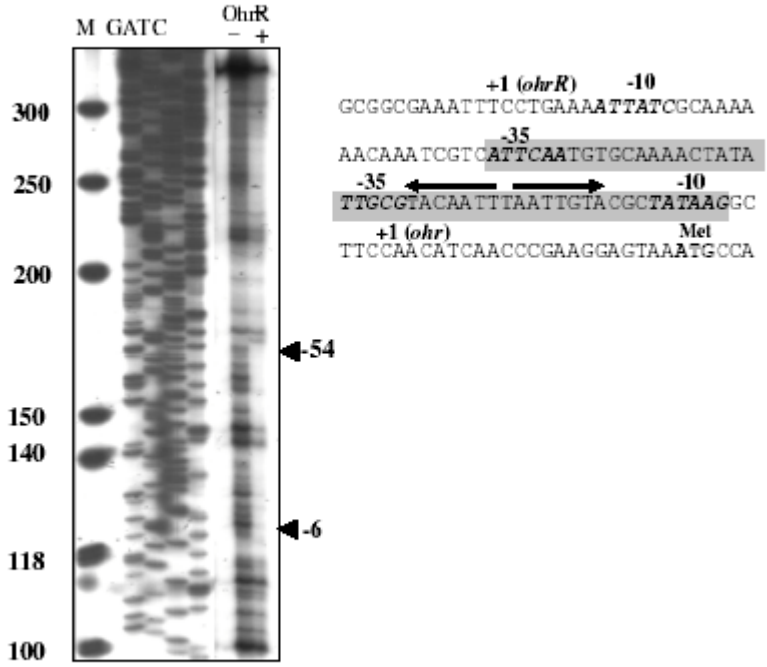

Fig. 7. DNase I protection assay of OhrR binding to the *ohr-ohrR* intergenic region

Results of a DNaseI footprinting assay using purified OhrR and a ^{32}P-labeled probe spanning the *ohr-ohrR* intergenic region. Minus (-) represents the probe fragment treated with DNase I in the absence of OhrR. Plus (+) represents the probe fragment treated with DNase I in the presence of OhrR. Arrowheads and numbers indicate the limits of the protected sites and their corresponding position relative to the *ohr* transcription start (+1). The sequence of the *ohr-ohrR* intergenic region is also shown in which the OhrR protected region is shaded. Divergent arrows indicate the putative OhrR box. The -10 and -35 regions of *ohr* and *ohrR* promoters are shown in bold type.

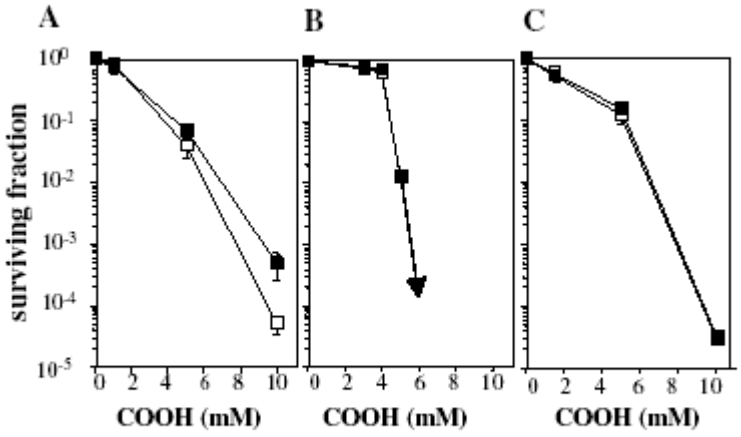

Fig. 8. Induced adaptive protection to CuOOH in *A. tumefaciens* requires functional *ohr* and *ohrR*

CuOOH induced adaptive response experiments were performed by incubating exponential phase cultures of *A. tumefaciens* (A), *ohr* mutant (B) and *ohrR* mutant (C) in 50 μM CuOOH for 30 minutes before treatment with the indicated concentrations of CuOOH for 30 min. Cells that survived various treatments were scored after 48 h incubation. The CuOOH survival curves against CuOOH concentration are plotted. (■), CuOOH-induced; (□), uninduced cultures. Values presented are the mean and SD of four replicate experiments.

Table 1
Bacterial strains, plasmids and primers used in this study

Strain/plasmid/primer	Description	Source
***A. tumefaciens* strains**		
NTL4	pTiC58-cured derivative of strain C58 Δtet_{C58}	(17)
bcp1	*bcp1*::pKNOCK-Gm	This study
bcp2	*bcp2*::pKNOCK-Gm	This study
prx1	*prx1*::pKNOCK-Gm	This study
prx2	*prx2*::pKNOCK-Gm	This study
prx3	*prx3*::pKNOCK-Gm	This study
ohr	*ohr*::pKNOCK-Km	This study
ohrR1	*ohrR1*::pKNOCK-Gm	This study
ohr bcp1	*ohr*::pKNOCK-Km, *bcp1*::pKNOCK-Gm	This study
ohr prx1	*ohr*::pKNOCK-Km, *prx1*::pKNOCK-Gm	This study
Plasmids		
pKNOCK	Broad-host-range suicide vector; RP4 *oriT*, R6K γ-*ori*	(7)
pBBR1MCS-4	Broad-host-range cloning vector; *rep, mob, lacZα*, Ap^R	(4)
pBcp1	pBBR1MCS-4 containing *A. tumefaciens bcp1*	This study
pBcp2	pBBR1MCS-4 containing *A. tumefaciens bcp2*	This study
pPrx1	pBBR1MCS-4 containing *A. tumefaciens prx1*	This study
pPrx2	pBBR1MCS-4 containing *A. tumefaciens prx2*	This study
pPrx3	pBBR1MCS-4 containing *A. tumefaciens prx3*	This study
pOhr	pBBR1MCS-4 containing *A. tumefaciens ohr*	This study
pOhrR1	pBBR1MCS-4 containing *A. tumefaciens ohrR1*	This study
Primers	**Sequences (5′-3′)**	
BT487	GAAGGAGTAAATGCCATG	
BT488	TAAGCCCGCTTTATCAGG	
BT532	ATCGGGTAAGTGAGGACC	
BT533	ATTCGCACCGCGACCGGC	
BT536	CTGCGCGTAAAGGGCAA	
BT537	GAGCGTGACGTCGAGAAC	
BT538	CGGAACAGCTTTTCGCGG	
BT539	TTCTTCGGCTTTCTCACG	
BT574	GGAGAAAGCACACTATGA	
BT575	TTCGTTAGCAGCTTAGCC	
BT907	ATCGACTTCAGCGCGCTC	
BT908	TTCGGCGATCTTGCCATC	
BT909	GCGAAAGAAAGGTAGAAT	
BT910	GGCTGGATGGCCCCTCAG	
BT911	CACCTCAATCTTGCGCTT	
BT912	CACCCGCTTCAAACATTG	
BT913	TGCCAGGGATGGAAATGC	
BT914	ATTGGCCAAGACACTCAT	
BT920	ATTGAATGACGATTTGTT	
BT921	CAATGTGCAAAACTATAT	
BT974	CTTATAGCGTACAATTAA	
BT975	AATTGTACGCAATATAGT	
BT976	TTTTGCGATAATTTCAG	
BT992	TGGAGCAGGACCATGGAC	
BT1046	TAAGAATGGGCTTGTTAAA	
BT1047	GTCTCCTCGTGCCTGTACT	
BT1173	CGCTTAGAGCGCACACCAA	
BT1174	TCTCGAAAATCGCGACGCC	
BT1236	TAATTGTACGCTATAAGG	
BT1317	AGAACAGGAGACAAGACATC	
BT1318	AACCGAGATCAGGCCGCAGC	
BT1319	CTATTGCCTTTCGGTCAACG	
BT1320	ACTGCTCCACCAGACCGTCA	

3.2 Atypical gene regulation and evaluation of the physiological role of *soxR* in *Agrobacterium tumefaciens*

Abstract

The physiological function of *soxR,* in *Agrobacterium tumefaciens* was evaluated. Lack of *soxR* decreased resistance to superoxide generators. *soxR* expression was autoregulated and inducible by superoxide generator. Gel shift assay revealed the binding of SoxR to its promoter. Thus, under uninduced condition SoxR binds to its promoter and represses expression. Upon exposure to superoxide anions, SoxR is active thereby activating its own expression. The genes directly regulated by SoxR were identified.

Introduction

*Agrobacterium tumefacien*s is a soil and phytopathogenic bacterium causing crown gall tumors in a variety of dicotylenous plants through the insertion of a segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of the infected cells (1). This microorganism is also widely used for the introduction of foreign genes into a variety of hosts including plants and fungi (7).

During plant-microbe interaction, a rapid production and accumulation of reactive oxygen species (ROS) known as the oxidative burst is an important part of the initial plant defense response against invading microorganisms (3). ROS are highly toxic to bacterial cells and bacteria have evolved several enzymatic and non-enzymatic mechanisms to protect themselves against them. Physiological adaptation to environmental changes is one of the important protective strategies used by bacteria when growing under oxidative stress conditions (6, 8, 19). The processes acquire various cellular activities controlled by a coordinating gene regulation. The well-defined key regulators of this response in prokaryotic cells are the oxidant sensing transcriptional regulators SoxR, OxyR and OhrR, which regulate the inducible expression of antioxidant genes in response to exposures to superoxide anion, H_2O_2 and organic peroxide, respectively (11, 15, 16).

The response to superoxide anion (and nitroactive stress) in *Escherichia coli* is a two-stage process (2). The presence of superoxide anion triggers the pre-existed SoxR iron-sulfur protein to be a potent transcription activator for the *soxS*, whose gene products then up-regulates transcription of at least 15 genes in the regulon (12, 15). Some of the activities induced through the *soxRS* regulon are directly responsible for diminishing or repair damage produced by ROS

(e.g. *sodA, zwf, nfo*). However, currently report in *Pseudomonas aeruginosa* revealed that *soxR* plays no physiological role in oxidative stress response (20, 22), although the gene is required for infection in animal model (10) and is inducible by paraquat (10). These findings suggest a diverse role of *soxR* in prokaryotes. This communication, we demonstrated the physiological function of *soxR* in superoxide stress in *A. tumefaciens* and showed that SoxR is a superoxide sensor and global transcriptional regulator.

Results and Discussions

The phenotypes of *soxR* mutant

The significance of *soxR* in term of oxidative stress protection was determined through the *A. tumefaciens soxR* mutant PW01 (17). The resistance levels toward various oxidants were determined using inhibition zone assay for exponential phase growing cells and plate sensitivity test for stationary phase cells (21). As shown in Fig.1A, during exponential phase the SoxR-deficient mutant was more sensitive to menadione (MD) and paraquat (PQ) by giving the zones of growth inhibition of 25 mm and 15 mm, respectively, compared with the zone of 18 mm and no detectable zone for the *A. tumefaciens* NTL4 parental strain (13). The mutants showed non-significant difference for the level of resistance to cumene and *t*-butyl hydroperoxides. MD and PQ are redox cycling agents capable of generating intracellular superoxide anions. Devoid of *soxR* rendered *A. tumefaciens* more susceptible to superoxide stress suggests the function of this gene in protection against superoxide anions. Stationary phase cells gave similar profile of oxidant resistance as exponential cells suggesting the relevance of *soxR* throughout the stages of growth (Fig. 1B).

We have shown previously that the level of iron-containing superoxide dismutase (SOD) in *A. tumefaciens* could be induced in response to the presence of sublethal concentration of MD in a *soxR*-dependent manner (17). Thus, increased sensitivity to superoxide generators in PW01 *soxR* mutant was likely due to loss of the ability to stimulate the expression of *sod*. It is noteworthy that *A. tumefaciens* possesses three putative genes encoding SOD. The importance of these *sod* genes is being investigated (Mongkolsuk *et al.*, manuscript in preparation). The altered phenotypes in PW01 could be restored by *trans* expression of *soxR* from pSoxR (pBBR1MSC-5 (5) containing *soxR* full-length amplified from *A. tumefaciens* NTL4 genomic DNA) (Fig. 1A, B).

The expression analysis of *A. tumefaciens* NTL4 *soxR*

In *A. tumefaciens*, *soxR* locates immediately down stream of *bfrA*, a gene encoding exogenous ferric siderophore receptor (Fig. 2A) (14). The transcript unit and level of *soxR* expression in response to oxidative stress were determined by using Northern blot analysis of total RNA extracted from oxidant-induced and uninduced cultures. The results revealed a single hybridized mRNA band of 600 bp in length, suggesting that the gene was transcribed as a monocistronic mRNA (Fig. 2B). The intensity of the hybridized signal from the MD-induced sample was 15-fold increased comparing with the uninduced control. No alteration in the signal intensity was detected in other oxidant induced conditions. The results indicate that *soxR* expression is inducible by challenging the bacterial cultures with superoxide generator. Generally, in *E. coli*, *soxR* is constitutively expressed and the level of existed SoxR is kept at relatively low level (9). Binding of active (oxidized form) or inactive (reduced form) SoxR to its box between -35 and -10 promoter region of *soxS*, a divergent gene, represses its own expression (9). Moreover, the level of *soxR* transcript is unaltered as cells are exposed to superoxide stress. The finding that the expression of *A. tumefaciens soxR* is inducible suggests the possibility that the function of *soxR* in superoxide response in this bacterium may differ from previous observation in *E. coli*. Similar observation has been reported in *P. aeruginosa* where *soxR* expression is inducible by treatment with paraquat and during infection (10).

Analysis of *soxR* promoter

First, the transcriptional start site of *soxR* was localized. Primer extension experiments were performed using RNA extracted from uninduced and menadione-induced cultures. As shown in Fig. 2C, majority of *soxR* transcript was initiated at C, 59 nucleotides upstream of the ATG, translational start codon. Analysis of the upstream region of the transcription starting revealed sequence motifs TTGACC and TATACC corresponded to the -35 and the -10 promoter regions, respectively. Interestingly, the two regions were separated by 19 bases and the intervening sequence between -35 and -10 promoter was merely identical (14 out of 18 matched) to a consensus sequence for SoxR binding in *E. coli* (Fig. 2C). Taken together, the result implied that SoxR may directly bind to and regulate its own promoter. In addition, the result was also consistent with the Northern blot analysis where menadione is capable of inducing *soxR* expression.

Next, *in vivo soxR* promoter was analyzed using pP_{soxR} plasmid containing *soxR* promoter-*lacZ* fusion. The *soxR* promoter activities in response to various oxidative stresses were determined in *A. tumefaciens* NTL4 and *soxR* mutant (PW01) and the results confirmed that menadione is a strong inducer of *soxR* promoter with the magnitude 10-fold comparing to

uninduced cells (Fig. 3A). The induction was abolished in the PW01 *soxR* mutant (Fig. 3A). Moreover, the menadione induction in PW01 could be restored by the *trans* expression of *soxR* from the plasmid vector, pSoxR (Fig. 3A). Pretreatment with other oxidants failed to have any effects to the levels of *soxR* transcript. When the basal levels of β-galactosidase activity in NTL4 and PW01 are taken into consideration, we found that lack of functional *soxR* (in PW01) increased the enzymatic activity to 1,085 U mg^{-1} protein (comparing to 667 U mg^{-1} protein in NTL4) while an over expression of SoxR (in *A. tumefaciens* NTL4 harboring pSoxR) repressed it (480 U mg^{-1} protein). All the evidences suggest that SoxR is regulated its own expression.

A 5' sequential deletion of *soxR* promoter in pP_{soxR} was performed to localized region required for *soxR* induction. We found that the levels of β-galactosidase activity in NTL4 carrying $pP_{soxR822}$ (deleted upstream of -43) could be induced by menadione pretreatment in a similar manner as observation in strain harboring pP_{soxR}. When a -35 promoter region was deleted in $P_{soxR837}$ (deleted upstream of -23), the level of the β-galactosidase activity was drastically dropped and could not be induced by menadione (data not shown). These data suggest that induction of *soxR* promoter required no additional sequence upstream of -35 promoter region.

***In vitro* binding of SoxR to *soxR* promoter**

Generally, *soxR* regulates the expression of *soxS*, the only known gene in *E. coli* that is mediated by *soxR*, by binding to the SoxR protein to *soxS* promoter. The presence of putative SoxR binding box together with the finding that the expression of *soxR* could be induced by superoxide generator in an *soxR* dependent manner suggest the possibility that SoxR may bind to its own promoter and controls its expression. To prove this hypothesis, *in vitro* DNA mobility shift assay was used to investigate the ability of SoxR protein to bind its promoter. Purified SoxR protein (20-100 ng) was incubated with radioactively labeled 270 bp of intact *soxR* promoter fragment in a binding buffer. The protein-DNA complexes were detected on 5% native-PAGE. The results in Fig. 3B showed that SoxR specifically bound to the *soxR* promoter. The unlabeled *soxR* promoter fragment acted as a competitor that could be overcome at high protein levels (80 ng). In addition, the DNA gel shift experiment was repeated by using deleted -35 region *soxR* promoter fragment (P_{837} fragment) instead of intact *soxR* promoter. The result illustrated that SoxR could not bind to the *soxR* promoter when the -35 region was deleted (data not shown). This result clearly shows that SoxR binds to its promoter and the binding requires the sequence spanning -35 and -10 region of its own promoter.

Characterization of the genes regulated by SoxR

Lack of *soxS* homolog in *A. tumefaciens* genome sequence implied that the mechanism responding to superoxide in this microorganism probably different from that reported in enteric bacteria. The finding that SoxR binds and regulates its own expression in a superoxide inducible fashion suggests the possibility that SoxR may control the expression of other superoxide responsive genes by directly binds to their promoter. Thus, the genes regulated under *soxR* were identified by searching a slightly degenerated consensus sequence CCTCAACTATAGTTGAGG of the SoxR binding site against *A. tumefaciens* genome sequence specially the flanking sequences between the annotated ORFs. The putative SoxR binding site was identified on the putative promoter regions of 3 ORFs, namely Atu4762, Atu4895 and Atu5152 that encode superoxide dismutase (*sodF*) and two conserve hypothetical proteins, respectively (Table 1). Atu4762 encodes superoxide dismutase (SOD) belonging to a manganese-iron family. Preliminary results indicated the expression of this SOD is directly controlled by SoxR (Mongkolsuk et al., manuscript in preparation). Atu4895 and Atu5152 are uncharacterized membrane proteins that share high score of identity in the primary structure (69%) and seem to be existed only in *A. tumefaciens*. Analysis of the putative amino acid sequence using TopPred program available at http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html (4) suggests that these proteins contain three transmembrane motifs (data not shown). Northern blot analyses were performed using RNA samples isolated from NTL4 and PW01 *soxR* mutant and hybridized with *atu5152* specific probe. The results revealed a positively hybridized band of 0.5 kb in the blotted RNA from menadione pretreated culture was highly induced (30-fold) relative to uninduced control and such induction disappeared in PW01 *soxR* mutant (Fig. 4A). This suggests that the expression of Atu5152 could be induced in response to superoxide stress in a *soxR* dependence and the transcripts were monocistronic (Fig. 1A). In vivo *atu5152* promoter-*lacZ* fusion was performed and the results supported Northern analysis data (Fig. 4B). Also, the level of β-galactosidase in PW01 *soxR* mutant was 3-fold higher than that in the NTL4 parental strain. Thus, under the uninduced stage, SoxR probably binds to *atu5152* promoter and represses its expression in a similar manner as observation in *soxR* promoter. The presence of superoxide stress triggers SoxR to an active from of transcriptional activator. To test this speculation, *in vitro* DNA mobility shift assay was performed to evaluate the ability of SoxR protein to bind the Atu5152 promoters. The results in Fig. 3C displayed that SoxR specifically bound to both promoters. These evidences indicate that Atu5152 are the SoxR target genes by which SoxR directly binds and probably regulates their expression in response to superoxide stress.

However, these genes have as yet uncharacterized roles in helping the organism cope with superoxide stress. Therefore, the further work is required to elucidate the role of these genes and the mechanism which these genes were regulated by SoxR. Our data illustrated the function of *A. tumefaciens* SoxR as a global transcriptional regulator that directly controls the expression of genes in the regulon.

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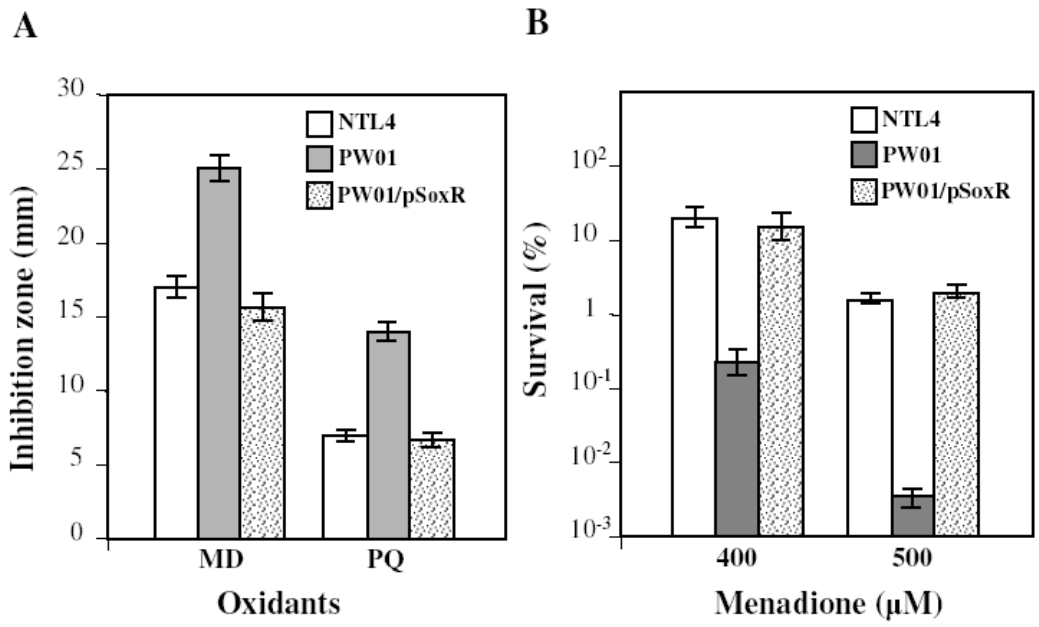

Fig. 1. Determination of the resistance levels toward superoxide generators in *A. tumefaciens* strains

A, the killing effects of superoxide generators on *A. tumefaciens* strains were done using an inhibition zone assay (21). The exponential phase cultures were mixed with molten top agar and overlaid onto LB plates. Sterile 6 mm-diameter paper discs soaked with 5 μl of 1.0 M paraquat (PQ) or 1.0 M menadione (MD) were placed on the surface of the cell lawn and the zones of growth inhibition were measured after 24 h of incubation at 30 ^{o}C.

B, the resistance level of the stationary phase cells to MD was determined using plate sensitivity assay (21). Serial dilutions of a stationary phase cells were made in 50 mM sodium phosphate buffer pH 7.0 and 10 μl of each dilution was spotted onto a LB agar plate containing 400 and 500 μM MD. The plates were incubated at 30^{o}C for 24 h before the results were read. Percentage survival is defined by the number of cells grown on plate containing MD divides by the number of cells grown on plate without MD and multiplies by 100.

NTL4, *A. tumefaciens* parental strain; PW01, *soxR* mutant; PW01/pSoxR, PW01 harboring pSoxR.

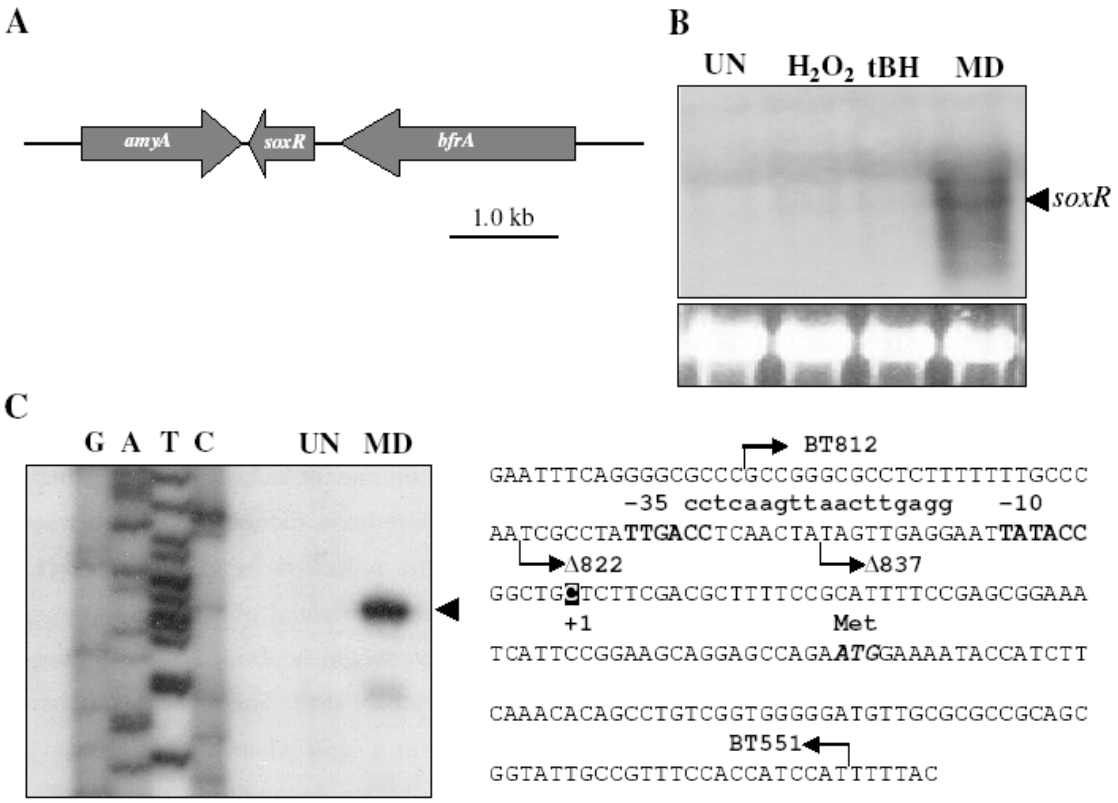

Fig. 2. Menadione induces *soxR* expression and localization of *soxR* promoter.

A, physical and transcription maps of *soxR* in *A. tumefaciens*. The arrows indicate the orientation and length of the transcripts.

B, Northern blot of the total RNA (10 μg) prepared from exponential phase cultures of *A. tumefaciens* cultivated under uninduced (UN) and induced with 250 μM H_2O_2 (H_2O_2), 200 μ M t-butyl hydroperoxide (tBH) and 200 μM MD for15 min was prepared and hybridized with [^{32}P]-labeled *soxR*-specific probe. The level of 23S rRNA as loading control is shown underneath the autoradiograph of the Northern blot.

C, primer extension of RNA extracted from uninduced (UN) and MD-induced cultures. The experiment was performed using [^{32}P]-labeled oligonucleotide primer BT551 (5' ATGGATGGTGGAAACGGC3'). C T A and G are sequence ladders. The arrowhead or +1 indicates the *soxR* transcription start site. Putative -35 and -10 regions are shown in bold capital letters. The translation initiation codon ATG is in bold italics. *E. coli* SoxR binding site is shown in small letters. Arrow and number indicate the primer starting position.

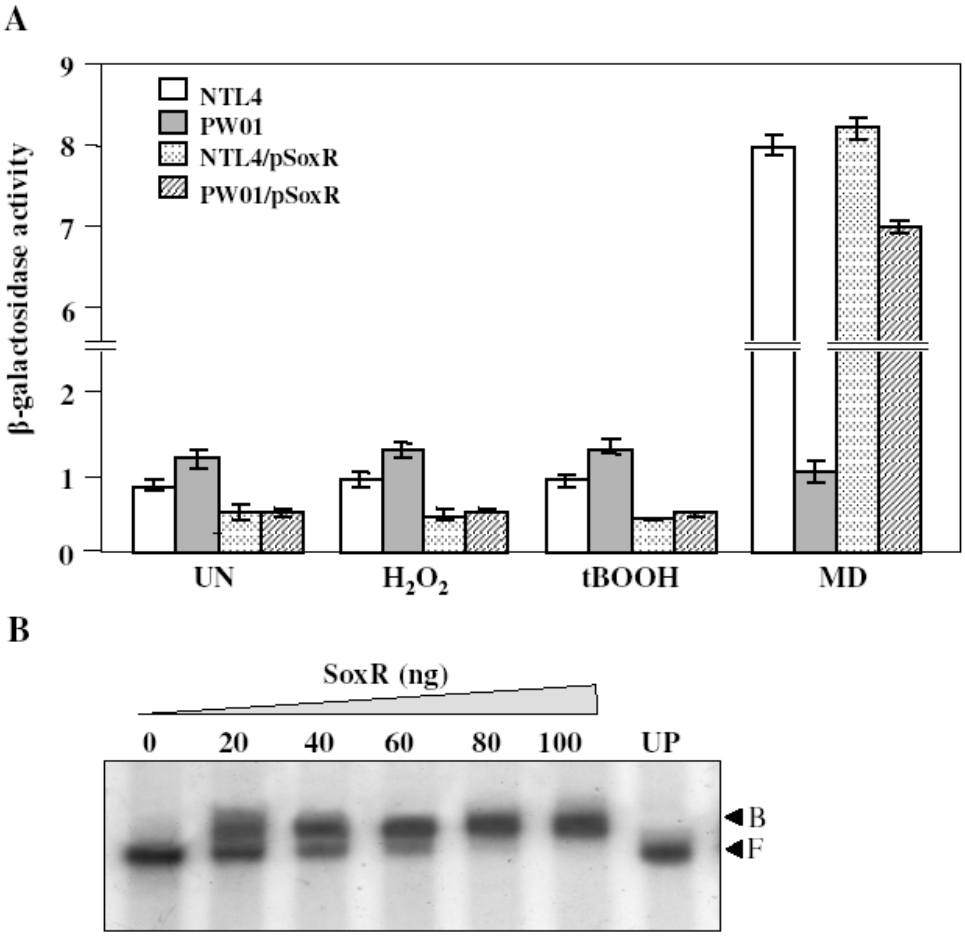

Fig. 3. *In vivo* promoter analysis and binding of SoxR to the promoter fragment

A, a 204-bp *soxR* promoter was amplified from *A. tumefaciens* genomic DNA using BT812 and BT551 oligonucleotide primers before being transcriptionally fused to a promoter-less *lacZ i*n low-copy-number plasmid pUFR027lacZ (18) to give pP_{soxR}. Exponential phase cultures *A. tumefaciens* strains treated with oxidants as Fig. 2C's legend were harvested for crude lysate preparation and β-galactosidase assay as described (18). NTL4, *A. tumefaciens* parental strain; PW01, *soxR* mutant; NTL4/pSoxR, NTL4 harboring pSoxR; PW01/pSoxR, PW01 harboring pSoxR.

B, *A. tumefaciens* SoxR was over-expressed *E. coli* BL21 using pETBlue-2 vector system (Novagen, USA) and purified through P-11 phosphocellulose ions exchange column chromatography eluted with 0.5 M KCl (15). The purity of SoxR protein was greater than 70% as judged from SDS-PAGE. Gel mobility shift reactions were performed by adding 3 fmol of labeled probe (^{32}P labelled 207-bp soxR promoter fragment) in 25 µl of reaction 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, 0.5 mg ml^{-1} polydI/dC). Purified SoxR (0-100 ng) was added and the reaction was incubated at 25^{o}C for 15 min. Protein-DNA complexes were separated by electrophoresis on 6 % non-denaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer

(TBE) at 4 ºC. UP indicates reactions containing 1 µg unlabeled *soxR* promoter in addition to SoxR (80 ng). F indicates free probe; B, indicates bound probe.

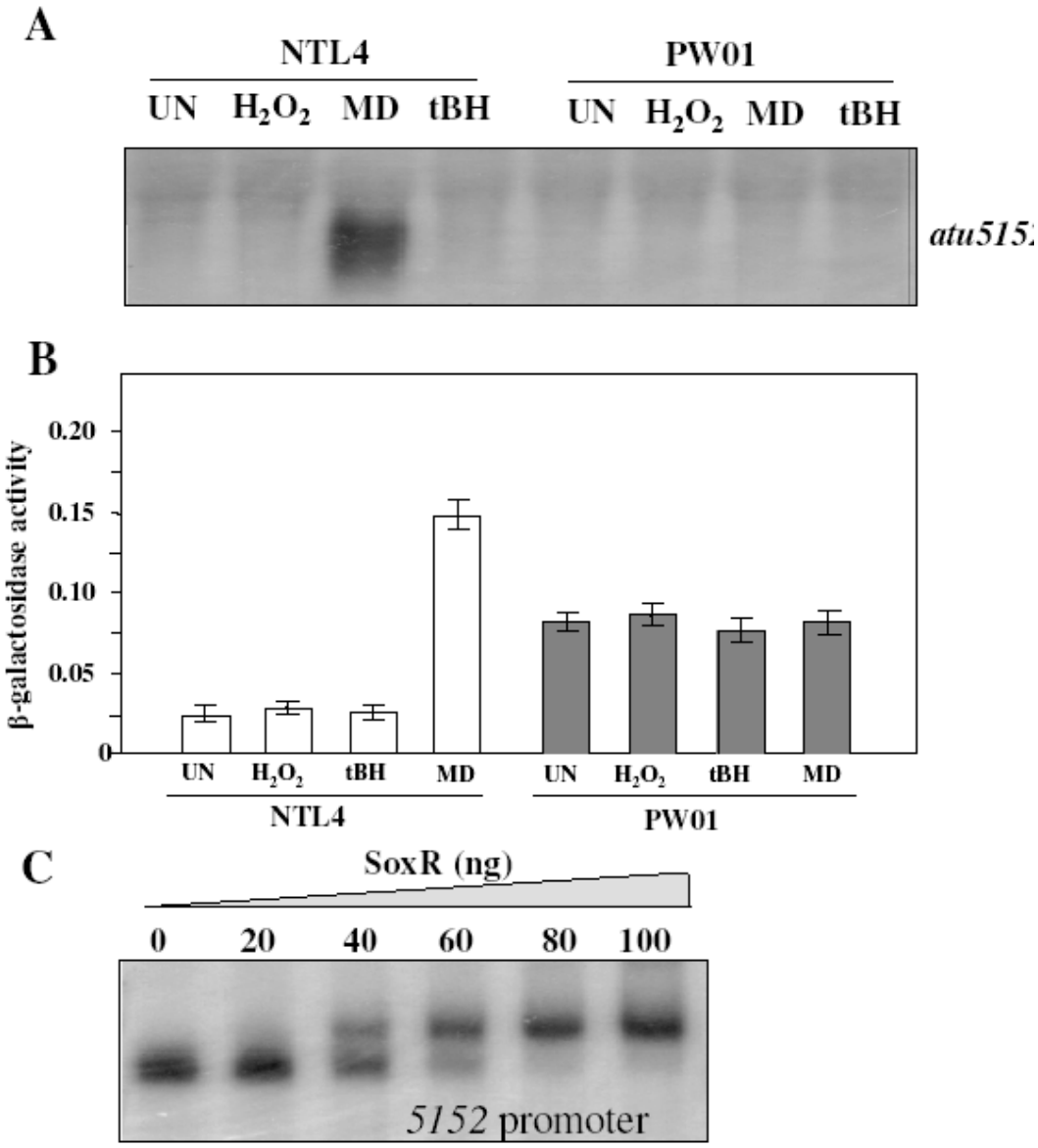

Fig. 4. *soxR* directly regulates *atu5152*

A, Northern blot analysis was performed as described in Fig. 2A's legend except hybridization was performed with [^{32}P]-labeled *atu5152* probe. The 210-bp probe was amplified from A. tumefaciens genomic DNA using BT882 (5'TTGCCATCTGGACACGCT3') and BT883 (5'AAAGATCAGGCAATGAATA3').
B, a 212-bp *atu5152* promoter was amplified from *A. tumefaciens* genomic DNA using BT1125 (5'AAACCTCTCATCTCGCCC3') and BT1126 (5'CAATACGGCTCCAACCAG3') primers and fused to *lacZ i*n pUFR027lacZ (18). Exponential phase cultures *A. tumefaciens* NTL4 and PW01 *soxR* mutant treated with oxidants as Fig. 2C's legend. Crude lysates reparation and β-galactosidase assay were performed as described (18).

C, Gel mobility shift assay was followed the method described in Fig. 3C's legend except the [^{32}P]-*atu5152* promoter fragment was used instead.

Table. Putative SoxR binding site sequences identified in *A. tumefaciens* genome

ORF	SoxR binding site sequence	Products
Atu3915	CCTCAACTATAGTTGAGG	SoxR
Atu4762	CCTCAACCGTGATTGAGG	Superoxide dismutase
Atu4895	CCTCAACTCAAGTTGAGG	Conserve hypothetical protein
Atu5152	CCTCAACTAGAGTTGAGG	Conserve hypothetical protein

CHAPTER IV

4.1. Novel organic hydroperoxide sensing and responding mechanisms for OhrR, a major bacterial sensor and regulator of organic hydroperoxide stress

Abstract

Xanthomonas campestris pv. phaseoli, OhrR belongs to a major family of multiple cysteine-containing bacterial organic hydroperoxide sensors and transcription repressors. Site-directed mutagenesis and subsequent *in vivo* functional analyses revealed that changing any cysteine residue to serine did not alter the ability of OhrR to bind to the P1 *ohrR-ohr* promoter but drastically affected the organic hydroperoxide sensing and responding mechanisms of the protein. *Xanthomonas* OhrR requires two cysteine residues, C22 and C127, to sense and respond to organic hydroperoxides. Analysis of the free thiol groups in wild type and mutant OhrRs under reduced and oxidized conditions indicate that C22, the organic hydroperoxide-sensing residue, was first oxidized by organic hydroperoxide. This led to the formation of an unstable OhrR-cysteine^{22} sulfenic acid intermediate in an NBD-labeled, oxidized, C127S, C131S mutant OhrR that was detected by UV-Visible spectra analysis. In wild type OhrR, the OhrR-sulfenic acid intermediate rapidly reacts with the thiol group of C127 forming a disulphide bond. The HPLC-MS analysis of tryptic digest fragments of alkylated, oxidized OhrR and native PAGE analyses confirmed the formation of reversible intermolecular disulfide bonds between C22 and C127. Oxidation of OhrR led to cross-linking of two OhrR monomers resulting in inactivation of its repressor function. Evidence present here provides insight into a new organic hydroperoxide sensing and responding mechanism for OhrRs of the multiple cysteine family, the primary bacterial transcription regulator of the organic hydroperoxide stress response.

INTRODUCTION

Xanthomonas campestris strains comprise a group of soil bacteria and plant pathogens. In the environment and during plant microbe interactions, *Xanthomonas* sp. are exposed to reactive oxygen species (ROS) including H_2O_2, lipid hydroperoxide and superoxide anions generated as by-products of aerobic metabolism, exposure to chemicals in the environment and from plant active defense responses. (6, 11).

Organic hydroperoxides are highly toxic due to their ability to directly oxidize macromolecules and participate in the generation of reactive lipid radicals. In many bacteria, organic hydroperoxide metabolism remains poorly characterized. In *Xanthomonas* and other bacteria, the best-characterized organic hydroperoxide detoxification systems involve peroxiredoxins, such as alkyl hydroperoxide reductase (AhpR), and organic hydroperoxide resistance (Ohr) thiol peroxidases (2, 4, 10, 17). Both enzymes directly catalyze the reduction of organic peroxides to, less toxic, organic alcohols. AhpR and Ohr have similar biochemical actions though they differ in their physiological roles and gene expression patterns. The expression of *ahpC* (the catalytic subunit of AhpR) is regulated by OxyR, a peroxide sensor and transcription regulator (13, 20) whereas *ohr* is controlled by the organic peroxide-inducible transcription repressor, OhrR (4, 15, 21).

The ability to sense and respond to changes in peroxide levels is crucial for bacterial survival under peroxide stress. Both OxyR and OhrR are involved in the sensing of organic hydroperoxide but the latter is probably more sensitive to changes in organic hydroperoxide levels than the former. Oxidation of OxyR leads to changes in its structure and function from a transcription repressor, as in reduced OxyR, to an activator, as in oxidized OxyR. Reduced OhrR binds to its target site and represses gene expression, while organic hydroperoxide-dependent oxidation of OhrR results in inactivation of its repressor function resulting in transcription from the target promoter. Mechanisms by which *Bacillus subtilis* OhrR senses and is inactivated by organic hydroperoxide have been postulated and they involve the oxidation of a sensing cysteine by hydroperoxide to a protein-cysteine sulfenic acid (C-SOH) that inactivates the repressor (5).

Here, we have classified OhrRs into two families based on the number of cysteine residue they contain. *X. campestris* pv. phaseoli OhrR belongs to the major family that contains multiple cysteine residues. In addition, we present evidence for a novel organic hydroperoxide sensing and responding mechanism for this major family of OhrRs.

Materials and Methods

Materials

Bacterial culture media were obtained from Difco. Restriction endonucleases, DNA modification enzymes, and isopropyl-1-thio-β-D-galactopyranoside were purchased from Promega. Organic solvents (high pressure liquid chromatography (HPLC) grade) and water (optima grade) were obtained from Fisher. Acrylamide/bis (40%) solution was obtained from Bio-Rad, USA. Cumene hydroperoxide, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NDB chloride), and 4-vinylpyridine were obtained from Aldrich. L-1-(Tosylamino)-2-phenylethyl chloromethyl ketone-treated trypsin was obtained from Worthington. Pierce supplied the immobilized Tris[2-carboxyethyl] phosphine (TCEP) disulfide-reducing gel, Gel Code Blue stain, and trifluoroacetic acid ampules. All other chemicals and antibiotics were purchased from Sigma. Purified proteins were concentrated using Millipore YM10 regenerated cellulose ultrafiltration membranes. The Amersham Biosciences Heparin FF, Q-sepharose and Sephadex 200 columns, connected to an Amersham Biosciences PCC-500 fast performance liquid chromatography system, were used for protein purification. A Hewlett-Packard HP-8452 UV-visible and a Beckman DU 7500 diode array spectrophotometer were used for spectroscopic measurements.

Bacterial strains and growth conditions

All *X. campestris* strains were grown aerobically in Silva Buddenhagen (SB) medium (0.5% peptone, 0.5% yeast extract, 0.5% sucrose and 0.1% glutamic acid, pH 7.0) containing the appropriate antibiotics at 28^{o}C. Antibiotics were used at the following final concentrations; 15 μg ml^{-1} tetracycline, 30 μg ml^{-1} kanamycin and 15 μg ml^{-1} gentamicin. The organic hydroperoxide induction experiments were done with exponential phase *X. campestris* pv. phaseoli cultures (OD_{600} of 0.6) treated with 100 μM cumene hydroperoxide (CuOOH) for 30 min.

β-galactosidase assay

Crude bacterial cleared lysates were generated as previously described (16). Cleared lysates were used for total protein determination and enzyme assays. β-galactosidase was determined as described earlier (16).

Site-directed mutagenesis of OhrR

X. campestris. pv. phaseoli mutant OhrR proteins C127S; C131S; C22S,C127S; C22S,C131S; C127S,C131S; and C22S,C127S,C131S were constructed using a polymerase chain reaction (PCR) based site-directed mutagenesis method using primers complementary

to the coding and non-coding sequence of the template OhrR, but containing the desired mismatch to change a given C codon to S, as previously described (16). In order to generate the mutant C127S, the mutagenic forward primer BT355 (5'CAGCTGTTTTCGGCATCGGC3') and M13-Reverse primers; and M13-Forward and a mutagenic reverse primer BT356 (5'GCCGATGCCGAAAACACCTG3') were used in a PCR reaction with pBBRohrR (16) as DNA template. PCR products were digested with *Eco*RI + *Sac*I and cloned into the broad-host-range expression plasmid vector pBBR1 MCS-5 (8). The sequence of the mutated DNA was verified using an automated DNA sequencer. A similar protocol using different pairs of primers was used to produce other OhrR mutants. To generate the C131S mutation, the forward and complementary reverse primers used in the PCR reaction were BT357 (5'GCATCGGCCTCGTCGTTGGAC3') and BT358 (5'GTCCAACGACGAGGCCGCCGCTGC3'), respectively. C22S,C127S and C22S,C131S mutants were created using OhrR C22S plasmid (16) as the DNA template amplified with the BT355- BT356 and BT357-BT358 primer pairs, respectively. To create C127S,C131S; and C22S,C127S,C131S mutants, BT357-BT358 primers were used to amplify OhrR C127S; and C22S,C127S plasmids, respectively.

Purification of OhrR wild type and mutant proteins

The *ohrR* coding region was amplified from pBBRohrR with primers BT377 (5'ATTCTCGAGTCCCGCGCCAAGGCT3') and BT378 (CGAATTCGCCGATGGTCCC3') and the 590 bp *Nco*I and *Xho*I digested PCR product was ligated into the expression vector pETBlue-2 (Novagen), digested with the same enzymes, to create pETohrR. A similar protocol using different DNA templates was used to generate pETC22S and pETC127S,C131S for the high expression of OhrRC22S and OhrRC127S,C131S, respectively. The PCR fragment sequence was confirmed by DNA sequencing. *Escherichia coli* BL21(DE3)/ pLacI (Novagen) harboring either pETohrR, pETC22S or pETC127S,131S was cultured in a 10-L BioFlo 2000 Biofermentor (New Brunswick Scientific, USA), containing LB broth plus 50 μg ml^{-1} ampicillin at 37°C, until the OD_{600} reached 0.8. Isopropyl β-D-thiogalactoside (IPTG) was added to final concentration of 1 mM to induce OhrR expression. The culture was grown for an additional 2 h before the cells were harvested by centrifugation at 5000×*g* for 10 min at 4°C. The bacterial pellet was disrupted using a Bead Beater (BioSpec Products, USA). The crude extract was subsequently treated with 2.5% (w/v) streptomycin sulfate to precipitate nucleic acids prior to being subjected to 20% and 65% $(NH_4)_2SO_4$ precipitations. The 20% to 65 % $(NH_4)_2SO_4$ pellet was suspended in resuspension buffer (20 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 5% glycerol, 0.1

mM PMFS, 2 mM DTT, and 25 mM NaCl) and applied to a HiPrep 16/10 Heparin FF column (Amersham Bioscience). Bound proteins were eluted with a NaCl gradient (0.025-1.0 M). OhrR in eluted fractions was identified using 15% SDS-PAGE. Fractions containing OhrR were pooled and loaded onto a Q-sepharose column (Amersham Bioscience). Bound proteins were eluted with the same NaCl gradient as before. Fractions containing OhrR were pooled and concentrated by ultrafiltration. The purity of protein samples was determined using 15% SDS-PAGE. Finally, purified protein was aliquoted and stored at -20 °C.

Mass spectrometric analysis

Oxidized OhrR protein was prepared by treating purified protein with an equivalent concentration of cumene hydroperoxide (CuOOH). Protein samples were extensively dialyzed in deionized water (6 liters) in a Slide-A-Lyzer cassette (Pierce) prior to analysis by electrospray ionization mass spectrometry (ESI-MS; Micromass, Manchester, UK) precalibrated with horse heart myoglobin. The protein sample (1 μM), in 1% formic acid, was injected at a flow rate of 300 μl/h and positively charged ions in the *m*/*z* range of 800–1800 were analyzed using MassLynx software (version 3.5; Micromass).

Trypsin digestion of OhrR

Prior to performing trypsin digestion, the sulfhydryl groups in OhrR were blocked by two different methods using either 4-vinylpiridine or 2-bromoethylamine and N-ethylmaleimide (NEM). In the case of 4-vinylpiridine treatments, reduced and oxidized OhrR samples in MES buffer (250 mM of 2-(N-morpholino)-ethane sulfonic acid (MES), pH 6.5 and 1mM EDTA) were treated with 100 mM 4-vinylpiridine under denaturing conditions (8 M urea). In the case of 2-bromoethylamine treatments, protein samples in TE buffer (100 mM Tris pH 8.0 and 1mM EDTA) were incubated overnight with 100 mM 2-bromoethylamine pH 8.0 at room temperature under denaturing conditions (8 M urea). The free cysteine ($R\text{-}CH_2\text{-}SH$) residues were alkylated to form S-2-aminoethylcysteines ($R\text{-}CH_2\text{-}S\text{-}CH_2CH_2NH^{3+}$) that are susceptible to trypsin digestion. In order to drop the pH of the mixture and promote S^- formation on free cysteine residues, an equal volume of MES buffer was added to the reaction. Blocking of unmodified cysteine residues was performed by adding 100 mM N-ethylmaleimide (NEM) and incubation at room temperature for 90 minutes. After dialysis and solvent removal, exhaustive trypsin digestion of OhrR, in either oxidized or reduced (DTT-treated) form, was carried out by incubation at an enzyme to substrate ratio of 1:60 at 37 °C for 24 h. Trypsin digestion was carried out at pH 6.5 to minimize disulfide exchange (19).

Tryptic peptide separation using High Pressure Liquid Chromatography (HPLC) and mass spectrometric analysis.

Tryptic maps were generated by injecting samples into a Rainin Dynamax HPLC system equipped with an Vydac C18 column and were eluted with a 90-min gradient consisting of 5–100% Solvent B in Solvent A (0.1% trifluoroacetic acid in deionized, ultrapure H_2O; Solvent B was 70% acetonitrile with 0.08% trifluoroacetic acid in H_2O). The molecular mass of all fragments was determined by ESI-MS.

NBD chloride-sulfenic acid trapping.

The formation of Cys-SOH as a reaction intermediate in the cysteine-dependent oxidation of wild type and the mutant OhrRs, C127S,C131S and C22S, was done by labeling these proteins with NBD chloride as previously described (3), with some modifications. NBD chloride will react with both thiol and sulfenic acids in proteins to form thioether (R-S-NBD) and sulfenate ester (R-S-O-NBD) that can be distinguished by their UV-visible spectra at 420 nm and 347 nm, respectively. Each protein (60 μM) was treated with DTT (2 mM) for 1 h. Excess DTT was removed by ultrafiltration. Various proteins, in phosphate buffer pH 7.0, were stored in an equal volume of Tris [2-carboxyethyl] phosphine (TCEP), an efficient reductant of alkyl disulfides over a wide pH range. Oxidized protein was prepared by treating the purified protein with an equivalent amount of CuOOH and incubating at room temperature for 10 min. Both reduced and oxidized proteins were then treated with NBD-chloride (20 equivalents) under denaturing conditions (4 M Guanidine HCl) for 5 min at room temperature. TE buffer pH 7.0 was added to the solution to give a final concentration of 2.0 M Guanidine HCl. Excess NBD-chloride was removed by ultrafiltration and the absorbance of the proteins samples was measured (200-600 nm) on a Beckman DU 7500 diode array spectrophotometer (Fullerton, CA).

DTNB assay

The free thiol content of reduced and oxidized wild type and mutant OhrRs was determined using the DTNB assay. First, excess DTT in purified protein samples was removed by ultracentrifugation. The samples were then reacted with 100 μM DTNB (5,5'-dithiobis-2-nitrobenzoic acid) in DTNB buffer (0.1 M $(NH_4)_2SO_4$; 0.05 M Tris, pH 8.0; 0.5 mM EDTA, pH 8.0) under denaturing conditions (4 M Guanidine HCl). The 2-nitro-5-thiobenzoic acid (TNB) generated by the reaction was detected by its absorbance at 412 nm (ε =14,150 $M^{-1}cm^{-1}$) (3).

Non-reducing SDS-PAGE

In order to identify the disulfide linkages in reduced and oxidized OhrR, non-reducing SDS-PAGE was performed. Reduced protein was prepared by treating the purified OhrR with 20 equivalents of DTT and then excess DTT was removed by ultrafiltration. Oxidized OhrR was prepared by treating the protein with 1 equivalent of CuOOH. The NEM-treated oxidized protein was prepared by treating the oxidized OhrR with 100 mM of NEM. Protein samples were then subjected to electrophoresis on a SDS-polyacrylamide gel as described previously (9) with some modifications (22).

Results

Two families of OhrR

In bacteria, OhrR is the major sensor and regulator of organic hydroperoxide stress (5, 14, 16). Multiple alignments of OhrR deduced amino acid sequences from both Gram positive and Gram negative bacteria revealed an interesting pattern (Fig. 1). In all OhrRs, there was a highly conserved amino-terminal cysteine residue that corresponded to C22 of *X. campestris* pv. phaseoli and C15 of *B. subtilis* OhrR. This cysteine residue has been shown to be important for the repressor to respond to organic hydroperoxide in vivo and in vitro (5, 16). In *B. subtilis*, C15 has been shown to be the organic hydroperoxide-sensing residue that becomes oxidized by organic hydroperoxide resulting in inactivation of the repressor (5). Examination of other regions of these OhrRs revealed a striking difference. The majority of OhrRs had two or more additional cysteine residues located near their carboxy termini. The OhrRs from *Caulobacter crescentus*, *Brucella melitensis, Vibrio cholerae* and *Burkholderia mallei* had cysteine residues at positions corresponding to C127 in *Xanthomonas*. The OhrRs from *Acinetobacter calcoaceticus, Sinorhizobium meliloti,* and *Agrobacterium tumefaciens* possessed a cysteine residue at position C131 while those from *Azotobacter vinelandii, Clostridium acetobutylicum* and *Pseudomonas aeruginosa* contained an additional cysteine residue at C124 (Fig. 1). *X. campestris* pv. phaseoli and *Erwenia carotovora* OhrRs had cysteine residues at both 127 and 131. By contrast, a minor group of OhrRs consisting of those from *B. subtilis, Oceanobacillus iheyensis*, and *Streptomyces coelicolor* had only a single sensing cysteine residue, C22. These differences in the primary structure of OhrR raised the possibility that there could be differences in the mechanisms involved in sensing and responding to organic hydroeproxide between the different proteins.

C22 and C127 are required for OhrR sensing and responding to organic hydroperoxide

The studies thus far indicate that the reduced form of OhrR binds to target promoters and represses transcription (5, 15). In the presence of organic hydroperoxides, OhrR is inactivated and released from the promoter. It was of interest to know how *X. campestris* pv. phaseoli OhrR senses and responds to changes in organic hydroperoxide levels. Thus, the roles played by C22, C127 and C131 of *X. campestris* pv. phaseoli OhrR in the organic hydroperoxide sensing and inactivation mechanisms were investigated. A series of site-directed mutagenesis experiments were performed to replace both single and various combinations of residues C22, C127, and C131 with serine residues. The ability of these mutant OhrRs to de-repress and repress an OhrR regulated promoter in response to the presence or absence of organic hydroperoxide was then evaluated in vivo by introduction of the plasmid borne *ohrR* mutants (pBBR1MCS-5 (8)) into strain *Xp*P1lacZ, a mini-Tn5 P1lacZ chromosomal insertion mutant in *X. campestris* pv. phaseoli *ohrR* containing a promoterless *lacZ* transcriptionally fused downstream of the OhrR regulated P1 promoter of the *ohrR-ohr* operon (16). Analysis of β-galactosidase activity revealed that *Xp*P1lacZ cells containing plasmids carrying either the wild type or cysteine mutants of *ohrR* repressed P1 promoter activity under uninduced conditions to equal degrees (Fig. 2). However, CuOOH treatment of cells harboring various mutant *ohrRs* revealed novel and unexpected patterns. The mutation C22S in OhrR abolished the ability of the organic hydroperoxide, CuOOH, to derepress expression from the P1 promoter resulting in constitutively low β-galactosidase levels in the presence or absence of CuOOH. This is consistent with previous observations that C22 is required for organic hydroperoxide induction (16). In addition, *Xp*P1lacZ expressing OhrRC127S also failed to response to CuOOH treatment thereby implicating this residue in the sensing process (Fig. 2). By contrast, changing residue C131 in OhrR to S had no effect on CuOOH's ability to derepress the P1 promoter (Fig. 2).

Analysis of sulhydryl groups of reduced and oxidized OhrR

The site directed mutagenesis of *ohrR* revealed that residues C22 and C127 play essential roles in the protein's ability to sense and respond to organic hydroperoxide. However, the roles played by each of these C residues, in the process by which OhrR senses and is inactivated by CuOOH, was not entirely clear. Thus, we attempted to determine the function of different C residues under reduced and oxidized conditions. Wild type OhrR, along with the C22S and C127S-C131S mutants, were purified under reducing conditions in the presence of 2 mM DTT, to prevent the over-oxidation of free cysteine residues, as described in the materials and methods. First, the number of free thiol groups in reduced and oxidized wild type OhrR was determined by DTNB titration assay. The results showed that

the thiol content of OhrR in the reduced and oxidized (after CuOOH treatment) forms were 2.79 ± 0.22 and 0.82 ± 0.14, respectively (Table 1). As expected, reduced OhrR had three free thiol groups while upon CuOOH oxidation only one free sulfhydryl group was detected (Table 1). Various, mutant OhrRs were used to determine which cysteine residues lost thiol groups upon CuOOH oxidation. The DTNB titration assay of reduced and oxidized C22S OhrR gave similar values around 1.78± 0.16 of free sulhydryl groups. Hence, in the absence of C22, the CuOOH treatment had no effect on the remaining cysteine residues. By contrast, the thiol content of reduced and oxidized C127S-C131S OhrR was 1.07 ± 0.04 and 0.01 respectively. The loss of the one free thiol group in C127S-C131S mutant protein clearly indicated an important role for C22 in the CuOOH mediated oxidation of OhrR.

Oxidation of OhrR leads to formation of a sulfenic acid intermediate of the sensing C22

The hydroperoxide oxidation of cysteine residues could lead to formation of a cysteine sulfenic acid (C-SOH) or a more highly oxidized product of cysteine such as cysteine sulfinic acid (C-SO_2H) (3, 5). The previous observations raised the possibility that exposure to organic hydroperoxide likely led to the oxidation of C22 resulting in the formation of a protein C-SOH intermediate. Experiments were carried out to detect the presence of C-SOH, after CuOOH treatment of OhrR, using NBD chloride to trap the highly labile C-SOH (3); however, this assay failed to detect an R-SO-NBD derivative in CuOOH-oxidized OhrR (data not shown). This could have been due to rapid reaction of the protein-sulfenic intermediate (R-SH) with other C residues as suggested by the results of the free thiol groups assay in OhrR that indicated the loss of two thiol groups upon CuOOH oxidation. To eliminate this problem, the NBD chloride trapping experiment was repeated using CuOOH oxidized C127S, C131S OhrR. This should increased stability of the OhrR C22-SOH so that it could be trapped by NBD chloride. The results from ultraviolet/visible spectral scanning showed that NBD-chloride-labeled oxidized C127S, C131S OhrR exhibited a maximal absorbance at 347 nm, a typical characteristic of NBD-adducts of R-SOH (R-SO-NBD) (Fig. 3). While a peak at 420 nm in the NBD chloride treated reduced C127S, C131S OhrR, corresponding to an NBD-modified thiol adduct (R-S-NBD), was detected. NBD chloride assays using C22S OhrR with or without organic peroxide treatments showed a dominant peak at 420 nm indicating the lack direct oxidation of C127 and C131 to Cys-SOH by organic hydroperoxide (data not shown). These analyses confirmed the conversion of the OhrR C22-thiolate (R-S^-) to C22-sulfenic acid (R-SOH) upon oxidiation by CuOOH (Fig. 4). These results were consistent with the results of the DTNB assays of OhrR (Table 1) that identified C22 as the sensing cysteine and that the OhrR C127, C131 could not be directly oxidized by CuOOH.

HPLC/ESI-MS Peptide Mapping of reduced and oxidized OhrR

The loss of two free thiol groups following CuOOH treatment (Table 1) suggested the possible formation of a disulphide bond in oxidized OhrR. Thus, HPLC/ESI-MS was used to determine the existence and location of any disulfide-bonds, as well as the remaining free thiol group, in trypsin-digested reduced and oxidized OhrR. The free thiol groups in both reduced and oxidized OhrR were blocked with 4-vinylpiridine. The tryptic digest fragments of reduced and oxidized OhrR were analyzed by HPLC/ ESI-MS. Tryptic peptides with molecular masses 2829.32 ± 0.51 and 2276.31± 0.70 corresponding to the peptides surrounding C22 (residue 11-34) and C127, C131 (residue 118-137), respectively, were observed in reduced OhrR (Table 2). These two peptides were not detected in tryptic digests of oxidized OhrR protein. Moreover, a new tryptic peptide with mass of 4892 ± 0.63 was detected in the oxidized protein. The molecular mass of this peptide corresponded to the peptide in which C22 formed a disulphide bond with either C127 or C131 (Table 2). Unfortunately, residues C127 and C131 are located in the same tryptic peptide fragment, thus the MS analysis could not determine specifically which cysteine residues had formed a disulfide with C22. In order to identify the carboxy-terminal cysteine residue involved in the disulfide bond, the sulfhydryl of the free cysteine residue in the oxidized protein was modified with 2-bromoethylamine to generate S-2- aminoethylcysteine that is susceptible to trypsin digestion. In order to prevent unmodified thiol groups forming artifactual disulfide bonds as well as undergoing disulfide bond exchange after denaturation, the 2-bromoethylamine-treated oxidized OhrR was reacted with N-ethyl maleimide (NEM), to block the unmodified sulfhydryl groups. The tryptic map of this modified, oxidized OhrR was analyzed by HPLC/ESI-MS. Among the different peptides between oxidized and reduced OhrR, two important peptides from the oxidized OhrR, with molecular masses of 4117.05 ± 0.24 and 731.34 ± 0.00, were observed that corresponded to peptides containing disulfide linkages between C22 and C127 and the peptide SLDELR (residue 132-137), respectively. This indicated that C131 was modified by 2-bromoetylamine and rendered susceptible to trypsin digestion (Table 2). The evidence strongly indicated the presence of a disulfide linkage between C22 and C127 in oxidized OhrR.

Intermolecular disulfide bonding in oxidized OhrR

We extended the investigation by determining whether the disulphide bond that was formed in oxidized OhrR was inter or intra molecular using non-reducing SDS-PAGE (22). The results reveal that the majority of the reduced OhrR existed as a monomer of 18.5 kDa (Fig. 4). After the protein was oxidized by CuOOH treatment, the amount of dimeric OhrR

(37.0 kDa) significantly increased (Fig. 4, compare lanes R and O). When the oxidized protein was retreated with the thiol-depleting agent, NEM, most of the monomeric form was dimerized (Fig. 4, lane NEM). Consistent with the non-reducing SDS-page results, ESI-MS analysis of reduced and oxidized OhrR detected proteins with masses 18000.47 ± 1.83 (monomer) and 35997.97 ± 6.00 (dimmer), respectively, suggesting that the disulfide linkage formed in oxidized OhrR was intermolecular. The reversibility of the disulfide bond was tested by reducing oxidized OhrR with DTT. This resulted in the conversion of most of the dimeric OhrR to the monomeric form (Fig. 4, compare lanes O and DTT) indicating a reversible disulfide linkage.

Discussion

The expression of *ohr* is regulated by OhrR, a transcriptional repressor in the MarR super family (4, 21). One of the major questions regarding the organic hydroperoxide stress response is how does OhrR sense and respond to organic hydropreoxides. The data presented here indicate that the mechanism of sensing and responding to organic hydroperoxide proposed for *B. subtilis* OhrR, a member of the single cysteine family of OhrRs, does not apply to the majority of OhrRs. The analysis of OhrR primary amino sequence alignments clearly show that OhrR can be divided into two groups, a minor group of single cysteine OhrRs such as in *B. subtilis* and a major group containing multiple cysteine residues such as in *X. campestris* pv. phaseoli. In the multiple cysteine groups, the sensing cysteine located near the amino terminus is absolutely conserved while the second cysteine, near the carboxy terminus, is always located in the same general region of the protein, but its exact position varies. Mutational analysis of these cysteine residues in *X. campestris* pv. phaseoli OhrR proved that they are not required for binding of the repressor to the operator site. Nonetheless, in vivo functional analyses of cysteine mutants and wild type OhrRs indicated that both C22 and C127 are required in order for the regulator to sense and respond to organic hydroperoxide. This is a major mechanistic difference from the sensing and responding mechanism of the single cysteine OhrR family where oxidation of the single sensing cysteine is enough to inactivate the repressor. DTNB assays to detect free thiol groups and NBD chloride trapping assays to detect the presence of cysteine sulfenic acid groups in wild type OhrR and various cysteine mutants of OhrR, have yielded important information concerning the mechanism of organic hydroperoxide sensing by OhrR and the roles of different cysteine residues in the process. The loss of two free thiol groups upon CuOOH oxidation of OhrR supports the idea that more than one C residue is involved in sensing and responding to organic hydroperoxide. Moreover, the role of C22 as the sensing residue for CuOOH is

supported by the lack of alteration in the number of free thiol groups in C22S OhrR and the loss of one free thiol group in C127, 131S OhrR (containing only C22) after CuOOH treatment. Thus, C22 has to first be oxidized by CuOOH prior to forming a disulfide bridge with C127 that results in inactivation of the protein. The initial oxidation of C22 was independent of the C127 and C131 residues that could not be directly oxidized by CuOOH. Thus, C127 and C131 do not function as the initial organic hydroperoxide sensing residues. These in vitro results are consistent with the in vivo analyses of mutant OhrRs. The fact that the positions of the carboxyl terminal Cys residues are more varied but generally always in the same region of the protein suggest some structural flexibility in the region of OhrR that allows the C residue to react with the oxidized N-terminal sensing C residue.

Cysteine sulfenic acid (C-SOH) formation in proteins has been reported as the product of the reaction between cysteine thiols and peroxides such as H_2O_2, organic hydroperoxides and peroxynitrite (3, 5, 18). The role of sulfenic acid in the redox sensing pathways of prokaryotic cells involves the oxidation of specific transcription regulators such as *E. coli* OxyR and *B. subtilis* OhrR (5, 7). The detection of a cysteine sulfenic acid intermediate in oxidized C127S,C131S OhrR indicates that CuOOH oxidation of C22 leads to the formation of protein sulfenic intermediate. C-SOH is highly reactive and can, reversibly or irreversibly, generate other forms of modified cysteinyl groups. The irreversible oxidation of C-SOH gives rise to C-SO_2H and C-SO_3H, respectively (18). Nevertheless, C-SOH can be stabilized within the protein and recycled, via disulfide-bonded intermediates, back to C-SH by biological reductants (18). Condensation of C22-SOH in *Xanthomonas* OhrR with the proximal thiol group of residue C127 to form intra- or intermolecular disulfide bonds is the most likely explanation for the data presented here (18). The inability to detect a protein–sulfenic acid intermediate in wild type OhrR indicated that the C22-sulfenic acid is unstable and rapidly reacts with one of the N-terminal cysteines to form a disulphide bond.

Analysis of tryptic digestion fragments of oxidized OhrR labeled with 4-vinylpiridine using HPLC/ESI-MS confirmed that disulfide formation between Cys22 and one of the cysteine residues C127 or C131 did indeed occur. Treatment of oxidized OhrR with 2-bromoethylamine and NEM, prior to trypsin digestion and HPLC/ESI-MS analysis, indicated that the disulphide linkage occured between C22 and C127. Moreover, nondenaturing SDS-PAGE and ESI-MS analyses of reduced and oxidized OhrR indicated that the disulphide bond in oxidized OhrR is an intermolecular bond between C22 and C127 from different OhrR subunits. This is in good agreement with structural analyses of other MarR family members that suggest that reduced OhrR probably binds to its target site as a dimmer (1, 12, 23). The

results of nondenaturing SDS-PAGE of OhrR also indicated that the intermolecular disulphide linkage between C22 and C127 was easily reversed in the presence of the reducing agent DTT (Fig. 4).

The genetic and biochemical evidence presented here has lead to the development of a model for *Xanthomonas* OhrR mediated peroxide sensing and derepression of target promoters, such as the *ohrR* P1 promoter, that likely applies to other members of the multiple cysteine family of OhrRs. Initially, exposure of promoter-bound dimeric OhrR to organic peroxide would result in the oxidation of the redox-sensing residue C22 to form a transient OhrR-C22-SOH intermediate. C22-SOH in each OhrR subunit rapidly reacts with the thiol group of residue C127 in the opposite subunit of the dimmer to form intermolecular disulphide linkages. Disulphide bond formation between the two subunits induces a change in the conformation of the OhrR dimmer such that it is no longer capable of binding DNA. The repressor is then released from the promoter thus allowing transcription of *ohrR*. The fate of the covalently linked OhrR subunits is not known. However, the fact that the disulphide bonds are easily reversed by a reducing agent, combined with gel mobility shift data indicating that this re-reduction restores DNA binding activity (16), raises the possibility that covalently linked OhrR subunits are recycled via reduction by cellular reducing agents. While many of the details as to how OhrRs sense organic peroxide remain to be elucidated, it is clear that the sensing mechanisms of the single and multiple cysteine families of OhrR are distinct.

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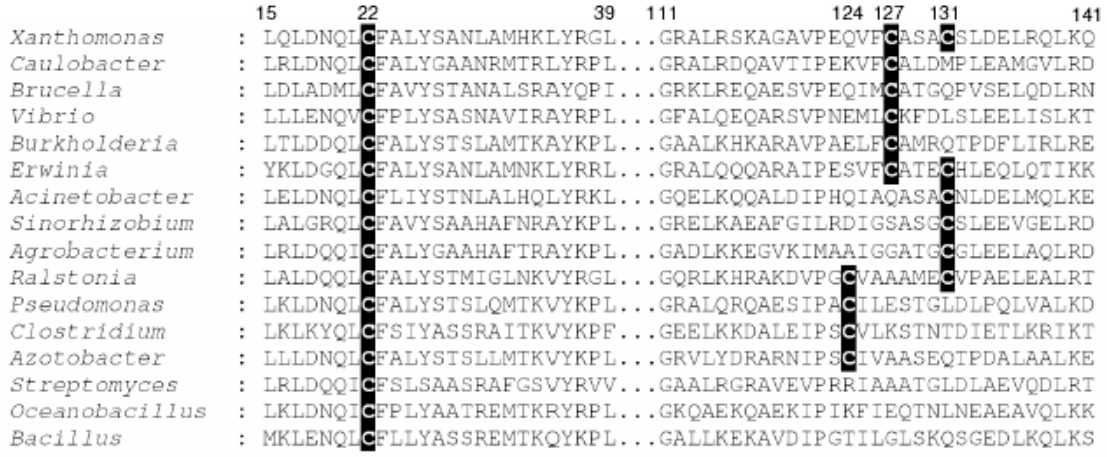

FIG. 1. Alignment of the deduced aminmo acid sequences of OhrRs from various bacteria. *Xanthomonas, X. campestris* (AAK62673): *Caulobacter*, *C. crescentus* (AAK22899); *Brucella*, *B. melitensis* (ALL53650), *Vibrio*, *V. cholerae* (AAF96901); *Burkholderia*, *B. mallei* (AAU46183); *Erwenia*, E. *carotovora* subsp. *atroseptica* (CAG76066); *Acinetobacter*, A. *calcoaceticus* (GAG69726); *Sinorhizobium, S. meliloti* (CAC45533); *Agrobacterium*, *A.tumefaciens* (AAL41860); *Ralstonia*, *R. solanacearum* (CAD18257); *Pseudomonas*, *P. aeruginosa* (AAG06237); *Clostridium*, *C. acetobutylicum* (AAK79536); *Azotobacter*, *A. vinelandii* (EAM06258); *Bacillus*, *B. subtilis* (CAA05594); *Oceanobacillus*, *O. iheyensis* (BAC15414); *Streptomyces, S. coelicolor* (CAB87337). Numbers indicate the position of amino acid residues corresponding to *X. campestris* pv. phaseoli OhrR. Cysteine residues are shaded.

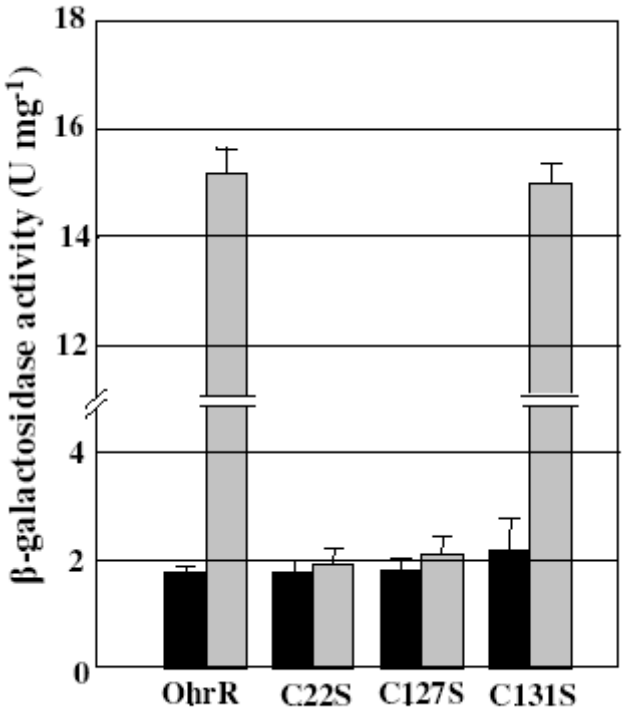

FIG. 2. Organic hydroperoxide-dependent regulation of the *X. campestris ohrR* P1 promoter by wild type and mutant OhrRs.

β-galactosidase activities of exponential phase cultures of various *Xp*P1lacZ transcriptional fusion strains (15, 16) expressing wild type and C22S, C127S and C131S mutant OhrRs that were either induced with 200 μM CuOOH for 30 min (gray bars) or were untreated (black bars). The values presented are the mean and SD of three independent experiments.

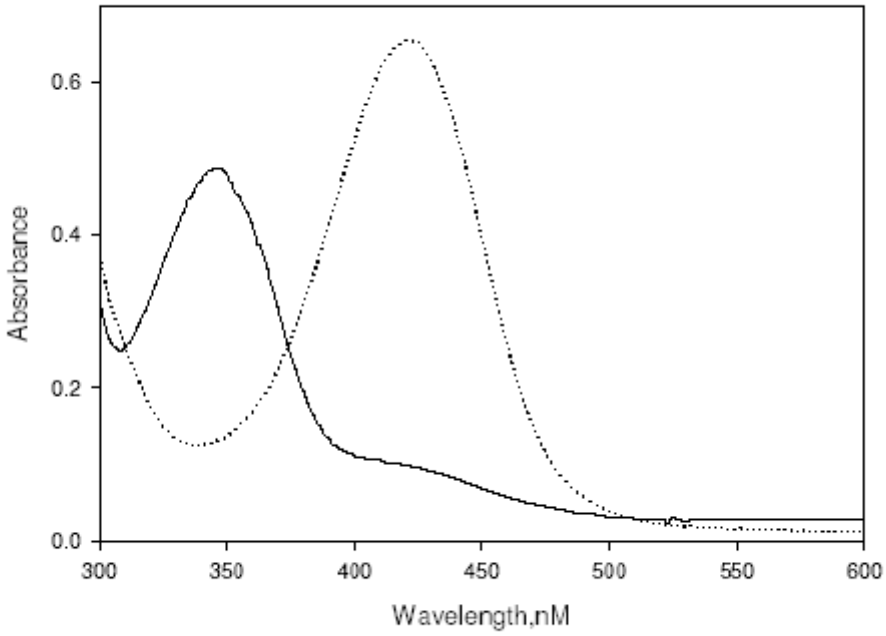

FIG. 3. Spectra of NBD-chloride labeled OhrR mutant proteins

Reduced C127S,C131S OhrR was treated with (dashed line) or without (solid line) an equivalent amount of CuOOH prior to reaction with 20 molar equivalents of NBD-chloride. The NBD-labeled proteins were spectrophometrically scanned from 300nm to 600 nm.

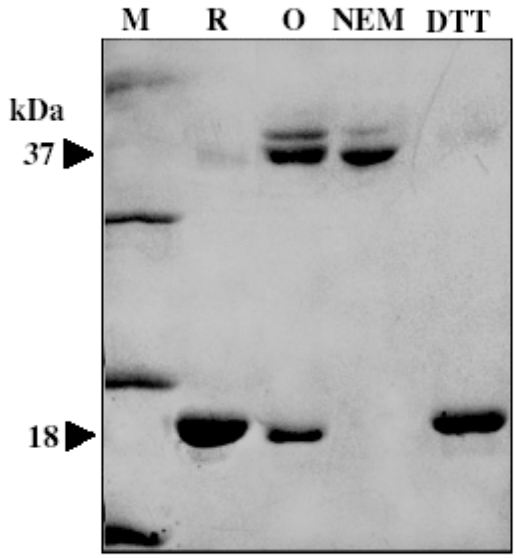

FIG. 4. Disulfide bond formation between OhrR monomers

Purified OhrR prepared under various conditions were separated in non-reducing SDS-PAGE. R, reduced OhrR; O, oxidized OhrR; NEM, oxidized OhrR treated with N-ethyl maleimide; DTT, oxidized OhrR treated with DTT. Arrows indicate the position of monomers (18 kDa) and dimmers (37 kDa). Protein bands were stained with Coomasie blue. M represents protein molecular weight markers.

Table 1

DTNB analysis of free thiols in OhrR and mutant proteins under denaturing conditions

OhrR (remaining Cysteine)	Free thiols	
	Reduced[a]	**Oxidized**[b]
OhrR (C22, C127, C131)	2.79 ± 0.22	0.82 ± 0.14
C22S (C127, C131)	1.89 ± 0.11	1.78 ± 0.16
C127131S (C22)	1.07 ± 0.04	0.010 ± 0.001

[a]Proteins were prepared under reducing conditions

[b]Proteins were oxidized by exposing to 1 equivalent cumene hydroperoxide

Table 2

The masses of trypsin digest fragments of OhrR under oxidized and reduced conditions

	Residues	Sequence	Cysteine	Expected Mass	Observed mass
Reduced fragment 1	11-34	TDTLLQLZ[a]FALYSANLAMHK	C22	2829.29	2829.32 ± 0.51
Reduced fragment 2	118-137	AGAVPEQVFZ[a]ASAZ[a]SLDELR	C127C131	2276.62	2276.31 ± 0.70
Oxidized fragment	11-34 with 118-137	TDTLLQLCFALYSANLAMHK AGAVPEQVFCASACSLDELR	C22 C127C131	4893.62	4892 ± 0.63
Modified oxidized fragment	11-34 with 118-131	TDTLLQLCFALYSANLAMHK AGAVPEQVFCASAC*[b]	C22 C127	4117.76	4117.05 ± 0.24
The rest fragment after modified	132-137	SLDELR	-	731.38	731.34 ± 0.00

[a]Z is free Cys modified with 4-vinylpiridine

[b]C* is alkylation of free Cys residue with 2-bromoethylamine to S-2-aminoethylcysteine which was digested with trypsin

4.2. Chemical modulation of physiological adaptation and cross protective responses against oxidative stress in soil bacterium and phytopathogen, *Xanthomonas*.

Abstract

Soil bacteria need to adapt quickly to changes in the environmental conditions. Thus, physiological adaptation plays an important role in microbial survival, especially under stressful conditions. Here, the abilities of chemicals and pesticides to modulate physiological adaptive and cross protective responses, that make the bacteria more resistant to oxidative stress, are examined in the soil bacterium and phytopathogen, *Xanthomonas*. The genetic basis for the observed stress resistance, as well as the regulatory mechanisms controlling gene expression during the process, has begun to be elucidated.

Introduction

Bacteria, like other living organisms, alter their physiological processes in a manor dictated by outside environmental factors. These adaptations, in response to different stimuli, are often preceded by alterations in the pattern of gene expression resulting in increased survivability under unfavorable conditions. For the purpose of this review, physiological adaptation is defined as the ability of a chemical or a stress to induce protection against a subsequent exposure to the same chemical or stress. By contrast, physiological cross protection occurs when exposure to a chemical or a stress subsequently induces protection to unrelated chemicals or stresses. Here, we examine the ability of low-level exposure to various chemical inducers to stimulate adaptations that result in increased resistance to hyper stress conditions, such as exposure to oxidants, in the soil bacterium and phytopathogen, *Xanthomonas*.

By definition, an oxidative stress condition arises from an excess of reactive oxygen species (ROS), including superoxide anions, H_2O_2, and organic hydroperoxides, which are highly toxic to biological macromolecules. Bacteria have evolved both enzymatic and non-enzymatic mechanisms to remove these ROS [3]. Questions remain as to where and when *Xanthomonas* would encounter ROS and oxidative stress. Aerobic respiration generates

large quantities of reactive oxygen species. Also, it has been shown that plants increase the production and accumulation of ROS as a part of the active defense response to microbial infection [5]. Thus, during interactions with plants, *Xanthomonas* must overcome toxic ROS in order to proliferate and multiply. As a soil bacterium, *Xanthomonas* is also exposed to environmental pollutants, some of them are strong oxidants. Widely used herbicides such as paraquat, as well as some metal pollutants, are potent generators of ROS, *in vivo*.

The simplest bacterial adaptation to stress probably occurs during the stationary phase of growth. During stationary phase, cells become starved for nutrients. Many gram-negative bacteria enter into a physiological state during stationary phase that allows them to remain viable for a long period of time. Interestingly, these stationary phase cells also become hyper-resistant to many types of stresses including oxidative stress. In some bacteria, general stress resistance is mediated, at least in part, by the product of the stationary phase sigma factor gene, *rpoS* [4]. In *Xanthomonas*, we have observed that stationary phase cells are hyper-resistant to H_2O_2, organic peroxides and superoxide generators, but not to either pH or heat stress [12]. Once stationary phase induced stress resistance is established, *de novo* protein synthesis is no longer required. At present, the regulatory mechanism(s) controlling the establishment of this general stress resistance phenotype in *Xanthomonas* is unknown, but the response appears to be independent of nutrient levels or the presence of cell derived metabolites in the stationary phase medium [12]. Some of the characteristics of the stationary phase induced oxidative stress resistance response in *Xanthomonas* are unlike that observed in other bacteria suggesting that a wide range of mechanisms may be employed by different bacteria to protect themselves from stresses during stationary phase.

The inducible adaptive response

Actively growing bacteria are generally more susceptible to stresses. Most bacteria have inducible stress protective systems to deal with a variety of environmental assaults. One of the most studied of these systems is the oxidative stress defense system. In *Xanthomonas*, as well as in other bacteria, exposure to low concentrations of the oxidative stress inducer, H_2O_2, induces high-level resistance to a subsequent challenge with lethal concentrations of H_2O_2 (Fig.1) [8]. This inducible physiological adaptive response is not unique to H_2O_2. In several bacteria, other oxidants have been shown to be capable of inducing an adaptive protective response [2]. In *Xanthomonas*, as in many gram negative bacteria, OxyR is one of the major peroxide sensing transcription regulators involved in controlling the oxidative stress response. Normally, the OxyR dimer exists in a reduced form that represses expression of genes in its regulon. Upon exposure to peroxides, the reactive sensing cysteine residue, C199

of OxyR becomes oxidized resulting in the subsequent formation of an intermolecular disulphide bond [13]. Oxidized OxyR is then able to interact with RNA polymerase and activate transcription of the genes in its regulon [11]. The role of OxyR in the *Xanthomonas* adaptive response to oxidative stress has been investigated using an *oxyR* mutant [8]. The results show that the regulation of this stress response is complex. Inactivation of *oxyR* renders cells highly susceptible to all oxidants confirming the importance of this global regulator in the oxidative stress response. H_2O_2-induced adaptive protection against H_2O_2 killing is clearly dependent on a functional *oxyR* as shown by the loss of the response in the *oxyR* mutant (Fig. 1). *oxyR* is also a regulator of genes involved in peroxide metabolism [11]. Thus, changes in the levels of enzymes such catalase (involved in H_2O_2 degradation) and alkyl hydroperoxide reductase (involved in organic peroxide and H_2O_2 degradation) in response to various inducers have also been investigated. As expected, the expression of catalase (KatA) and alkyl hydroperoxide reductase (AhpC) is highly induced (at least 5-10-fold over uninduced levels) by pre-treatments with low levels of peroxides in an OxyR dependent manner (Fig. 2). During exponential phase, the relative levels of these enzymes show a direct correlation with the level of resistance to H_2O_2. Thus, the H_2O_2 induced adaptive response is most likely a result of the increased expression of *katA* and *ahpC*. Ohr, a thiol peroxidase first discovered in *Xanthomonas* [9], belongs to a new class of genes involved in organic peroxide metabolism. The enzyme metabolizes organic peroxide much more efficiently than H_2O_2 [1]. *ohr* is regulated by OhrR, an organic peroxide sensing transcription repressor [10]. OhrR is efficiently oxidized by organic peroxide but, only poorly by H_2O_2 [6]. Inactivation of *ohr* resulted in an increased sensitivity to organic peroxide, but not to other oxidants. Preliminary data suggest that the *ohrR-ohr* system may have a role in adaptation to exposure to complex organic peroxides. While it is clear that peroxides elicit a strong adaptive response in *Xanthomonas*, it is interesting that no physiological adaptive response to superoxide generators has been observed in this organism [7].

The inducible cross protective response.

Superoxide generators such as paraquat and menadione are strong inducers of cross protective responses to H_2O_2 and organic peroxide killing (Fig. 1). The genetic basis of the regulation of the induced cross protective response is also complex. Unlike the H_2O_2 adaptive response, the superoxide generator (menadione) induced cross protection against H_2O_2 and organic peroxide is OxyR independent (Fig. 1) [8]. Interestingly, the superoxide generator, menadione is the most potent inducer of both catalase and *ahpC* expression and can increase enzyme expression by up to 10-fold (Fig. 2). This induction mechanism is dependent on the

oxidation of OxyR, possibly by H_2O_2 generated through the dismutation of superoxide anions. By contrast, menadione treatment has no effect on *ohr* expression. Nonetheless, in the absence of OxyR, menadione could still induce high levels of resistance to H_2O_2 and organic peroxides. Thus, menadione induced physiological cross protection against H_2O_2 and organic peroxide involved activation of genes other than catalase, *ahpC* and *ohr* that could efficiently metabolize or repair peroxides induced cellular damage. Current work involves the identification of novel menadione regulated genes that have the ability to confer high levels of peroxide resistance. The regulators responsible for the cross protection responses have not been identified. Inducible cross protective responses to different types of peroxides have been observed in *Xanthomonas*. It is known that *oxyR* is involved in the regulation of these cross protective responses since an organic peroxide such as *tert*-butyl hydroperoxide is able to induce protection against H_2O_2 killing in an *oxyR*-dependent manor [8]. In addition to being a H_2O_2 sensor, OxyR has also been shown to be able to sense organic peroxides. The protein could be oxidized and activated *in vivo* by organic peroxide leading to the activation of *ahpC* and *katA* expression. Up regulation of genes involved in peroxide metabolism is responsible for the cross protective responses that are induced by organic peroxide. Although, organic peroxide is a strong inducer of *ohr*, it is unlikely that the thiol peroxidase enzyme is also involved in H_2O_2 protection due to the enzymes low substrate affinity for H_2O_2 [1]. However, H_2O_2 only induces low-level cross protection to organic peroxide killing. H_2O_2 activation of OxyR and the resulting increased *ahpC* expression levels is not sufficient to confer high-level cross protection to organic peroxide in *Xanthomonas*. This is consistent with the observation that the *ohrR*-*ohr* system has the major role in protecting *Xanthomonas* from organic peroxide toxicity but is only weakly induced by H_2O_2 [6].

Conclusion

Exposure of *Xanthomonas* to pollutants, that have the ability to generate strong oxidants such as the herbicide paraquat and metals, could stimulate both adaptive and cross protective physiological responses to oxidative stress in the bacteria. These responses are, in part, mediated by the oxidant-dependent activation of the global peroxide sensing transcription regulators OxyR, OhrR that in turn, activate/derepress genes in their respective regulons. Since increased production and accumulation of ROS are an important part of the plant active defense response to microbial invasion, environmental conditions that render bacterial phytopathogens more resistant to these ROS could alter both the frequency and

severity of crop disease. Therefore, exposure to environmental pollutants could affect crop yields in ways that are not immediately apparent.

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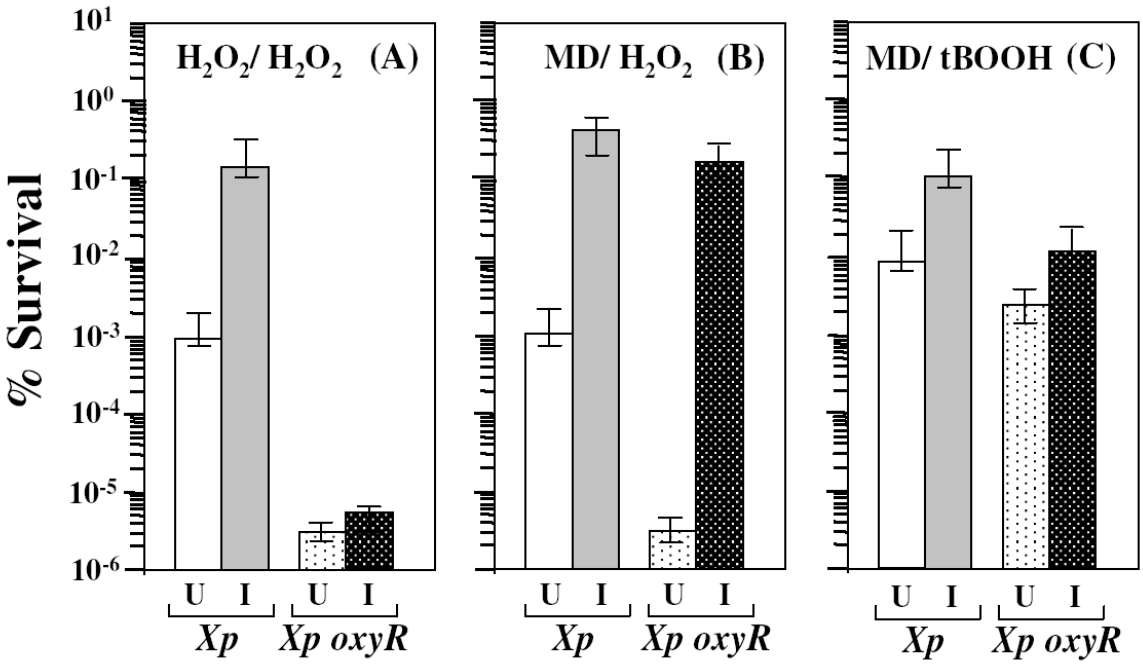

Fig.1. Inducible adaptive and cross protection responses to peroxide killing in *Xanthomonas*.

X ,campestris pv. *phaseoli* (*Xp*) and an *oxyR* mutant (*Xp oxyR*) were grown in SB medium [8]. Each culture was induced with a non-lethal concentration (30 μM) of either H_2O_2 or menadione (MD) for 30 min prior to exposure to a lethal concentration (100 μM) of either H_2O_2, menadione (MD) or *tert*-butyl hydroperoxide (tBOOH) for 30 min. Surviving cells were counted after 24 h incubation. The percent survival was calculated as the colony forming units (cfu) obtained after treatment divided by the cfu obtained from an untreated culture. Uninduced (U) cultures (i.e. those that received no pretreatment) were used as controls.

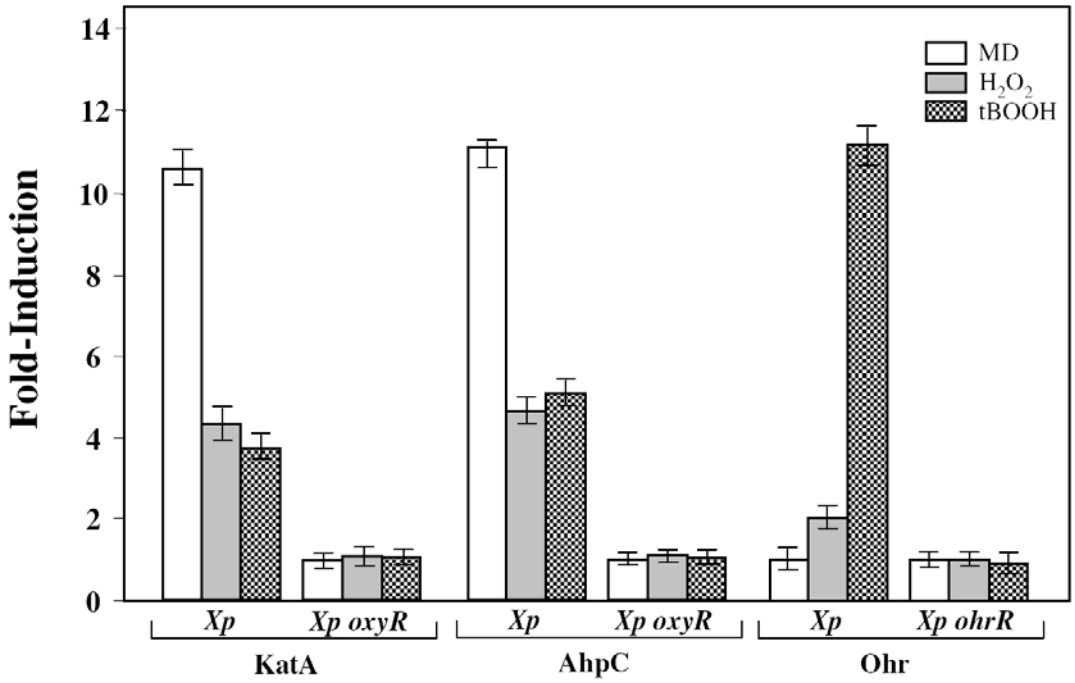

Fig. 2. Induction of *Xanthomonas* peroxide metabolizing enzymes following treatment with oxidants.

Catalase (KatA) activity was assayed as previously described [8]. AhpC and Ohr protein levels were measured by Western blot using antibodies specific to each protein as described previously [9]. The fold-induction is defined as the induced level divided by the uninduced level. *Xp* and *Xp oxyR* were grown in SB medium until exponential phase. Induced cultures received 100 μM of a given oxidant. Uninduced cultures received no oxidant treatment.

4.3. The unique glutathione reductase from *Xanthomonas campestris* : gene expression and enzyme characterization.

Abstract

The glutathione reductase gene, *gor*, was cloned from the plant pathogen *Xanthomonas campestris* pv. *phaseoli.* Its gene expression and enzyme characteristics were found to be different from previously studied homologues. Northern blot hybridization, promoter-*lacZ* fusion and enzyme assay experiments revealed that its expression, unlike in *E. coli*, is OxyR-independent and constitutive upon oxidative stress conditions. The deduced amino acid sequence shows a unique NADPH binding motif where the most highly conserved arginine residue, which is critical for NADPH binding, is replaced by glutamine. Interestingly, a Search of the available Gor amino acid sequences from various sources, including other *Xanthomonas* species, revealed that this replacement is specific to the genus *Xanthomonas*. Recombinant Gor enzyme was purified and characterized and was found to have a novel ability to use both, NADPH and NADH, as electron donor. A *gor* knockout mutant was constructed and shown to have increased expression of the organic peroxide-inducible regulator gene, *ohrR*.

Introduction

Glutathione is a major cellular free thiol-containing compound that is present in animals, plants, fungi, and a large number of prokaryotic species. Glutathione is synthesized by glutathione synthetase and functions as an important cellular antioxidant that can react with a variety of compounds containing electrophilic centers. Apart from its function as an antioxidant, glutathione is also responsible for the maintenance of the intracellular thiol redox status and thus contributes to the function of many biological processes within the cell [1, 2]. For most of its functions glutathione must be in the reduced form. Glutathione reductase (Gor) is the enzyme that reduces the oxidized form of glutathione, glutathione disulfide (GSSG), to reduced glutathione (GSH). In *E. coli*, high steady-state levels of glutathione maintain a strong reducing environment in the cell [3]. Glutathione can react with H_2O_2 , O^-_2, or $HOO^{\cdot}$ to form stable glutathione radicals that will then dimerize to form glutathione disulfide. Finally, glutathione reductase can then transfer an electron to glutathione disulfide, to re-form reduced glutathione [4]. Typically, reduction of GSSG to GSH is catalyzed by

Gor, which in most cases exhibits a marked preference for NADPH over NADH as the electron donor. One of the most important functions of glutathione is to reduce disulfide bridges in proteins caused by oxidative stress. Although formation of the disulfide bonds is easily reversible, their presence can drastically alter protein function.

Glutathione reductase is a member of an important class of flavoprotein enzymes, the disulfide oxidoreductases, containing two active-site electron acceptors: FAD, and a redox-active disulfide. The other members of this class include lipoamide dehydrogenase [5], mercury reductase [6], trypanothione reductase [7] and thioredoxin reductase [8]. These proteins share extensive amino acid sequence similarities, in particular, sequences surrounding the redox active cysteine residues, implying that they have arisen by divergent evolution from a common ancestor [9]. Glutathione reductase is the most important enzyme in maintaining a high intracellular ratio of reduced:oxidized glutathione (approximately 500:1) [10]. Gor is involved in redox cycles that are important in maintaining the anti-oxidative capacity of cells engaged in a wide variety of functions in which reactive oxygen species may be produced and is considered to be a key enzyme involved in maintaining the redox status of the cell during oxidative stress.

Xanthomonas belongs to an important family of plant bacterial pathogens. The bacterial enzymes and genes involved in the oxidative stress response and in regulating cellular redox status are likely to play important roles in disease development. Therefore, study of the glutathione reductase in this phytopathogen would certainly yield crucial information relating to pathogenesis and how *Xanthomonas* adapts to the host plant environment during infection.

This work reports that *Xanthomonas campestris* glutathione reductase has an atypical NADPH binding motif, in which the most highly conserved arginine residue, that is critical for NADPH binding, is replaced by glutamine. This unique change is specific only to Gor from the *Xanthomonas* genus. Furthermore, recombinant *Xanthomonas* Gor was found to have the ability to utilize both NADPH and NADH as electron donors. A *gor*-disrupted *Xanthomonas* mutant displayed increased expression of the organic peroxide-responsive regulator gene (*ohrR*).

Materials and methods

Bacterial cultures and media. X. campestris pv. *phaseoli* was grown aerobically at 28^{o}C in SB medium as previously described [11, 12]. All *Escherichia coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37 ^{o}C.

Nucleic acid extraction and analysis, cloning, and nucleotide sequencing. Genomic DNA extraction from *X. campestris* was performed according to the method of Mongkolsuk et al [13]. Total RNA was isolated by hot-phenol method [13]. Molecular cloning, gel electrophoresis, and nucleic acid hybridizations were performed as previously described [14]. Nucleotide sequences were determined using an automated sequencer, model 310 (Applied Biosystems). *E. coli* and *Xanthomonas* were genetically transformed by a chemical method [14] and by electroporation [13], respectively.

In vitro transcription-translation analysis. Plasmid pGR1800 was used as a template for the expression of cloned gene products using a coupled in vitro transcription-translation *E. coli* S30 extract system (Promega). A ^{14}C-methylated protein molecular weight standard (Amersham) was used as a standard marker.

Construction of chromosomal gor promoter::cat transcriptional fusion strains. The 540-bp *gor* promoter fragment was generated by PCR amplification using pZL-G1 as the template and primers corresponding to the 5′ region starting 340-bp upstream of the translation start site (5′ CGCGAGCGCCTGCGCATCGG 3′) and 3′ region (5′ CGCTGGCCAACTCGATCTTGC3′). A *Bam*HI-*Hin*cII *gor* promoter fragment was ligated into *Bam*HI-*Eco*ICRI digested pUC18*Sfi*I *cat*, subsequently the *Sfi*I fragment containing the *gor* promoter and *cat* reporter was excised and ligated into the minitransposon pUT-Tn5 [15] to create pUT-Pgor which was then conjugally transferred into *Xanthomonas* and a stable kanamycin–resistant transconjugant was selected and named *X. campestris* strain TnPgor.

Amplification and sequencing of the conserved region of Gor in Xanthomonas species. Two oligonucleotide primers (5′ CACATCGTGATCGCCACCGG 3′ and 5′ GCCGCAATCGCCACCGGTGT 3′) corresponding to the conserved amnino acid regions HIVIATG and TPVAIAA were synthesized and used to PCR amplify *gor* gene internal fragments from *X. vessicatoria, X. translucens,* and *X. hyacinthi* chromosomal DNA. The 560-bp fragments were cloned into pDrive (Qiagen) and their DNA sequence was determined.

High-level production and purification of Gor. High levels of *gor* expression for Gor purification were achieved using a His-tagged gene fusion expression vector system (Qiagen) in *E. coli*. Oligonucleotide primers corresponding to the 5′ (5′ CGGCATGCATGAGTGCGCGTTA 3′) and 3′ (5′ CGAAGCTTCGCAACCAACCAT 3′) noncoding regions of the *Xanthomonas gor* locus were used to amplify *gor* from pZL-G1. The resulting 1400-bp PCR product was then digested with *Sph*I and *Hin*dIII, gel purified, and cloned into pQE30 vector (Qiagen). A clone that expressed high levels of the fusion protein was obtained and named pQEG. A 200-ml culture of *E. coli* harboring pQEG was

grown at 37 °C to an optical density at 600 nm of 0.6 and induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. The following purification steps were all done at 4 °C. The cells were subsequently pelleted, and the pellet was resuspended in sonication buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). The suspension was then sonicated for a total of 10 min, with periodic cooling intervals. His-tagged Gor fusion protein was purified using nickle affinity columns according to the manufacturer's recommendations. The purified fusion protein was eluted with 100 mM imidazole in sonication buffer and the homogeneity of the eluted protein fractions was judged by SDS-PAGE. The eluted fractions containing the pure protein were pooled and dialyzed overnight against 20 mM Tris-HCl, pH 7.0 to remove imidazole.

Molecular weight determination of Gor. Protein concentration was measured by the dye binding method [16]. Determination of the molecular weight under denaturing conditions in the presence of SDS was performed as previously described [17]. For molecular weight determination under non-denaturing conditions, the addition of reducing agent (mercaptoethanol) to the protein sample and sample heating were omitted. The native molecular weight of recombinant His-tagged Gor was determined by gel filtration chromatography on a FPLC Akta Purifier (Pharmacia) using a Superdex 75 HR10/30 column (Pharmacia).

Gor enzyme assay. Gor activity was measured by monitoring the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) to thiobis(2-nitrobenzoic acid) by GSH which is produced by Gor according to a previously described method [18].

Disruption of gor gene. A *gor* insertion mutant was created by single recombination of plasmid, pBX170, into the chromosomal copy of *gor*. Specifically, a 1800-bp *Sph*I-*Hin*dIII fragment from pZl-G1 was gel purified and cloned into similarly digested pUC18 resulting in pGR1800. A 800-bp fragment was deleted from pGR1800 by digestion with *Bst*EII, *Hin*dIII and gap-filled with Klenow polymerase and ligated to form pBX1000. pBX1000 was further deleted by removing a 830-bp *Xba*I fragment followed by religation to form pBX170. Therefore, pBX170 contains a 170-bp gene-internal *gor* fragment in pUC18. Plasmid pBX170 was then electroporated into *X. campestris* and ampicillin resistant/*gor* disrupted mutants were selected. The correct integration of pBX170 into *gor* was verified by southern hydridization (data not shown).

ohrR promoter assay. A previously described mini-Tn5 pP1lacZ construct, in which the *ohrR* promoter has been placed in front of a promoterless *lacZ* gene, was used as an

indicator to measure the cellular redox status [19]. β-galactosidase activity assays were carried out as previously reported [20].

Nucleotide sequence accession number. The nucleotide sequence of the *X. campestris* pv. *phaseoli gor* gene has been deposited in GenBank under accession number AY742859.

Results and discussion

Cloning of the X. campestris pv. *phaseoli gor gene and its expression.*

Analysis of multiple amino acid sequence alignments of many Gor proteins revealed the presence of two conserved regions, VGCVPKK and GYIAVE [21], which were suitable for the application of reverse genetics and PCR gene cloning techniques. Degenerate oligonucleotide primers corresponding to the conserved regions were synthesized, taking into account the fact that *Xanthomonas* frequently uses G or C in the last position of codons. One primer corresponding to amino acid region VGCVPKK (5′ GTXGGXTGYGTXCCXAAZAA 3′) and the second primer corresponding to amino acid region GYIAVE (5′ YTCXACXGCZATZTAXCCIXC 3′) (where X represents G and C, Y represents C and T, Z represents A and G, I represents inosine) were used to amplify a 400-bp gene-internal portion of the *X. campestris* pv. *phaseoli gor* gene, which was cloned, sequenced and used as a probe to screen an *X. campestris* pv. *phaseoli* genomic library constructed in a ZipLox vector (BRL life technology). A number of positively hybridizing clones were isolated, and plaques were purified. One positive clone, pZL-G1, was completely sequenced. Analysis of the nucleotide sequence revealed the presence of an open reading frame with a predicted amino acid sequence that shared high homology with Gor from a number of different sources. The *gor* gene was then subcloned into pGR1800, and in vitro transcribed and translated using the *E. coli* S30 system (Promega). A 50-kDa protein band was detected (Fig.1A) that corresponded to the calculated molecular mass of Gor verifying that the cloned *gor* could be in vitro translated to yield a full length protein. Next, we examined the transcription pattern of *gor* in *X. campestris* using Northern blot hybridization experiments. The results, shown in Fig. 1B, revealed that *X. campestris* pv. *phaseoli gor* is transcribed as a 1.5-kb monocistronic mRNA. The level of *gor* mRNA was unaltered when cells were exposed to the oxidative stress inducing agents; diamide, paraquat, *N*-ethylmaleimide (NEM), cadmium and nickel (Fig. 1B). *gor* promoter activity was also monitored in exponential phase cells of the *X. campestris* strain TnPgor, a strain that contains a chromosomal *gor::cat* transcriptional fusion, that had been exposed to 2 mM of either menadione, H_2O_2, *tert*-butyl hydroperoxide (t-BOOH),

cumene, or paraquat for 30 min. Consistent with the mRNA analysis, no significant change in *gor* promoter activity was observed in the presence of any of the oxidants tested (data not shown). Moreover, exposure of cells to 10 μM paraquat for up to 24 h resulted in no increase in *gor* promoter activity (data not shown). This is in contrast to the situation in the yeast, *Schizosaccharomyces pombe*, where *gor* expression has been shown to increase upon exposure to oxidants such as; organic hydroperoxide, diamide and the superoxide generator, menadione [22].

The constitutive expression of *X. campestris gor* raised the question of whether *gor* is in the OxyR regulon as is the case in *E. coli* [23]. To answer this question Gor enzyme and promoter activities were measured in *X. campestris* wild type, an *X. campestris oxyR* knockout mutant, and an *X. campestris oxyR5* strain that has spontaneous mutations at G197 and L301 of OxyR that render it constitutively active [24, 25]. Both Gor enzyme activity and *gor* promoter activity were not significantly different in the three strains (data not shown) indicating that *X. campestris gor* expression differs from *E. coli gor* in that it is not regulated by OxyR. Similar OxyR-independent expression of *gor* has thus far only been observed in the photosynthetic bacterium *Rhodobacter capsulatus,* where *gor* expression was found to not be induced by H_2O_2 [26].

Enzyme kinetic study and the coenzyme binding motif analysis of Gor.

A His-tagged Gor protein fusion was constructed as described in materials and methods. His-tagged *Xanthomonas* Gor was expressed at high level in *E. coli* harboring pQEG and purified using nickle affinity column chromatography. The purity of each eluted protein fraction was determined by SDS-PAGE (Fig.2). Both SDS- and non-denaturing PAGE indicated that the recombinant *Xanthomonas* Gor enzyme ran as a single band of approximately 50 kDa. This was confirmed using gel filtration column chromatography by FPLC that indicated that the enzyme was active as a monomer of 50 kDa in size (data not shown). This is atypical of the known Gor from various sources which are generally dimeric enzymes [27]. The only monomeric Gor reported to date is from the photosynthetic alga *Chlamydomonas reinhardtii* [28].

The kinetic parameters of the recombinant *Xanthomonas* Gor catalyzed reduction of oxidized glutathione were determined (Table 1). Interestingly, the K_m for NADH of *Xanthomonas* Gor was 55.5 μM which is approximately 3.5- and 36-fold lower than those of human erythrocyte [29] and *E. coli* [30] Gor, respectively. Surprisingly, *Xanthomonas* Gor utilized both NADH and NADPH with nearly equal affinity (K_m of 52.6 μM for NADPH

versus 55.5 μM for NADH) (Table 1). This was unusual given that the Gor enzymes that have been studied in detail either use NADPH exclusively or show only a very low affinity for NADH [27]. Only Gor from *Chromatium vinosum* has thus far been reported to preferentially utilize NADH (K_m of 60 μM for NADH versus a K_m of 3,000 μM for NADPH) [31]. The deduced amino acid sequence of *Xanthomonas* Gor was compared with other Gor sequences from various sources including bacteria, plant and human using the Clustal W program [32], in order to identify sequence differences that might explain the enzymes unique NADH/NADPH specificity (data not shown). *Xanthomonas* Gor showed a high degree of sequence identity with Gor sequences from *E. coli* (45%), *Haemophilus influenzae* (45%), human (44%), and *Pseudomonas aeruginosa* (40%). All active site amino acid residues, as well as those involved in FAD binding and GSSG binding, were conserved among the different Gor homologs [27]. Most Gor homologs contained the highly conserved NADPH binding site sequence ($GxGYIAx_{18}Rx_5R$) where the first arginine residue (R200) in the Rx_5R motif is virtually 100% conserved. However, *X. campestris* Gor was found to have a unique NADPH binding site sequence ($GxGYIAx_{18}Qx_5E$) in which the highly conserved arginine residues are replaced by glutamine (Q200) and glutamic acid (E206) (Fig. 3). While this unique NADH/NADPH binding sequence is likely the reason for *Xanthomonas* Gor's ability to utilize both electron donors, the mechanism by which this is made possible remains unknown. A previous study of human glutathione reductase found that NADH also binds to Gor but with less affinity than NADPH, (ie. a 60-fold higher K_m, than that for NADPH) due to its lack of a 2′-phosphate group [30] that can interact with the positively charged residues R218 and R224 [30]. In

E. coli Gor, replacement of R218 and R224 with M and L, respectively, substantially decreased the enzyme's affinity for NADPH and resulted in a catalytically less favourable configuration for bound NADPH [30]. In the NADH-dependent enzymes, like dihydrolipoamide dehydrogenase, conserved E residues replace the R residues in equivalent positions of the NADH binding motif where they were suggested to be involved in binding the 2′-OH group of the ribose moiety of NADH [30]. Therefore, E206 in *Xanthomonas* Gor may facilitate NADH ribose group binding thus allowing the enzyme to use NADH as a cofactor. Rationalizing how the Qx_5E motif facilitates NADPH binding is more difficult since Q200 is an uncharged residue and E206 is negatively charged, so both do not favor binding of the negatively charged phosphate group of NADPH.

A comparison of a total of 86 deduced Gor amino acid sequences, that included those identified from 209 completed microbial genomes as well as all the Gor protein sequences

deposited in the SwisProt database, revealed that the Q200x$_5$E206 NADH/NADPH binding motif was present only in Gor from two *Xanthomonas* species, *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*, while all other Gor sequences contained the highly conserved NADPH binding motif (GxGYIAx$_{18}$Rx$_5$R) in which R200 was absolutely conserved among Gor from all sources except *Xanthomononas*. In order to determine if the Qx$_5$E sequence motif was shared between other members of the genus, *Xanthomonas* DNA fragments spanning the Qx$_5$E region within *gor* in *X. vessicatoria, X. translucens*, and *X. hyacinthi* were amplified by PCR, cloned and sequenced. The sequences from all three *Xanthomonas* species contained the Qx$_5$E binding motif indicating that the Gor NADH/NADPH binding specificity is common to members of the genus. *X. campestris* Gor also differed from Gor of other organisms in respect to its specific activity, that was comparatively low relative to the specific activities of Gor isolated from other sources [30, 33, 34]. Presumably, the relatively low specific activity of *Xanthomonas* Gor may be compensated for by the enzyme's unique ability to utilize both NADH and NADPH.

Increased expression of an organic peroxide-inducible regulator gene (ohrR) in gor mutants.

In order to define the physiological role of *Xanthomonas* atypical Gor, the expression of the well characterized organic peroxide inducible *ohrR* promoter system [19, 35-37] was used as an indicator of the cellular redox state in *Xanthomonas* wild type and *gor* mutant strains. The organic hydroperoxide resistance protein (Ohr) was first identified in *X. campestris* [12] and its expression is regulated by a novel transcription repressor, OhrR (Fig. 4A) [37]. Expression of the *ohrR-ohr* operon is highly induced by organic peroxide through the oxidation of a highly conserved cysteine residue that prevents the protein from binding to its target promoter region [19, 37]. Thus, expression of the *ohrR-ohr* operon is a sensitive indicator of oxidative stress that is induced either by exposure to organic oxidants in the external environment or those generated as a result of internal cellular processes. The question of whether *Xanthomonas* atypical Gor affects the cells ability to respond to oxidative stress was investigated through the use of a highly sensitive *ohrR* promoter-*lacZ* fusion system. A mini-Tn5 pP1lacZ construct was transferred to both wild type and *gor* disrupted mutant strains of *X. campestris* pv. *phaseoli* and their response toward organic peroxide exposure was determined and compared (Fig. 4B). In the absence of peroxide, *ohrR* promoter in *Xanthomonas* lacking Gor exhibited marginally higher β-galactosidase activity (Fig. 4B, uninduced) when compared to the wild type level indicating that the absence of Gor enzyme causes the intracellular environment to become more oxidized and the OhrR mediated

derepression of the *ohrR* promoter. The situation became more pronounced when both strains were exposed to higher concentrations of organic peroxide (t-BOOH). The *ohrR* promoter in *gor* mutants responded more strongly to all concentrations of t-BOOH (Fig.4B, 25-100 μM t-BOOH). Complementation of the *gor* mutant *gor* P1lacZ with a plasmid-borne *gor* in strain *gor* P1lacZ/pGor reduced *ohrR* promoter activity to the level in the wild- type background. The result demonstrated that *Xanthomonas* Gor indeed plays a key anti-oxidative stress role in maintaining the reduced cellular redox state.

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Table 1

Kinetic Parameters of Glutathione Reductase from *Xanthomonas*

Parameter		**NADPH**	**NADH**
K_m	(μM)	52.6	55.5
k_{cat}	(min^{-1})	2,250	1,950
k_{cat}/K_m	($min^{-1}\mu M^{-1}$)	42.8	35.1
V_{max}	(units/ml)	39.2	39.2
Specific activity	(units/mg)	45	39

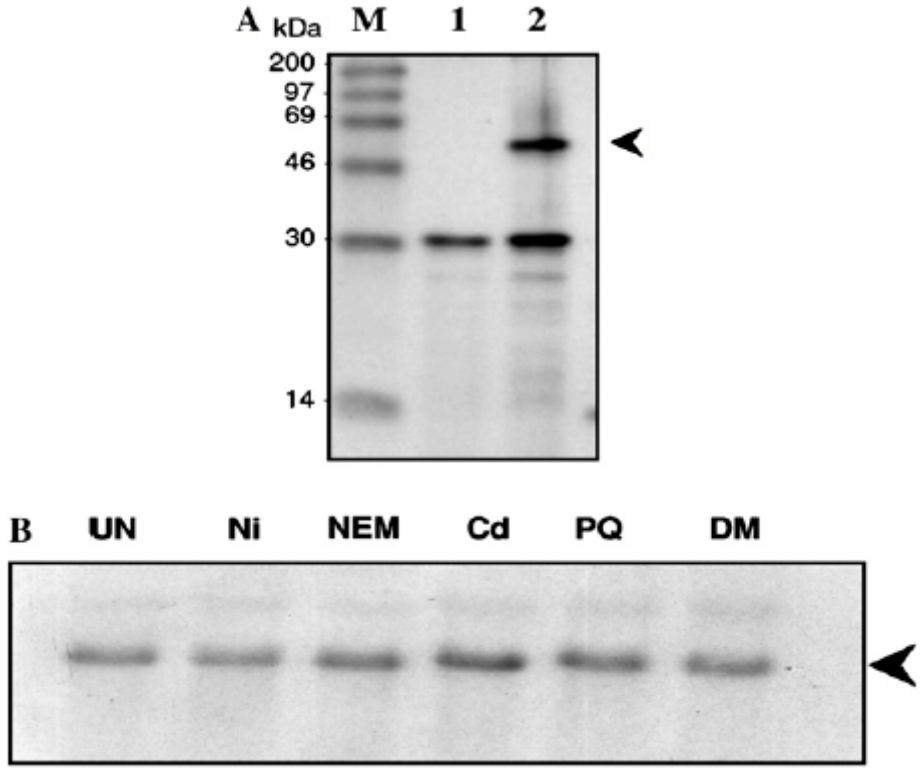

Fig. 1. In vitro translation products (A) and constitutive expression (B) of *gor.* (A) In vitro transcription-translation of pGR1800-encoded proteins with *E. coli* S-30 extracts. Lane M, protein molecular mass markers; lane 1, pUC18; lane 2, pGR1800. The arrow indicates in vitro
translation products of *gor*. The second band at around 30 kDa is the product of the ampicillin resistance gene. (B) Northern blot of total RNA isolated from *X. campestris* uninduced (UN) or induced with 0.2 mM of Ni ($NiCl_2$), NEM (*N*-ethylmaleimide), Cd ($CdCl_2$), or PQ (paraquat), and
2 mM DM (diamide). The membrane was probed with a radioactively labeled *gor* DNA fragment.
Ten micrograms of total RNA was loaded in each lane. The arrow indicates the 1.5 kb-mRNA of *gor*.

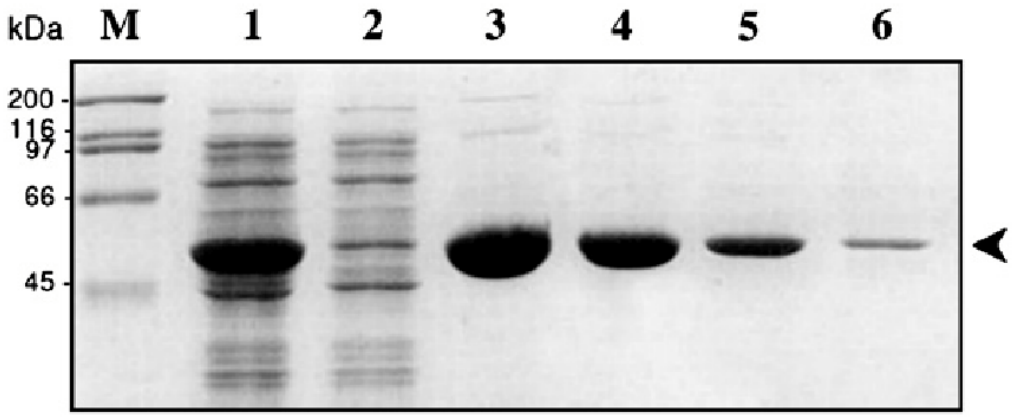

Fig. 2. SDS-PAGE of Gor at different stages of purification. Lane M, molecular mass standard; lane 1,
crude extract; lane 2, after nickle affinity column; lane 3-6, eluted fractions. Each lane was loaded with
5 μl of protein. Arrow indicates Gor protein bands.

Fig. 3. Nucleotide sequence and predicted amino acid sequence of *Xanthomonas gor*. The putative -35, -10 promoter regions, and ribosome binding site (RBS) are underlined. Regions of residues important for GSSG binding are shown in bold letters. Residues involved in NADPH binding are in italic and bold. Q and E residues that replace the most conserved R at the NADPH binding sites are marked by white letters on a black blackground.

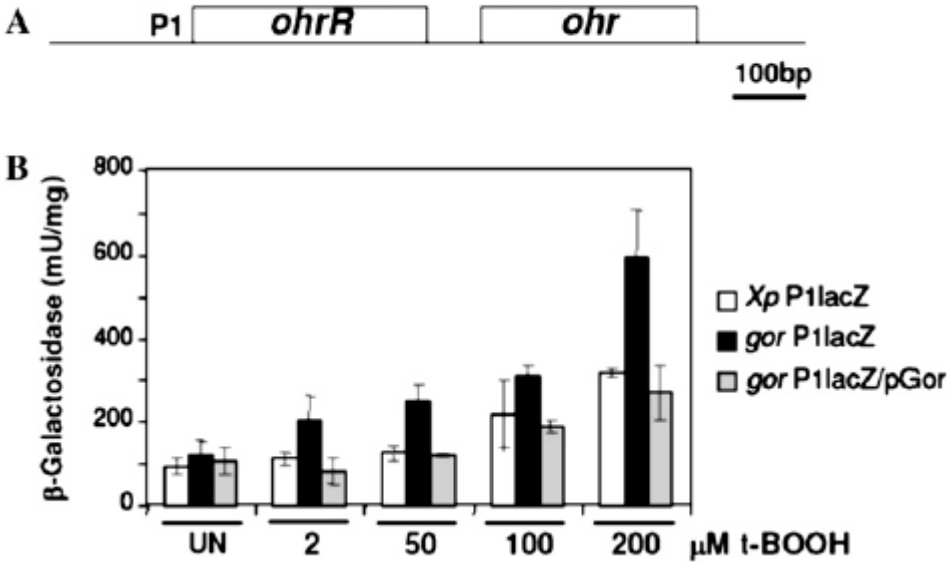

Fig. 4. Diagram of the genetic organization of *ohrR* and *ohr* (A). Expression of the *ohrR* promoter in *Xanthomonas* wild type and *gor* mutants when exposed to organic hydroperoxide (B). β-Galactosidase activities in crude extracts of an *ohrR-lacZ* fusion in parental (*Xp*P1lacZ) and *gor* mutant (*gor* P1lacZ), as well as the complemented strain (*gor* P1lacZ/ pGor) when uninduced (UN), or induced with 2, 50, 100, or 200 μM of t-BOOH. Each value shown is the mean of three separate experiments and error bars indicate standard error of the mean.

CHAPTER V

DpsA protects the human pathogen *Burkholderia pseudomallei* against organic hydroperoxide

Abstract

The human pathogen, *Burkholderia pseudomalle*, is able to survive and multiply in hostile environments such as within macrophages. In an attempt to understand its strategy to cope with oxidative stress, the physiological role and gene regulation of a nonspecific DNA-binding protein (DpsA) was investigated. Expression of *dpsA* increases in response to oxidative stress through increased transcription from the upstream *katG* (catalase–peroxidase) promoter, which is OxyR dependent. *dpsA* is also transcribed from its own promoter, which is activated by osmotic stress in an OxyR-independent manner. DpsA-deficient mutants are hypersensitive to *tert*-butyl hydroperoxide, while overexpression of DpsA leads to increased resistance to organic oxidants. *B. pseudomallei* DpsA can also protect *Escherichia coli* against organic hydroperoxide toxicity. The mechanism of DpsA-mediated resistance to organic hydroperoxides was shown to differ from that of alkyl hydroperoxide reductase.

Introduction

Aerobic microorganisms have evolved a variety of mechanisms to protect their DNA from oxidative damage caused by reactive oxygen species generated during metabolism or present in the environment. In *Escherichia coli*, the best studied bacterium, conditions of either oxidative or nutritional stress cause the production of high levels of the nonspecific DNA-binding protein Dps, which effectively protects DNA against oxidants (Martinez and Kolter *1997*). *E. coli* Dps and its homologue in *Bacillus subtilis*, MrgA, are highly abundant in stationary-phase cells, where the proteins bind DNA without apparent specificity to form extremely stable complexes (Almiron et al. *1992*; Chen and Helmann *1995*). Dps forms spherical dodecamers, homologous to ferritins, that sequester and protect DNA from damage due to oxidative stress, nucleases, UV light, and acid stress (Wolf et al. *1999*; Choi et al. *2000*). *dps* is also expressed during exponential growth, following exposure to low doses of

H_2O_2, as part of the OxyR regulon, suggesting that it is critical for survival during oxidative stress (Altuvia et al. *1994*).

Burkholderia pseudomallei is a facultative intracellular human pathogen that can cause a fatal disease, melioidosis. Bacterial pathogens are frequently exposed to reactive oxygen species during the course of infection. Exposure to oxygen radicals, in the form of superoxides, hydrogen peroxides, and organic hydroperoxides, can result from release of lysosomal contents within inflammatory cells or their generation by bacterial cellular metabolism (Buettner *1993*; Storz and Toledano *1994*). The organic hydroperoxide *tert*-butyl hydroperoxide (*t*-BOOH) has been shown to cause DNA base damage in cultured mammalian cells through its ability to react with metals to generate the highly reactive *tert*-butoxyl radical (Altman et al. *1994*). Recently, an iron-binding Dps-like protein, Dpr, from *Streptococcus mutans* was found to prevent iron-dependent hydroxyl radical formation (Fenton reactions); the protein incorporates up to 480 iron and 11.2 zinc atoms per molecule (Yamamoto et al. *2002*). Therefore, it is possible that the metal-binding ability of Dpr might protect cells against organic hydroperoxide toxicity by preventing hydroxyl radical formation via the Fenton reaction. Elimination of alkyl hydroperoxides is particularly important, because they are highly toxic molecules that can initiate a lipid peroxidation chain reaction that propagates free radicals, leading to DNA and membrane damage (Halliwell and Gutteridge *1984*; Akaike et al. *1992*). Alkyl hydroperoxide reductase (AhpR) and organic hydroperoxide resistance protein (Ohr) are the two major microbial enzymes that have been shown to be involved in the detoxification of organic hydroperoxides through the reduction of physiological lipid peroxides, such as linoleic acid hydroperoxide, thymine hydroperoxide and nonphysiological alkyl hydroperoxides, to their nontoxic alcohol forms. Genetic analysis of several bacteria has shown that mutations in the genes encoding these two enzymes lead to an organic-hydroperoxide-hypersensitive phenotype (Storz et al. *1989*; Mongkolsuk et al. *1998*; Fuangthong et al. *2001*).

In its natural environment, whether in a human host or an external environment during transmission, it is likely that *B. pseudomallei* is faced with growth-limiting or potentially lethal conditions, such as nutrient limitation and osmotic and oxidative stress. A facultative intracellular bacterium, *B. pseudomallei* is able to grow under conditions that are usually detrimental to the development of most microorganisms. However, while the ability of *B. pseudomallei* to resist many kinds of stresses is of particular clinical importance, there is currently very little information demonstrating how *B. pseudomallei* overcomes the various stresses it encounters during infection.

In this work, we reveal the protective role of DpsA against exogenous toxic organic hydroperoxides, as well as characterize its gene regulation in response to oxidative and osmotic stresses.

Materials and methods

Bacterial strains and growth conditions

The clinical isolate *B. pseudomallei* P844, *E. coli* DH5α, and their derivatives were grown in Luria-Bertani (LB) medium. *B. pseudomallei oxyR* (R957), *katG* (G221), and *oxyR katG* (RG27) knockout strains were described in previous studies (Loprasert et al. *2002*, *2003b*). *E. coli* TA4315 is an AhpC-deficient strain (Storz et al. *1989*). Plasmid pAhpC consists of a *B. pseudomallei ahpC* fragment in pBBR-Cm (Kovach et al. *1995*) and was constructed as previously described (Loprasert et al. *2003a*). Pseudomonas agar base supplemented with SR103E (cetrimide, fucidin, and cephaloridine) (Oxoid) was used after conjugation as a selective medium to inhibit growth of *E. coli*. M9 minimal medium was supplied by Gibco BRL. All cultures were grown at 37°C. Tetracycline (60 μg/ml), chloramphenicol (40 μg/ml), specinomycin (100 μg/ml), and erythromycin (100 μg/ml) were used, when required.

Northern analysis

Extraction of total RNA, using a modified hot acid phenol method, and Northern blot analysis of mRNA were carried out as previously described (Mongkolsuk et al. *1996*), using a 500-bp *Sph*I-*Pst*I fragment spanning *dpsA* as the probe. Mid-exponential-phase cultures of *B. pseudomallei* were induced by the addition of menadione to a final concentration 200 μM. Both induced and uninduced cultures were then incubated for an additional 15 min prior to the isolation of total RNA.

Construction of a chromosomal *dpsA::lacZ* transcriptional fusion

A minitransposon containing the *dpsA* promoter fused to *lacZ*, pUT-*dpsA*, was constructed using the vector pUT-*Tn5lacZ1* (de Lorenzo et al. *1990*). In brief, the kanamycin resistance gene was removed and replaced with the trimethoprim resistance gene (*dfr*) of pGSTp (Shalom et al. *2000*). A 600-bp *Eco*RV–*Eco*RI fragment, containing the 5′ end of *dpsA* and 400 bp of upstream sequence, was blunt ended and inserted into the *Sma*I site upstream of *lacZ* in pUT-*Tn5lacZ1*. The resulting plasmid, pUT-*dpsA*, was then conjugally transferred into *B. pseudomallei* and stable trimethoprim resistant transconjugants were selected. Using this method, the *dpsA::lacZ* construct was integrated into the chromosome of

both *B. pseudomallei* wild type and *oxyR* mutant strains (Loprasert et al. *2002*) creating P844D and R957D.

Induction of *dpsA* promoter by salts

NaCl or KCl (500 mM final concentration) was added to overnight cultures (OD_{600}=0.5) growing in M9 medium containing low (0.05%) glucose. The induced and uninduced cultures were grown for 1 h at 37°C before being harvested for β-galactosidase assays.

β -Galactosidase assays

Cell lysates were prepared using B-PER (bacterial protein extraction reagent, Pierce) and assayed for β -galactosidase activity using *o*-nitrophenyl- β -D-galactoside (ONPG) as the substrate as previously described (Steers et al. *1965*).

Construction of *dpsA* and *dpsA oxyR* mutants

The *dpsA* knockout mutant, D18, was created by insertion of a tetracycline resistant plasmid into *dpsA*. A 180-bp *Eco*RI-*Pst*I fragment from the coding region of *dpsA* was ligated into the suicide vector pKNOCK-Tc (Alexeyev *1999*) to create pD180, which was then transferred from *E. coli* S17-1 λ*pir* into *B. pseudomallei* by conjugation. Mutants (D18) containing a single-crossover within *dpsA* were selected on Pseudomonas agar containing 60 μg/ml tetracycline. A *dpsA oxyR* double mutant was created by conjugation between D18 (*dpsA*) and R957 (*oxyR*), resulting the Tc^r Cm^r *dpsA oxyR* mutant strain DR17. Both D18 and DR17 were shown by Southern analysis to contain the desired gene disruptions.

Complementation of the *dpsA* and *dpsA oxyR* mutants

The *dpsA* structural gene and its ribosome-binding site (500 bp) were amplified by PCR and cloned into pGEM-T (Promega) to generate pGEM-D. The primers were D303 (5′ AAGGAGTTTCGAGGATGG3′) and D304 (5′TCACGCGAGCAGCGAACG3′). *dpsA* was then removed from pGEM-D and cloned into the *Apa*I-*Spe*I site of pBBR-Sp (specinomycin resistant), which was created by replacing *cat* (chloramphenicol resistance gene) of pBBR-Cm with the Sp^r gene from pKRP13 (Reece and Phillips *1995*), to create pDps, which was then mobilized into the *dpsA* mutant DR18 by conjugation. In order to complement the *dpsA oxyR* mutant DR17, pUT-*oxyR-ery* (erythromycin resistant) was mobilized into the chromosome of DR17 which harbors pDps, creating DR17R/pDps (*dpsA oxyR TnR*/pDps).

Growth on oxidant agar plates

Cultures of the desired strains grown overnight in M9 low glucose medium were adjusted to OD_{600}=1.0 and serially diluted. Ten microliters of each dilution was spotted onto LB agar

containing 150 μM *tert*-butyl hydroperoxide (*t*-BOOH) and the extent of growth was observed after 24 h of incubation at 37°C.

Survival in oxidant medium

Overnight cultures in LB medium were subcultured (starting OD_{600}=1.0) into fresh modified M9 (0.2% casamino acids, 0.4% glucose) with and without 150 μM *t*-BOOH and the optical density was measured after 7 h of incubation at 37°C with shaking. The relative growth was calculated by comparing the optical density of treated cultures with comparable untreated cultures.

Growth inhibition zone assays

To test the susceptibility of *E. coli* strains to organic hydroperoxides, disk inhibition assays were done as previously described (Mongkolsuk et al. *1998*). Briefly, bacterial cells from an exponential-phase culture (10^8 cells) were added to 3 ml of warm top LB agar. The mixture was then overlayed onto an LB agar plate. When the agar had set, 6-mm paper discs containing 6 μl of 250 μM *t*-BOOH were placed on the cell lawn. Zones of growth inhibition were measured after a 24- h incubation.

Reduction of organic hydroperoxide assay

The reduction of organic hydroperoxide in the growth medium was measured at different times by a reaction using chromogen xylenol orange, ammonium ferrous sulfate, and sulfuric acid as previously described (Shea and Mulks *2002*).

Results and discussion

Regulation of *dpsA* expression by OxyR

We have previously shown that *dpsA* is co-transcribed with *katG* upon exposure to oxidative stress (Loprasert et al. *2003b*) (Fig. 1A). To test whether the global peroxide sensor OxyR is a regulator of *dpsA* expression, the relative amounts of *dpsA* mRNA in *oxyR*, *katG*, and *oxyR katG* double mutant strains were determined by Northern blot analysis. A lack of OxyR in *oxyR* (R957) and *oxyR katG* (RG27) mutants abolished the induction of *dpsA* following treatment with the superoxide generator menadione (Fig. 1B lane M of *oxyR* and *oxyR katG*). Transcripts of *katG–dpsA* (3.5 kb) and *dpsA* (0.6 kb) were not induced in OxyR-deficient strains when cells were exposed to oxidant (menadione). In the *katG* mutant, a *katG–dpsA* transcript was not detected while *dpsA* mRNA was highly induced, indicating that OxyR can activate *dpsA* expression from the *dpsA* promoter (Fig. 1B lane M of *katG*). It is worthwhile noting that, in the wild-type following oxidant treatment, a transcript of

approximately 3.5 kb (*katG–dpsA*) was highly induced while the 0.6-kb mRNA of *dpsA* showed no increase. *dpsA* transcripts were apparent only when *katG* was disrupted, suggesting that OxyR may preferably activate *dpsA* via the *katG* promoter instead of the downstream *dpsA* promoter. Arrangement of *katG* and *dpsA* in an operon would certainly benefit cells by allowing a prompt increase in expression of both genes in response to oxidative stress. While KatG detoxifies the peroxide threat, DpsA would simultaneously protect DNA from peroxide-induced damage.

Inducible transcription of *dpsA* by osmotic stress

To determine whether *dpsA* could be induced by oxidative and osmotic stresses, cells were treated with 0.5–10 mM H_2O_2, 500 mM NaCl or 500 mM KCl and *dpsA-lacZ* expression was monitored. Under these conditions, H_2O_2 did not cause any significant induction of the *dpsA* promoter (data not shown). Expression of *dpsA-lacZ* in wild-type (P844D) cells was induced eightfold and sixfold by NaCl and KCl, respectively, compared to an uninduced control. In the *oxyR*-disrupted mutant R957D, the level of *dpsA-lacZ* induction increased to 18-fold and 12-fold, respectively, for NaCl and KCl (Fig. 2A). Therefore, salt induction of *dpsA* expression does not require OxyR. To confirm that the *dpsA* promoter is indeed induced by salt, the relative amounts of *dpsA* mRNA in the wild- type strain under uninduced and NaCl-induced conditions were determined by Northern blot analysis. A 0.6-kb *dpsA* transcript was highly induced when cells were exposed to 500 mM NaCl for 1 h (Fig. 2B). These results are similar to those obtained in *E. coli,* where NaCl was also found to induce expression of the genes controlled by OxyR, including *dps*, in a RpoS-dependent manner (Michan et al. *1999*). However, it is worth noting that *B. pseudomallei dpsA* responds more strongly to osmotic stress when OxyR is absent whereas expression in an OxyR-deficient strain of *E. coli dps* showed no effect. This leads us to speculate that in *B. pseudomallei* reduced OxyR might normally bind and repress the *dpsA* promoter in the same manner that it has previously been shown to bind to, and repress expression of, the *katG* promoter in uninduced *B. pseudomallei* (Loprasert et al. *2003b*). Therefore, a lack of OxyR would certainly facilitate the RpoS dependence of the *dpsA* promoter by RNA polymerase. Expression of *dps* was also shown to be induced by general stress, e.g. heat shock, exposure to high salt or ethanol, and after glucose starvation in *B. subtilis* (Antelmann et al. *1997*).

***t*-BOOH sensitivity of *dpsA* mutants**

The physiological role of DpsA in *B. pseudomallei* was determined by testing the sensitivity of the various mutants to organic hydroperoxide stress. DpsA-deficient mutants

exhibited hypersensitivity to *t*-BOOH. The *dpsA* mutant D18 did not grow well on 150 μM *t*-BOOH-containing agar while the growth of *dpsA oxyR* double mutant DR1, was even poorer. In both strains, growth was restored to the wild-type level after complementation with the *dpsA*-containing plasmid pDps (Fig. 3A). The ability of the wild-type and mutant strains to grow in M9 minimal liquid medium containing 150 μM *t*-BOOH was also studied (Fig. 3B) and the results are in good agreement with those determined on agar plates. In the wild-type strain P844, overexpression of DpsA from pDps (strain P844/pDps) increased relative growth following *t*-BOOH exposure (75% compared to 55% for wild-type). In strain D18, which lacks a functional *dpsA*, the relative growth following *t*-BOOH exposure was reduced to 36%. When either DpsA or AhpC was overexpressed in the *dpsA* mutant strains D18/pDps and D18/pAhpC the relative rate of growth was restored to the wild-type level. This restoration of *t*-BOOH resistance in the *dpsA* strain expressing AhpC was expected since AhpC reduces and detoxifies *t*-BOOH (Storz et al. *1989*). Dps-deficient *E. coli* mutants have been shown to be hypersensitive to H_2O_2 (Almiron et al. *1992*), *N*-ethylmaleimide (NEM) (Ferguson et al. *1998*), and acid stress (Choi et al. *2000*). *B. subtilis mrgA* mutants are sensitive to H_2O_2 (Chen and Helmann *1995*). To our knowledge, this is the first report demonstrating that Dps protects cells from organic hydroperoxide toxicity. We have previously found that a *B. pseudomallei katG* mutant shows increased sensitivity to H_2O_2, menadione, NEM, and sodium hypochlorite (Loprasert et al. *2003b*). In order to rule out the possibility that KatG expression might be reduced in the *dpsA* mutant strain, the sensitivity of this strain to each of the aforementioned oxidants was measured. It was found that the *dpsA* mutant had the same sensitivity, as determined by growth inhibition zone assays, to H_2O_2 (0.5 M), menadione (100 mM), NEM (0.1 M), and sodium hypochlorite (0.6%) as the wild-type (data not shown).

Protection of *E. coli* against *t*-BOOH by *B. pseudomallei* DpsA

To test whether the protective property of DpsA to organic hydroperoxide is specific to *B. pseudomallei*, *dpsA* was overexpressed in the organic-oxidant-sensitive *E. coli* strain TA4315 (Storz et al. *1989*) which lacks functional *ahpC*. Growth inhibition studies clearly demonstrated that both *B. pseudomallei* DpsA and AhpC could protect *E. coli* against *t*-BOOH toxicity, suggesting that this is a common property of DpsA (Fig. 4A). Since *B. pseudomallei* AhpC also conferred protection to *t*-BOOH, we were interested in finding out whether both AhpC- and DpsA-mediated protection involve the same or different mechanisms. It has been well documented that AhpC is a reductase that catalyzes the reduction of alkyl hydroperoxide to alcohol (Storz et al. *1989*). The levels of *t*-BOOH in the culture medium during growth of *E. coli* strain TA4315 expressing either *B. pseudomallei*

AhpC or DpsA from plasmid were therefore determined. As anticipated, the AhpC-expressing strain (TA4315/pAhpC) completely reduced the *t*-BOOH in the culture medium within 20 min, whereas the DpsA-expressing strain (TA4315/pDps) showed no significant reduction of *t*-BOOH levels relative to strain TA4315 carrying plasmid vector pBBR (Fig. 4B). This indicates that the mechanisms of protection employed by DpsA and AhpC during organic hydroperoxide exposure are distinct. It is likely that the binding of DpsA to DNA acts as a physical barrier to organic-oxidant-induced DNA damage in a manner analogous to that observed for hydrogen peroxide protection (Wolf et al. *1999*). By contrast, AhpC enzymatically detoxifies the organic hydroperoxides. Analysis of *ahpC* expression in *Legionella pneumophila* and *Salmonella typhimurium* showed that *ahpC* levels increased several-fold during intracellular growth of the bacteria (Francis et al. *1997*; Rankin et al. *2002*), suggesting that physiological organic peroxide stress is an important threat to intracellular pathogens. Moreover, *B. pseudomallei* has also been shown to contain AhpC and KatG (catalase–peroxidase) which have the capacity to relieve a portion of the reactive nitrogen and oxidative stresses (Loprasert et al. *2003a*, *b*).

We have uncovered a novel physiological role for DpsA in the protection against organic hydroperoxide stress. The protein acts as an additional system that can be used by *B. pseudomallei* to guard against attack by host immune systems. This study demonstrates that DpsA is a key component of the stress protection system important for the survival of the infectious pathogen *B. pseudomallei*.

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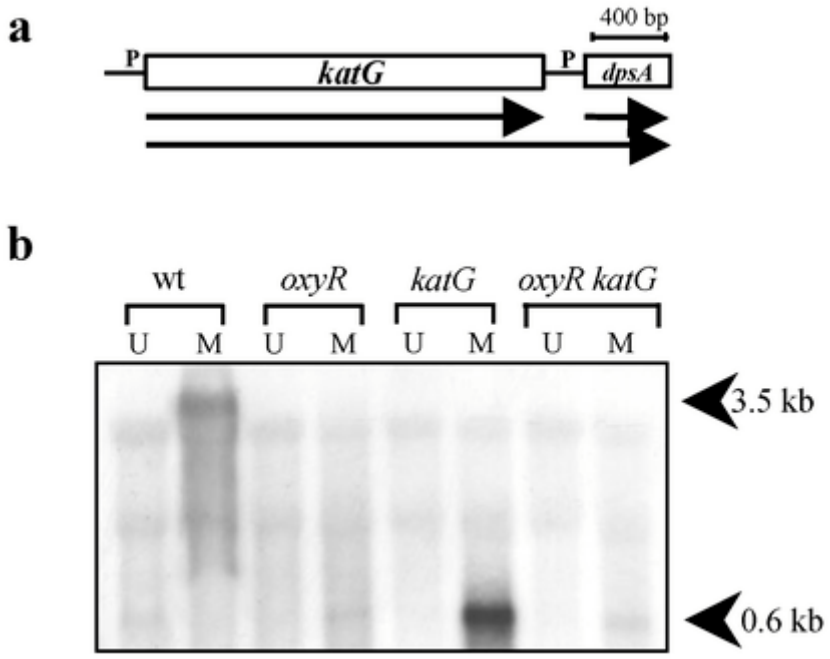

Fig. 1 Gene organization and transcriptional regulation of *katG dpsA* operon expression in response to oxidative stress. **a** The genetic organization of *katG* and *dpsA*. *Arrows* indicate the direction and extent of transcription, *P* promoter regions. **b** Northern analysis of *dpsA* mRNA prepared from *Burkholderia pseudomallei* P844 cells (wt), *oxyR* (R957), *katG* (G221), and *oxyR katG* (RG27) mutants under uninduced (U) and menadione-induced (M) conditions. *Arrowheads* indicate hybridizing mRNAs and their sizes (kb) are shown

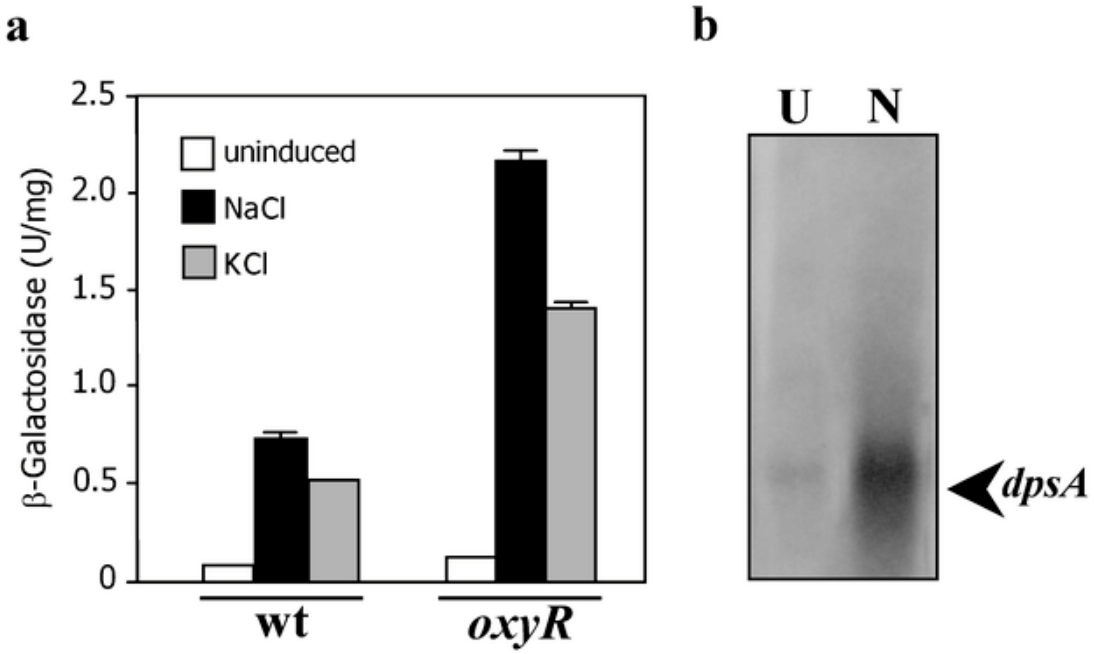

Fig. 2 Regulation of the *dpsA* promoter in response to osmotic stress. **a** β -Galactosidase activities in crude extracts of *dpsA-lacZ* fusion parent P844D (wt), and *oxyR* mutant R957D (*oxyR*) prepared from uninduced cells and cells induced with NaCl and KCl. Each value shown is the mean of three separate experiments: *error bars* standard error from the mean. **b** Northern analysis of *dpsA* mRNA prepared from *B. pseudomallei* P844 cells under uninduced (U) and NaCl-induced (N) conditions

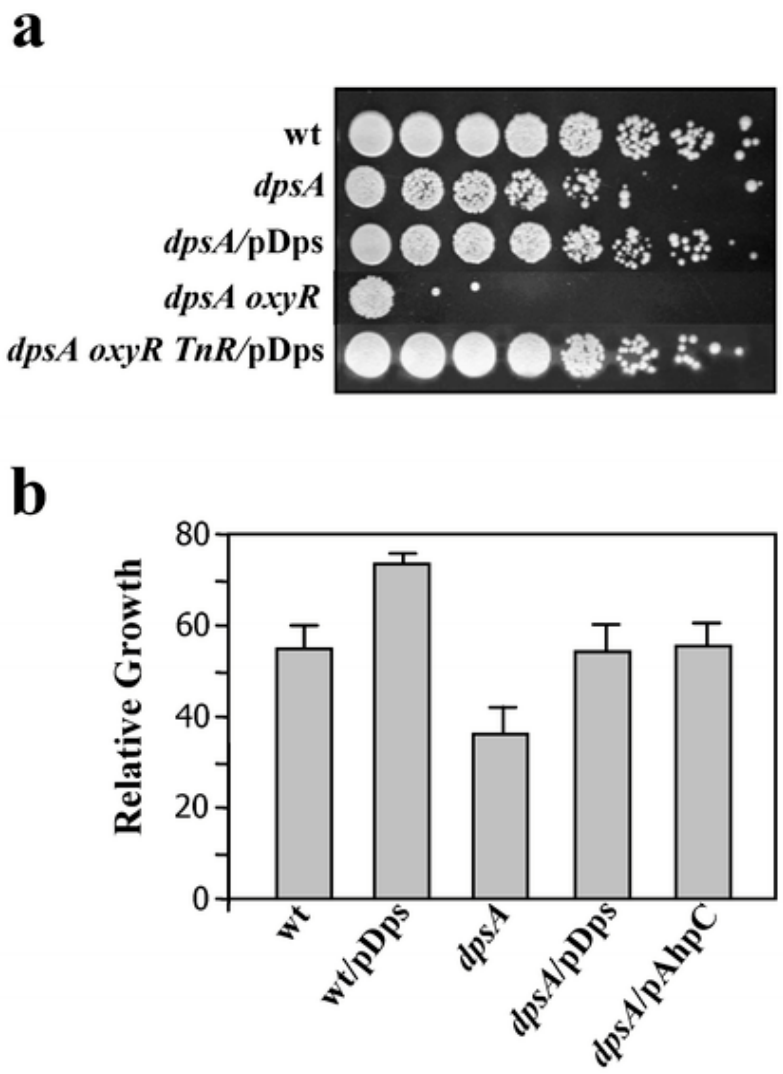

Fig. 3 Sensitivity of *B. pseudomallei* to *t*-BOOH. **a** Growth on *t*-BOOH-containing agar plate of serially diluted *B. pseudomallei* P844 (wt), *dpsA* mutant (*dpsA*), *dpsA* mutant complemented with *dpsA* on plasmid pDps (*dpsA*/pDps), *dpsA oxyR* mutant (*dpsA oxyR*), and the *dpsA oxyR* mutant complemented strain (*dpsA oxyR TnR*/pDps). **b** Relative growth of various strains in *t*-BOOH-containing M9 medium. *B. pseudomallei* P844 (wt), overexpressed DpsA (wt/pDps), *dpsA* mutant (*dpsA)*, complemented *dpsA* mutant (*dpsA/*pDps), and *dpsA* mutant with AhpC plasmid pAhpC (*dpsA*/pAhpC). Each value shown is the mean of three separate experiments; *error bars* standard error from the mean.

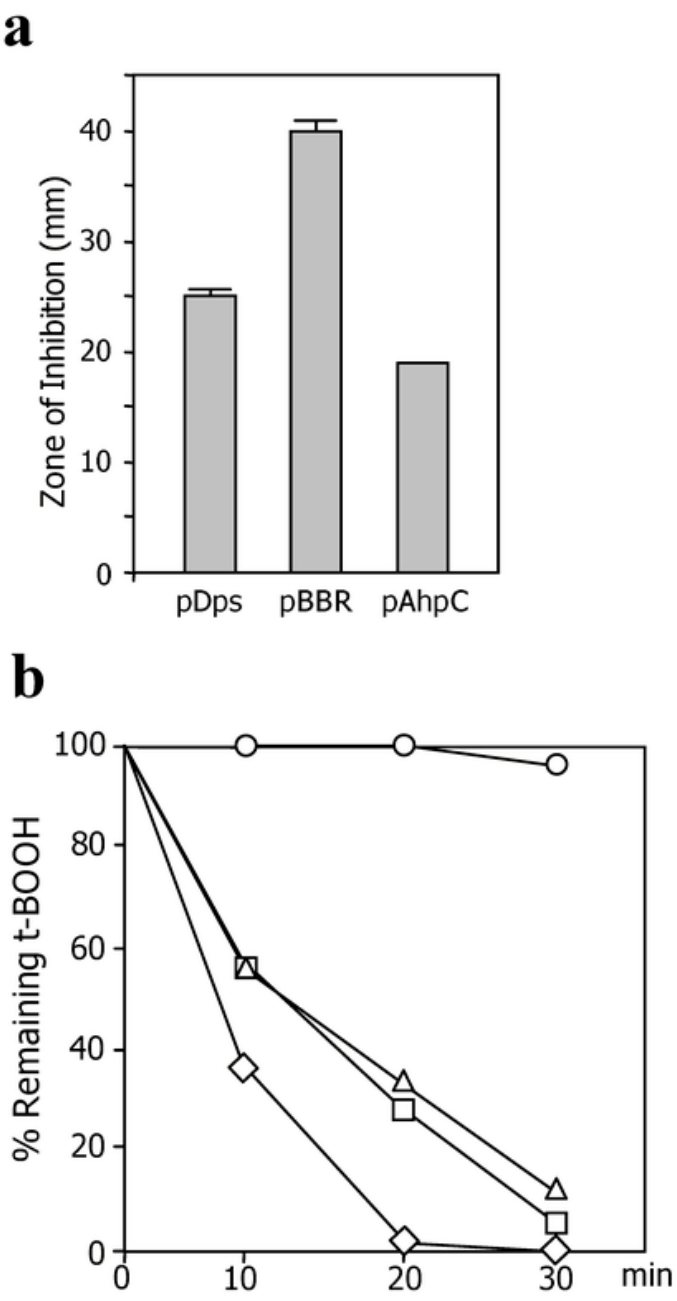

Fig. 4 Growth inhibitory zone and *t*-BOOH degradation assays. **A** Determination of the levels of resistance to *t*-BOOH killing displayed by *E. coli* TA4315 harboring pDps, pBBR vector, and pAhpC. **B** Measurement of the remaining *t*-BOOH after degradation by *Escherichia coli* TA4315 strains harboring pDps (*triangles*), pBBR vector (*squares*), and pAhpC (*diamonds*). Control (circles) is LB without cells. Each value shown in **a** and **b** is the mean of three separate experiments; *error bars* standard error from the mean.

สรุปผลงานตลอดโครงการ

1 ผลงานตีพิมพ์แล้วทั้งหมดของโครงการ

กลุ่มวิจัยดำเนินงานวิจัยในแบคทีเรีย 5 ชนิด คือ

1. แบคทีเรียก่อโรคพืช *Agrobacterium tumefaciens*
2. แบคทีเรียก่อโรคพืช *Xanthomonas campestris*
3. แบคทีเรียก่อโรคในกุ้ง *Vibrio harveyi*
4. แบคทีเรียก่อโรคในคน *Burkholderia pseudomallei*
5. แบคทีเรียก่อโรคในคน *Pseudomonas aeruginosa*

สามารถสรุปผลงานตีพิมพ์แยกเป็นชนิดของแบคทีเรียดังนี้

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3. การพัฒนาบุคลากร

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4. นางสาวนิศานาถ เจริญลาภ (ศ. ดร. ศกรณ์ มงคลสุข เป็นอาจารย์ที่ปรึกษา) (โครงการกาญจนาภิเษก)
   หลักสูตรเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล
5. นางสาวปนัดดา แสนคำ (ศ. ดร. ศกรณ์ มงคลสุข เป็นอาจารย์ที่ปรึกษา) (โครงการกาญจนาภิเษก)
   หลักสูตรเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล
6. นางสาวธิชากร จิตตาวุฒิโภคา (ศ. ดร. ศกรณ์ มงคลสุข เป็นอาจารย์ที่ปรึกษา) (โครงการกาญจนาภิเษก)

หลักสูตรเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล

M. Sc

สำเร็จการศึกษาแล้ว

1. นางสาวแก้วกัญญา นาคจรุง (ศ. ดร. ศกรณ์ มงคลสุข เป็นอาจารย์ที่ปรึกษา)
   หลักสูตรเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล

อยู่ระหว่างการศึกษา

1. นายสุรเวช ฤทธิรุ่งรัตน์ (ศ. ดร. ศกรณ์ มงคลสุข เป็นอาจารย์ที่ปรึกษา)
   หลักสูตรเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล (
2. นางสาวพัชรา งอกงาม (ศ. ดร. ศกรณ์ มงคลสุข เป็นอาจารย์ที่ปรึกษา)
   หลักสูตรเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล
3. นายเอกพล มหาวิหกานนท์ (ศ. ดร. ศกรณ์ มงคลสุข เป็นอาจารย์ที่ปรึกษา)
   หลักสูตรเทคโนโลยีชีวภาพ มหาวิทยาลัยมหิดล

3.2 การพัฒนาของนักวิทยาศาสตร์ในโครงการ

1. ดร. สุวิทย์ ล้อประเสริฐ

ผลงานของ ดร. สุวิทย์ ล้อประเสริฐ ในปี 2002 สามารถตีพิมพ์ในวารสารระดับนานาชาติที่มีค่า impact factor สูงมาก จำนวน 2 เรื่อง โดยเป็น first author และ corresponding author จำนวน 1 เรื่อง ในปี 2003 ดร. สุวิทย์ มีบทความในวารสารที่มี impact factor สูงมาก จำนวน 3 เรื่อง โดยเป็น first author และ corresponding author จำนวน 2 เรื่อง ส่วนในปี 2004 และ 2005 นี้ มีผลงานตีพิมพ์จำนวนในวารสาร impact factor สูงมาก จำนวน 2 เรื่อง โดยเป็น first author และ corresponding author ทั้งหมด จึงนับได้ว่า ดร. สุวิทย์ สามารถพัฒนาศักยภาพในการดำเนินงานวิจัยและตีพิมพ์ผลงานวิจัยในระดับสูง นอกจากนี้ในปี 2548 ดร. สุวิทย์ ยังได้รับทุนพัฒนาองค์ความรู้ใหม่จากสำนักงานกองทุนสนับสนุนการวิจัย

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[2] **Loprasert, S.*,** Sallabhan, R., Whangsuk, W. and Mongkolsuk, S. (2002) The *Burkholderia pseudomallei oxyR* gene: expression analysis and mutant characterization. Gene. 296, 161-169.

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[4] **Loprasert, S.*,** Sallabhan, R., Whangsuk, W. and Mongkolsuk, S. (2003) Compensatory increase in *ahpC* gene expression and its role in protecting *Burkholderia pseudomallei* against reactive nitrogen intermediates. Arch Microbiol. 180, 498-502.

[5] **Loprasert, S.*,** Whangsuk, W., Sallabhan, R. and Mongkolsuk, S. (2003) Regulation of the katG-dpsA operon and the importance of KatG in survival of Burkholderia pseudomallei exposed to oxidative stress. FEBS Lett. 542, 17-21.

[6] **Loprasert, S.*,** Whangsuk, W., Sallabhan, R. and Mongkolsuk, S. (2004) DpsA protects the human pathogen *Burkholderia pseudomallei* against organic hydroperoxide. Arch Microbiol. 182, 96-101.

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2. ดร. ไพบูลย์ วัฒนวิบูลย์

ในปี 2002 ดร. ไพบูลย์ มีผลงานตีพิมพ์ในวารสารระดับนานาชาติที่มี impact factor สูงจำนวน 4 เรื่อง โดยเป็นชื่อแรก 2 เรื่อง และเป็น corresponding author 1 เรื่อง ในปี2003 มีผลงานตีพิมพ์เป็นจำนวนทั้งสิ้น 7 เรื่อง โดยเป็นชื่อแรก 3 เรื่อง และเป็น corresponding author 4 เรื่อง สำหรับในปี 2004 มีผลงานตีพิมพ์ทั้งหมด 2 เรื่อง โดยเป็น co-corresponding author ทั้ง 2 เรื่อง ส่วนในปี 2005 นี้ มีผลงานตีพิมพ์แล้ว 6 เรื่อง โดยเป็น first author/co-corresponding author 1 เรื่อง และเป็น co-corresponding author 3 เรื่อง จึงเห็นได้อย่างชัดเจนว่า ดร. ไพบูลย์ มีการพัฒนาความสามารถในการดำเนินงานวิจัยในระดับสูงขึ้น

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

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[4] **Vattanaviboon, P.**, Whangsuk, W., Panmanee, W., Klomsiri, C., Dharmsthiti, S. and Mongkolsuk, S.* (2002) Evaluation of the roles that alkyl hydroperoxide reductase and Ohr play in organic peroxide-induced gene expression and protection against organic peroxides in *Xanthomonas campestris*. Biochem Biophys Res Commun. 299, 177-182.

[5] Banjerdkij, P., **Vattanaviboon, P**. and Mongkolsuk, S.* (2003) Cadmium-induced adaptive resistance and cross-resistance to zinc in *Xanthomonas campestris*. Curr Microbiol. 47, 260-262.

[6] Chauvatcharin, N., **Vattanaviboon, P.,** Switala, J., Loewen, P.C. and Mongkolsuk, S.* (2003) Cloning and characterization of *katA*, encoding the major monofunctional catalase from *Xanthomonas campestris* pv. phaseoli and characterization of the encoded catalase KatA. Curr Microbiol. 46, 83-87.

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3. รศ. ดร. สุนีย์ กอรปศรีเศรษฐ์

ในปี 2002 ดร. สุนีย์ มีผลงานตีพิมพ์เป็นชื่อแรก ในวารสารระดับนานาชาติจำนวน 1 เรื่อง หลังจากเข้าร่วมเป็นนักวิจัยในโครงการ ทำให้ในปี 2005 นี้ มีผลงานที่รอตีพิมพ์ในวารสารระดับนานาชาติที่มี impact factor สูง จำนวน 2 เรื่อง โดยเป็น first author และ corresponding author จึงเห็นได้อย่างชัดเจนว่า ดร. สุนีย์สามารถพัฒนาศักยภาพในการดำเนินงานวิจัยในระดับสูงขึ้น

[1] **Korbsrisate, S.,** Dumnin, S., Chawengkirttikul, R., Gherunpong, V., Eampokalap, B., Gongviseisoog, C., Janyapoon, K., Lertpocasombat, K. and Shimada, T. (2002) Distribution of *Aeromonas hydrophila* serogroups in different clinical samples and the development of polyclonal antibodies for rapid identification of the genus *Aeromonas* by direct agglutination. Microbiol Immunol. 46, 875-879.

[2] **Korbsrisate, S.**, Vanaporn, M., Kerdsuk, P., Kespichayawattana, W., Vattanaviboon, P., Kiatpapan, P. and Lertmemongkolchai, G. (2005) The *Burkholderia pseudomallei* RpoE (AlgU) operon is involved in environmental stress tolerance and biofilm formation. FEMS Microbiol Lett. in press.

[3] Suparak, S., Kespichayawattana, W., Haque, A., Easton, A., Damnin, S., Lertmemongkolchai, G., Bancroft, G.J. and **Korbsrisate, S.** (2005) Multinucleated giant cell formation and apoptosis in infected host cells is mediated by *Burkholderia pseudomallei* type III secretion protein BipB. J Bacteriol. 188, in press.

4. ดร. ผดุงศรี ดัปส์

หลังจากสำเร็จการศึกษาและเข้าร่วมโตรงการวิจัย ดร. ผดุงศรี ได้เริ่มดำเนินงานวิจัยและมีการพัฒนามาโดยลำดับ โดยในช่วง 3 ปีที่ผ่านมาสามารถตีพิมพ์ผลงานวิจัยในวารสารนานาชาติที่มี impact factor สูงมาก โดยเป็น first author 1 เรื่อง

[1] **Dubbs, P**., Dubbs, J.M. and Tabita, F.R. (2004) Effector-mediated interaction of CbbRI and CbbRII regulators with target sequences in *Rhodobacter capsulatus*. J Bacteriol. 186, 8026-8035.

5. ดร. กรประภา เครือวัลย์

หลังจากสำเร็จการศึกษาและเข้าร่วมโตรงการวิจัย ดร. กรประภา สารถพัฒนางานวิจัยมาโดยลำดับ ทำให้ในช่วง 3 ปีที่ผ่านมาสามารถเสนอผลงานวิจัยตีพิมพ์ในวารสารนานาชาติ โดยเป็น first author 1 เรื่อง

[1] **Kruawal, K.,** Sacher, F., Werner, A., Muller, J. and Knepper, T.P. (2005) Chemical water quality in Thailand and its impacts on the drinking water production in Thailand. Sci Total Environ. 340, 57-70.

6. ดร. รจนา สุขชวลิต

หลังจากสำเร็จการศึกษา ดร. รจนา ได้เข้าร่วมโตรงการวิจัยในปี 2003 ดร. รจนา ได้เริ่มดำเนินงานวิจัย และมีผลงานวิจัยที่สามารถตีพิมพ์ในวารสารนานาชาติที่มี impact factor สูง โดยเป็น first author/ corresponding author 1 เรื่อง

[1] **Sukchawalit, R.*,** Prapagdee, B., Charoenlap, N., Vattanaviboon, P. and Mongkolsuk, S.* (2005) Protection of *Xanthomonas* against arsenic toxicity involves the peroxide-sensing transcription regulator OxyR. Res Microbiol. 156, 30-34.

7. รศ. ดร. ดวงรัตน์ อินทร

ดร. ดวงรัตน์ ได้เข้าร่วมโตรงการวิจัยในปี 2004 ดร. ดวงรัตน์ เป็นอาจารย์และนักวิจัยที่ทุ่มเท สามารถดำเนินงานวิจัยได้ผลเป็นที่น่าพอใจ และสามารถตีพิมพ์ในวารสารนานาชาติ โดยเป็น first author/ corresponding author 1 เรื่อง

[1] **Inthorn, D.*,** Sombatjind, S., Wongsirikul, D., and Wantawin, C. 2005. Cadmium removal by immobilized and free cells of Noctoc paludosum and Rivularia sp. Asian J Microbiol Biotec Env Sc. 7:155-162.

Output ของทีมวิจัยแต่ละคน (First author or Corresponding author)

ผลงาน	Expected (ทั้งโครงการ)	Done (ถึงงวดปัจจุบัน)
จำนวนผลงานตีพิมพ์ในวารสารวิชาการนานาชาติรวมทั้งโครงการ	**>14**	**26**
หัวหน้าโครงการ		
ศ. ดร. ศกรณ์ มงคลสุข	8	**12**
ผู้ร่วมวิจัย		
ดร. สุวิทย์ ล้อประเสริฐ	3	**4**
ดร. ไพบูลย์ วัฒนวิบูลย์	3	**6**
Young Scientists		
ดร. สุนีย์ กอปรศรีเศรษฐ์	1	**2**
ดร. ยุวดี มหาคุณกิจเจริญ	1	
ดร. ผดุงศรี ดัปส์	1	
ดร. กอบชัย ภัทรกุลวณิชย์	1	
ดร. รจนา สุขชวลิต	1	**1**
ดร. มยุรี เฟื่องทอง		
ดร. ดวงรัตน์ อินทร		**1**
นักศึกษาปริญญาเอก		
นางสาววราวรรณ เอี่ยมพึ่งพร	(1)	(1)
นางสาวทัศนี ชูเชื้อ	(1)	(1)
นางเบญจภรณ์ ประภักดี	(1)	(2)

APPENDIX

REPRINTS

[1] Banjerdkij, P., Vattanaviboon, P. and Mongkolsuk, S. (2003) Cadmium-induced adaptive resistance and cross-resistance to zinc in *Xanthomonas campestris*. Curr Microbiol. 47, 260-262.

[2] Carpena, X., Loprasert, S., Mongkolsuk, S., Switala, J., Loewen, P.C. and Fita, I. (2003) Catalase-peroxidase KatG of Burkholderia pseudomallei at 1.7A resolution. J Mol Biol. 327, 475-489.

[3] Eiamphungporn, W., Nakjarung, K., Prapagdee, B., Vattanaviboon, P. and Mongkolsuk, S. (2003) Oxidant-inducible resistance to hydrogen peroxide killing in *Agrobacterium tumefaciens* requires the global peroxide sensor-regulator OxyR and KatA. FEMS Microbiol Lett. 225, 167-172.

[4] Loprasert, S., Sallabhan, R., Whangsuk, W. and Mongkolsuk, S. (2003) Compensatory increase in *ahpC* gene expression and its role in protecting *Burkholderia pseudomallei* against reactive nitrogen intermediates. Arch Microbiol. 180, 498-502.

[5] Loprasert, S., Whangsuk, W., Sallabhan, R. and Mongkolsuk, S. (2003) Regulation of the *katG-dpsA* operon and the importance of KatG in survival of Burkholderia pseudomallei exposed to oxidative stress. FEBS Lett. 542, 17-21.

[6] Nakjarung, K., Mongkolsuk, S. and Vattanaviboon, P. (2003) The *oxyR* from *Agrobacterium tumefaciens*: evaluation of its role in the regulation of catalase and peroxide responses. Biochem Biophys Res Commun. 304, 41-47.

[7] Vattanaviboon, P., Eiamphungporn, W. and Mongkolsuk, S. (2003) Atypical adaptive and cross-protective responses against peroxide killing in a bacterial plant pathogen, *Agrobacterium tumefaciens*. Curr Microbiol. 47, 323-326.

[8] Vattanaviboon, P., Panmanee, W. and Mongkolsuk, S. (2003) Induction of peroxide and superoxide protective enzymes and physiological cross-protection against peroxide killing by a superoxide generator in *Vibrio harveyi*. FEMS Microbiol Lett. 221, 89-95.

[9] Loprasert, S., Whangsuk, W., Sallabhan, R. and Mongkolsuk, S. (2004) DpsA protects the human pathogen *Burkholderia pseudomallei* against organic hydroperoxide. Arch Microbiol. 182, 96-101.

[10] Prapagdee, B., Eiamphungporn, W., Saenkham, P., Mongkolsuk, S. and Vattanaviboon, P. (2004) Analysis of growth phase regulated KatA and CatE and their physiological roles in determining hydrogen peroxide resistance in *Agrobacterium tumefaciens*. FEMS Microbiol Lett. 237, 219-226.

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News & Notes

Cadmium-Induced Adaptive Resistance and Cross-Resistance to Zinc in *Xanthomonas campestris*

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Abstract. Cadmium (Cd) and zinc (Zn) are environmental pollutants affecting both soil and water. The toxicity resulting from the exposure of *Xanthomonas campestris*, a soil bacterium and plant pathogen, to these metals was investigated. Pretreatment of *X. campestris* with sub-lethal concentrations of Cd induced adaptive protection against subsequent exposure to lethal doses of Cd. Moreover, Cd-induced cells also showed cross-resistance to lethal concentrations of Zn. These induced protections required newly synthesized proteins. Unexpectedly, Zn-induced cells did not exhibit adaptive protection against lethal concentrations of Zn or Cd. These data suggested that the increased resistance to Cd and Zn killing probably involved other protective mechanisms in addition to ion efflux.

Heavy metals are important environmental pollutants that have been both intentionally and accidentally released into the environment by agriculture and industry. Abundant inputs of phosphate fertilizers to crop fields can result in an increased accumulation of Cd and other metal ions in the soil [11]. Most heavy metals are highly toxic to ecosystems even at very low concentrations. However, trace amounts of metals such as Zn, cobalt, and nickel are essential nutrients that are required at nanomolar concentrations but become highly toxic at micro- or millimolar concentrations [7]. In general, exposure of bacteria to low levels of one stress can induce a subsequent increase in resistance to the same (adaptive) or unrelated (cross-protection) stress [6]. These responses are necessary strategies for bacterial survival in stressful environments. Presently, little is known regarding these metal-induced physiological responses. Exposure of bacteria to heavy metals has been shown to lead to altered expression of genes involved in metal transport as well as genes involved in other stress responses such as heat shock and oxidative stress [1, 12]. This suggests that global changes in gene expression are required to protect bacteria from metal toxicity.

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Cd-Induced Adaptive and Cross-Protection Responses

Cd and Zn are redox-inactive metals. The mechanism(s) by which *Xanthomonas* defends against metal toxicity is not understood. We have observed that *Xanthomonas* displays atypical adaptive resistance and cross-protection responses to oxidative stress [6, 13]. Thus, the ability of low concentrations of metal ions to induce adaptive resistance and cross-protection against subsequent exposure to high concentrations of the same and non-related metal ions was investigated. *X. campestris* pv. phaseoli cultures were pretreated with a sub-lethal concentration (75 μM) of Cd for 30 min before being challenged with lethal concentrations of Cd and Zn. The results show that pre-exposure to Cd induced a 50-fold increase in the resistance level to subsequent exposure to lethal concentrations of Cd when compared with uninduced cultures (Fig. 1A). *Xanthomonas'* ability to adapt to Cd would permit the bacteria to survive in an environment where

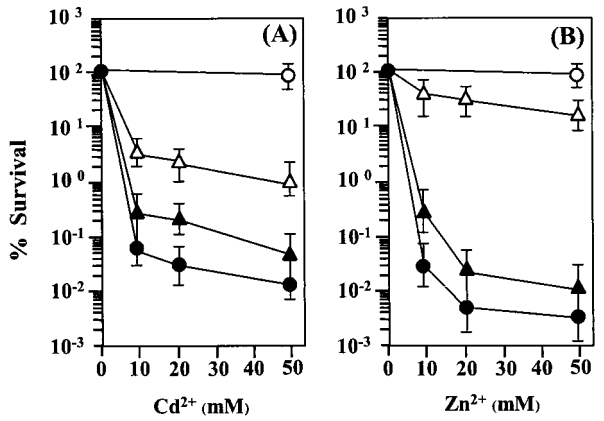

Fig. 1. The effects of Cd pretreatment on the survival of *X. campestris* pv. phaseoli after killing treatments with Cd and Zn. *X. campestris* pv. phaseoli was grown aerobically in Silva-Buddenhagen (SB) medium [6] at 28°C. The heavy metal-induced adaptive and cross-protection experiments were performed by adding either 75 μM $CdCl_2$ or 200 μM $ZnSO_4$ to exponential phase cultures and growing for an additional 30 min before aliquots of cells were removed and treated with indicated concentrations of either Cd or Zn for 30 min. After treatment, cells were removed and washed once with fresh SB medium before determining the survival cells by plating appropriate dilutions on SB agar plates. Colonies were counted after 48 h. Percentage survival was defined as the number of colony forming units (cfu) recovered after treatment divided by cfu prior to treatment multiplied by 100. The survival curves of Cd killing treatments (A) and Zn killing treatments (B) of *X. campestris* pv. phaseoli uninduced (▲), Cd-induced (△), and Cd-induced in the presence of chloramphenicol (●); (○) represents the effect of chloramphenicol without Cd or Zn killing treatments. Values presented are the means and standard deviations of three replicates.

the level of Cd is high. Similar Cd-induced adaptive protection has been observed in other microorganisms [7, 9]. Next, we determined whether pre-exposure to low concentrations of Cd could induce cross-protection against lethal concentrations of Zn. Induction experiments were performed as previously described, with the exception that the uninduced and induced cultures were subsequently treated with lethal concentrations of Zn. The results show that Cd-induced cells were over 1000-fold more resistant to exposure to lethal concentrations of Zn than uninduced cells (Fig. 1B). The ability of Cd to cross-protect against Zn toxicity has not been observed in other bacteria. These results indicated that a low level pre-exposure to Cd induced high and moderate levels of protection against Zn and Cd toxicity, respectively.

Adaptive Resistance and Cross-Protection Responses Required Nascent Polypeptide Synthesis

In general, the induced adaptive resistance and cross-protection against stresses in microbes require de novo protein synthesis [6]. The induction experiments were repeated; however, 100 μg mL^{-1} chloramphenicol (a concentration of the protein synthesis inhibitor that has been previously determined to inhibit protein synthesis in *Xanthomonas* [6]) was added to the bacterial cultures prior to pretreatment with Cd. The induced and uninduced cultures were then treated with lethal concentrations of either Cd or Zn. Both responses were eliminated by the presence of chloramphenicol during the induction step (Fig. 1A, B). The results clearly show that the Cd-induced adaptive resistance and cross-protection responses required de novo protein synthesis. The requirement for nascent polypeptide synthesis implies that the observed responses probably resulted from the ability of Cd to induce the expression of genes involved in metal toxicity protective pathways. In addition, these findings implied that Cd either induced genes in a separate protective pathway against Zn toxicity or that the induced Cd and Zn resistance is due to a common protective pathway such as the chemiosmotic antiporter (Czc efflux system) [8]. Cd toxicity is believed to result from the depletion of glutathione followed by the inactivation of enzymes through reactions between their sulfhydryl groups, leading to elevated production of reactive oxygen species and eventually cell death [10]. Thus, an alternative mechanism for the induced protection against Cd killing could involve induced expression of genes in the oxidative stress protective regulons [3].

Lack of Zn-Induced Adaptive Resistance and Cross-Protection to Cd Killing Treatment

Cd and Zn are known as potent inducers that stimulate the expression of genes encoding efflux pump systems including the *czcCBA* operon [4, 8]. This operon also exists in *X. campestris* [2]. Thus, if induction of a *czc*-like cation efflux system plays a significant role in the protection of *X. campestris* from Cd or Zn toxicity, Zn-induced cells might also be expected to display increased resistance to both Zn and Cd killing treatments. In order to investigate this hypothesis, exponential phase cultures were pretreated with sub-lethal concentrations of Zn prior to exposure to lethal concentrations of Zn and Cd. Unexpectedly, induction with low concentrations of Zn failed to induce both adaptive and cross-protective resistances to Zn and Cd, respectively (Fig. 2A, B). These results suggest that cation efflux is unlikely to be the major system *X. campestris* uses to protect itself from Cd and Zn toxicity.

At present the mechanism(s) responsible for lethal toxicity of Zn in bacteria is still unclear. Unlike Cd,

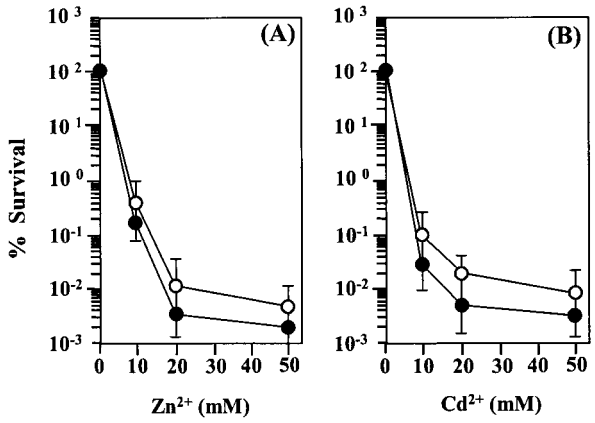

Fig. 2. The effects of Zn pretreatment on the survival of *X. campestris* pv. phaseoli after killing treatments with Zn and Cd. The cultivation conditions, induction, and killing with Cd and Zn were performed as described in Fig.1 legend. The survival curves of Zn killing treatments (A) and Cd killing treatments (B) of *X. campestris* pv. phaseoli in uninduced (○) or Zn-induced (●) cultures. Values presented are means and standard deviations of three replicates.

which induces oxidative stress in bacterial cells [5], our preliminary data suggested that Zn does not induce an oxidative stress response (data not shown). Although low concentrations of Zn could act as an antioxidant by displacement of redox active metal ions [10], the actual mechanism of Zn toxicity is currently being investigated.

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Atypical Adaptive and Cross-Protective Responses Against Peroxide Killing in a Bacterial Plant Pathogen, *Agrobacterium tumefaciens*

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Abstract. Physiological adaptive and cross-protection responses to oxidants were investigated in *Agrobacterium tumefaciens.* Exposure of *A. tumefaciens* to sublethal concentrations of H_2O_2 induced adaptive protection to lethal concentrations of H_2O_2. Similar treatments with organic peroxide and menadione did not produce adaptive protection to subsequent exposure to lethal concentrations of these oxidants. Pretreatment of *A. tumefaciens* with an inducing concentration of menadione conferred cross-protection against H_2O_2, but not to *tert*-butyl hydroperoxide (tBOOH), killing. The menadione induced cross-protection to H_2O_2 was due to the compound's ability to highly induce the peroxide scavenging enzyme, catalase. The levels of catalase directly correlated with the bacterium's ability to survive H_2O_2 treatment. Some aspects of the oxidative stress response of *A. tumefaciens* differ from other bacteria, and these differences may be important in plant/microbe interactions.

Agrobacterium tumefaciens is a soil-borne phytopathogenic bacterium infecting many dicotyledons and causing crown gall tumors worldwide [18]. *A. tumefaciens* is also widely used to generate genetically engineered plants [2].

Aerobic growth generates large quantities of reactive oxygen species (ROS) including superoxide anions ($O_2^{\bullet -}$), peroxide (O_2^{2-}), and hydroxyl radicals ($^{\bullet}OH$) [7]. In addition, the rapid production and accumulation of reactive oxygen species (ROS) known as the oxidative burst has also been shown to be an important plant defense response in plant/microbe interactions [9]. ROS are highly toxic to bacterial cells through their detrimental effects on many biological macromolecules including DNA, proteins, and membrane. Consequently, bacteria have evolved several ways to protect themselves from oxidative damage through the evolution of both enzymatic and nonenzymatic systems that either directly detoxify or repair damage caused by ROS. Also, bacteria have protective pathways that involve stress-induced adaptive and cross-protective responses. Exposure to a sublethal concentration of one oxidant can induce protection against subsequent exposure to killing concentrations of the same oxidant (an adaptive response) or unrelated agents (a cross-protective response) [14]. These responses are highly conserved in many bacteria [3, 4, 14, 20, 21]. Oxidant-induced protective responses often result from the coordinated activation of gene(s) involved in both oxidant detoxification and damage repair [1]. An example of this is found in the plant pathogenic bacterium *Xanthomonas campestris,* which produces elevated levels of a monofunctional catalase (KatA), an alkyl hydroperoxide reductase (AhpCF), and an organic hydroperoxide resistance protein (Ohr) in response to exposure to ROS [13–15]. This increase in detoxification enzymes results in increased resistance to killing by oxidants.

At present little is known about the oxidative stress response of *A. tumefaciens.* Understanding this response could give insights into how *A. tumefaciens* survives in the environment and during interactions with its host. Here, we describe atypical peroxide inducible adaptive and cross-protective responses against peroxide killing in *A. tumefaciens.* The protective levels correlated with the inducer's ability to induce the expression of the peroxide scavenging enzyme, catalase.

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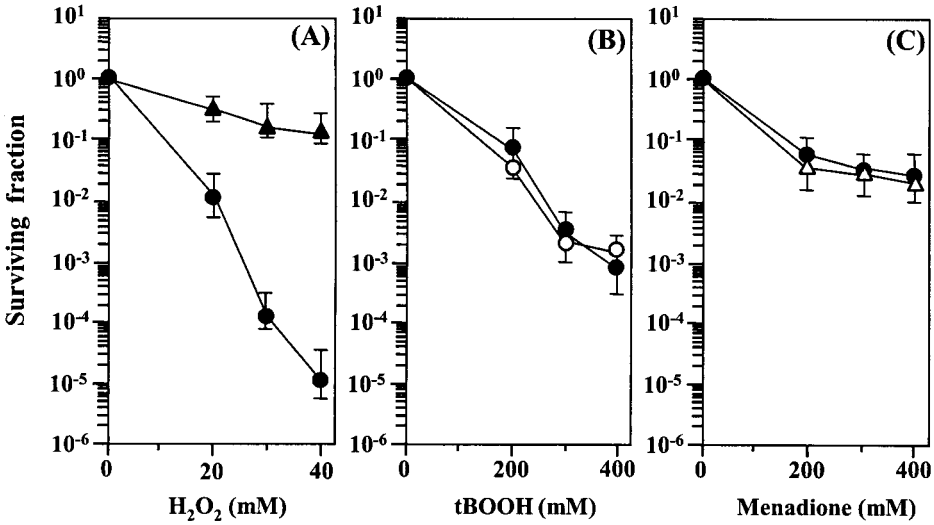

Fig. 1. Induced adaptive responses to peroxides in *A. tumefaciens.* The cultivation conditions, induction, and killing with peroxides were performed as described in Materials and Methods. (A) The H_2O_2 survival curves for uninduced (●), H_2O_2-induced (▲); (B) the tBOOH survival curves of uninduced (●), tBOOH-induced cultures (○); (C) the menadione survival curves of uninduced (●), menadione-induced (△). Values presented are means and standard deviations of three replicates.

Materials and Methods

Bacterial growth conditions. *Agrobacterium tumefaciens* NTL4 [11] was grown aerobically in LB medium at 30°C with continuous shaking at 150 rpm. To ensure synchronous growth, overnight cultures were inoculated into fresh LB medium to give an OD_{600} of approximately 0.1. Exponential phase (OD_{600} about 0.6, after 4 h of growth) cells were used in experiments as indicated.

Determination of resistance levels to oxidants. The peroxide-induced adaptive and cross-protective experiments were performed by adding either 250 μM H_2O_2, 250 μM *tert*-butyl hydroperoxide (tBOOH), or 200 μM menadione to exponential phase cultures. These cultures were grown for an additional 30 min before aliquots of cells were removed and treated with lethal concentrations of either H_2O_2 (20, 30, 40 mM), tBOOH (200, 300, 400 mM), or menadione (200, 300, 400 mM) for 30 min. After treatment, cells were removed and washed once with fresh LB before appropriate dilutions were plated on LB agar. Colonies were counted after 48 h of incubation at 30°C. Surviving fractions are defined as the number of colony-forming units (cfu) recovered after the treatment divided by cfu prior to treatment. All experiments were independently repeated at least three times, and representative data are shown.

Catalase activity assays. Preparation of cell lysates and catalase assays were performed as previously described [19]. Briefly, 20-mL cultures were pelleted and washed once with 50 mM sodium phosphate buffer (PB) pH 7.0. Bacterial suspensions in PB containing 1 mM PMSF, a protease inhibitor, were lysed by brief sonication, followed by centrifugation at 10,000 *g* for 10 min. Cleared lysates were used for catalase assays and total protein determination [19].

Results and Discussion

Adaptive responses against peroxide killing. Inducible protective responses are important for exponential phase cells since they are more susceptible to oxidant killing. We investigated the ability of *A. tumefaciens* to adapt to peroxides and superoxide stresses. *A. tumefaciens* cultures were pretreated with 250 μM H_2O_2 for 30 min before being exposed to killing concentrations of H_2O_2. The results show that the pretreatment increased protection against H_2O_2 killing 10,000-fold (Fig. 1A). By contrast, pretreatment of *A. tumefaciens* with sublethal concentrations of tBOOH or the superoxide-generating agent menadione, did not induce adaptive protection against subsequent exposure to lethal concentrations of these compounds (Fig. 1B, C). The adaptive response towards H_2O_2 treatment is highly conserved and has been observed in many microbes [6, 8, 14, 24]. Although adaptive responses against menadione treatment have been reported in a few microorganisms [5], many bacteria seem to lack this ability [14]. Similarly, adaptation to tBOOH is uncommon in bacteria. It appears that different microbes respond differently to stresses.

Cross-protective responses against peroxide killing. The ability of one type of stress to induce protection against an unrelated stress (cross-protection) is an important survival strategy for bacteria under stressful conditions. Thus, we investigated the ability of different peroxides and superoxide anions to induce cross-protection against unrelated peroxides. *A. tumefaciens* cultures were pretreated with inducing concentrations of menadione or tBOOH and subsequently were treated with lethal concentrations of H_2O_2. The results in Fig. 2A showed that pretreatment with tBOOH did not induce protection against H_2O_2 killing, while menadione pretreatment in-

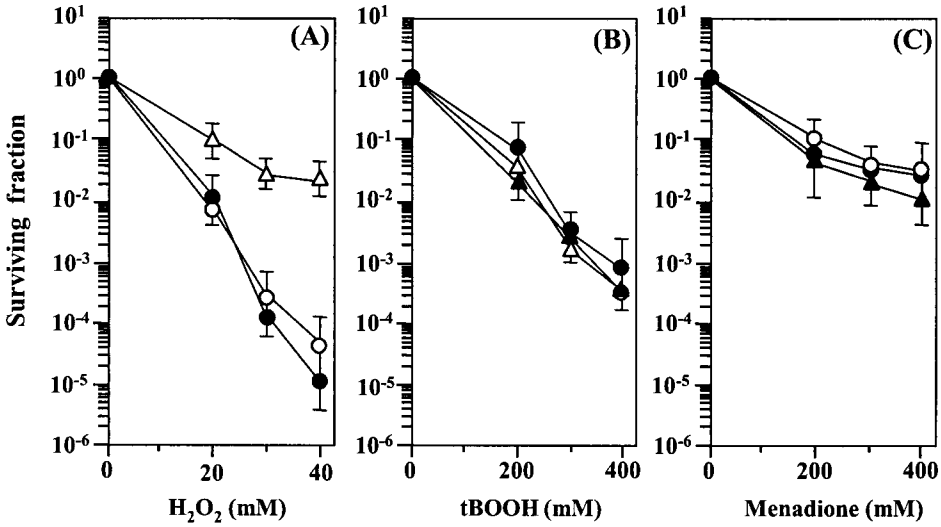

Fig. 2. Induced cross-protective responses in *A. tumefaciens.* The survival curves of exponential phase cultures of *A. tumefaciens* pretreated with H_2O_2 (▲), tBOOH (○), or menadione (△) prior to treatments with lethal concentration of H_2O_2 (A), tBOOH (B), and menadione (C) at indicated concentrations compared with the uninduced culture (●). The values presented are means and standard deviations of three replicates.

duced a 1,000-fold protection against H_2O_2 killing (Fig. 2A). The levels of menadione-induced cross-protection against H_2O_2 killing were lower than the protection levels attained by H_2O_2-adapted cells (Fig. 1A and 2A), suggesting that the adaptive mechanism could be the major protective response against H_2O_2 killing. This observation is in contrast to that of another bacterial phytopathogen, *Xanthomonas,* where menadione is severalfold more effective than H_2O_2 at inducing protection against H_2O_2 killing [12, 14]. The ability of menadione and tBOOH to induce cross-protective responses to oxidant killing was also investigated [14]. In all cases, under the experimental conditions used, no cross-protection was detected (Fig. 2B, C).

Induction of catalase in *A. tumefaciens*. The above data suggested that both adaptive and cross-protective responses against H_2O_2 killing are likely to be a result of H_2O_2 and menadione-induced expression of genes involved in H_2O_2 protection. In bacteria, catalase is the major H_2O_2 detoxification enzyme that catalyzes the conversion of H_2O_2 to water and oxygen. Catalase levels show a direct correlation with the resistance levels to H_2O_2 killing [14]. Exposure of many bacteria to oxidative stress induces the expression of either a bifunctional catalase-peroxidase or a monofunctional catalase [10, 12]. The increase in catalase activity is thought to be responsible for the adaptive and cross-protection responses against H_2O_2 killing. Thus, we measured the catalase activity in response to oxidant pretreatments. Exponential phase cultures of *A. tumefaciens* were pretreated with inducing concentrations of H_2O_2, menadione, or tBOOH, after which total catalase activity was determined. Pretreatment of *A. tumefaciens* with either H_2O_2 or menadione induced an increase in total catalase activity by 3.5 and 2.5-fold, respectively, compared with uninduced levels (Table 1). The magnitude of catalase induction was directly correlated with the ability of these compounds to induce protection against H_2O_2 killing (Fig. 1A and 2A). These findings suggested that catalase activity was the major factor determining the level of resistance to H_2O_2 killing in *A. tumefaciens.* Analysis of the bacterial genome sequence revealed two putative open reading frames identified as monofunctional catalase CatE (AAL46177) and a bifunctional catalase-peroxidase KatA (AAL45436). CatE shows a high degree of sequence identity with atypical growth phase-regulated, stationary phase catalases including *Sinorhizobium meliloti* KatE (76% [16]) and *X. campestris* pv. phaseoli KatE (56% [20]). *A. tumefaciens* KatA has been cloned, characterized, and shown to be a virulence factor in-

Table 1. The effects of peroxide pretreatment on total catalase activity in *A. tumefaciens*

Conditions	Catalase activity[a] (U · mg protein^{-1})	Induction level (fold)
Uninduced	7.5 ± 1.6	1.0
H_2O_2-induced (250 μM)	26.0 ± 2.5	3.5
Menadione-induced (200 μM)	19.0 ± 2.0	2.5
tBOOH-induced (250 μM)	8.0 ± 1.5	1.1

[a] Values represent means and standard deviations from three replicates.

volved in tumorigenesis in its host plant, and its expression could be induced by plant tissue sections and by acidic pH [22, 23]. The oxidant-dependent induction of total catalase activity was probably due to increased expression of *katA.* The proc is most likely mediated by the peroxide sensor and transcriptonal regulator, OxyR [17]. Although *oxyR* has not been characterized in *A. tumefaciens,* genome analysis of this bacterium revealed an *oxyR* homolog (AE009392) located divergently to *katA* (data not shown).

The inability of organic hydroperoxide to induce high catalase levels was interesting. In some bacteria, OxyR can be activated by both H_2O_2 and organic hydroperoxide [3, 13, 17, 19]. This implies that *A. tumefaciens* OxyR, unlike other bacterial OxyRs, cannot be activated by organic peroxide. This hypothesis is being investigated.

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Catalase-peroxidase KatG of *Burkholderia pseudomallei* at 1.7 Å resolution

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The catalase-peroxidase encoded by *katG* of *Burkholderia pseudomallei* (BpKatG) is 65% identical with KatG of *Mycobacterium tuberculosis*, the enzyme responsible for the activation of isoniazid as an antibiotic. The structure of a complex of BpKatG with an unidentified ligand, has been solved and refined at 1.7 Å resolution using X-ray synchrotron data collected from crystals flash-cooled with liquid nitrogen. The crystallographic agreement factors R and R_{free} are 15.3% and 18.6%, respectively. The crystallized enzyme is a dimer with one modified heme group and one metal ion, likely sodium, per subunit. The modification on the heme group involves the covalent addition of two or three atoms, likely a perhydroxy group, to the secondary carbon atom of the vinyl group on ring I. The added group can form hydrogen bonds with two water molecules that are also in contact with the active-site residues Trp111 and His112, suggesting that the modification may have a catalytic role. The heme modification is in close proximity to an unusual covalent adduct among the side-chains of Trp111, Tyr238 and Met264. In addition, Trp111 appears to be oxidized on $C^{\delta 1}$ of the indole ring. The main channel, providing access of substrate hydrogen peroxide to the heme, contains a region of unassigned electron density consistent with the binding of a pyridine nucleotide-like molecule. An interior cavity, containing the sodium ion and an additional region of unassigned density, is evident adjacent to the adduct and is accessible to the outside through a second funnel-shaped channel. A large cleft in the side of the subunit is evident and may be a potential substrate-binding site with a clear pathway for electron transfer to the active-site heme group through the adduct.

Keywords: crystal structure; catalase-peroxidase; KatG; isoniazid

Introduction

The bifunctional catalase-peroxidases are enzymes that degrade hydrogen peroxide either as a catalase ($2H_2O_2 \rightarrow 2H_2O + O_2$) or as a peroxidase ($H_2O_2 + 2AH \rightarrow 2H_2O + 2A$), thereby preventing cellular damage induced by breakdown products of H_2O_2 such as the highly reactive hydroxyl radical. The catalatic reaction, with a more rapid turnover rate, dominates over the peroxidatic reaction, making the enzyme appear to be a catalase rather than a peroxidase, despite the close sequence resemblance to plant peroxidases.[1] This resemblance to plant peroxidases was clearly illustrated when the change of a tryptophan residue to phenylalanine in the catalase-peroxidase distal heme pocket reduced the catalatic activity by 1000 fold (of *Escherichia coli* HPI) and increased the peroxidatic activity by threefold.[2–4] The crystal structure of the catalase-peroxidase from *Haloarcula marsimortui* has revealed a core structure very similar to that present in plant peroxidases.[5]

The catalase-peroxidases gained significant notoriety in 1992 when it was confirmed that mutation of *katG*, encoding the *Mycobacterium tuberculosis* KatG, imparted isoniazid (INH) resistance.[6] The obvious importance of isoniazid

Abbreviations used: INH, isonicotinic acid hydrazide, $C_6H_7N_3O$ (isoniazid); HMCP, *H. marismortui*; BpKatG, catalase-peroxidase from *B. pseudomallei*; EcKatG, catalase-peroxidase from *E. coli*; MtKatG, catalase-peroxidase from *M. tuberculosis*; CP, catalase-peroxidase; HRP, horseradish peroxidase; CCP, cytochrome *c* peroxidase; APX, ascorbate peroxidase.

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Table 1. Data collection and structural refinement statistics and structure analysis

	BpKatG_NAT[a]	BpKatG_INH[b]
A. *Data collection statistics*		
Unit cell parameters		
a (Å)	100.9	100.9
b(Å)	115.1	115.6
c(Å)	175.3	175.2
Resolution range (Å)	20–1.80 (1.86–1.80)	18–1.70 (1.73–1.70)
Unique reflections ($F > 0$)	181,754 (17,651)	221,727 (11,076)
Completeness (%)	95.6 (93.8)	99.2 (99.9)
R_{sym} (%)[c]	5.3 (45)	7.3 (58)
$\langle I/\sigma_{(I)}\rangle$	14.7 (2.4)	13.5 (2.7)
Redundancy	3.3 (3.0)	3.6 (3.6)
B. *Refinement statistics*		
Resolution	20–1.80 (1.85–1.80)	18–1.70 (1.75–1.70)
Working set	162,436 (11,553)	199,550 (14,281)
Free reflections	17,971 (1321)	22,115 (1573)
R_{cryst} (%)[d]	14.7 (23.6)	15.3 (23.5)
R_{free} (%)	18.3 (26.5)	18.6 (26.7)
C. *Structure analysis*		
No. non-hydrogen atoms		
BpKatG (subunit A)	5543	5543
Main-chain	2863	2863
Side-chain	2680	2680
BpKatG (subunit B)	5531	5531
Main-chain	2861	2861
Side-chain	2670	2670
Water molecules	2044	2295
Hemes (sub. A,B)	86	86
Sodium ions	2	2
PEO	4	4
rmsd from ideality		
Bond lengths (Å)	0.017	0.013
Bond angles (deg.)	1.46	1.30
Averaged *B* factor (Å^2)		
Subunit A	16.9	16.9
Main-chain	15.2	15.4
Side-chain	18.7	18.6
Subunit B	16.5	16.5
Main-chain	14.6	14.9
Side-chain	18.4	18.2
Water molecules	26.1	28.7
Hemes (sub. A and B)	14.9	15.3
Sodium ions	25.7	21.4
PEO	35.5	34.0

[a] Values in parentheses correspond to the highest-resolution shell.

[b] The BpKatG_INH corresponds to crystals where 100 mM INH was added to the cryobuffer (see Materials and Methods); however, there is no significant change, with respect to the data obtained without INH. The structure obtained without soaking with INH, though at lower resolution, is used in the present analysis as a control.

[c] $R_{sym} = \sum_{hkl} \sum_j |I_{hkl,j} - \langle I_{hkl}\rangle| / \sum_{hkl} \langle I_{hkl}\rangle$, where j extends to all the observed hkl symmetry-related reflections.

[d] $R_{cryst} = \sum||F_{obs}| - |F_{calc}|| / \sum|F_{obs}|$. R_{free} is as for R_{cryst} but calculated for a test set comprising reflections not used in the refinement

as an anti-tuberculosis drug and the prevalence of *katG*-induced INH-resistant *M. tuberculosis* strains causing tuberculosis led to great interest in determining the structure of a KatG with several groups worldwide attempting to solve its structure. Unfortunately, attempts to crystallize a number of different catalase-peroxidases were without success until very recently, when the crystallization of the enzymes from *Haloarcula marismortui*,[5,7] Synechococcus,[8] and *Burkholderia pseudomallei*[9] and of the C-terminal domain of *E. coli* HPI were reported.[10] Curiously, despite many years of study the actual *in vivo* peroxidatic substrate of the catalase-peroxidases has not been identified. INH is not a normal bacterial metabolite, and its reaction must arise from interaction in a binding site intended for the natural substrate.

The large size of the catalase-peroxidase subunits, containing two distinct, sequence-related domains, relative to the plant peroxidases may have been the result of a gene duplication and fusion event.[11] The N-terminal domain contains the heme and active-site residues, that when modified affect enzyme activity. The C-terminal domain has less sequence similarity, and does not have the conserved heme active-site motif characteristic of peroxidases. The heme occupancy for many catalase-peroxidases appears to be partial, originally determined to be 0.5 heme per subunit.[12] The presence of a heterogeneous mixture of dimers and tetramers in *E. coli* HPI with zero, one and two, or one, two and three hemes was confirmed recently,[2] and this may have been a major problem in crystal formation. In fact, the catalase-peroxidases from *H. marismortui* (HMCP) and *B. pseudomallei* (BpKatG) were purified with an apparently higher heme to subunit ratio, and this less heterogeneous protein proved amenable to crystallization.

Here, we describe the structure solution and refinement at 1.7 Å resolution of BpKatG from improved crystals.[9] The overall structure is very similar to that of the *H. marismortui* CP but with a number of important unreported features made possible by the higher-resolution data that provide exciting insights into the catalytic possibilities of the enzyme.

Results and Discussion

Overall description of the BpKatG structure

The electron density map defines main-chain and side-chain atoms of 1428 amino acid residues, two metal ions and two modified heme groups in two subunits and 2314 water molecules. The 34 N-terminal residues of both subunits do not appear in the structure, but the maps show clear continuity over the complete length from Asn35 to Ala748 in both subunits. Residue Asn35 is refined only about the main-chain atoms and the side-chain atoms are not included in the model. The model has crystallographic agreement R and R_{free} factors of 15.3 and 18.6% for 199,550 reflections in the resolution shell between 1.7 Å and 18.0 Å (Table 1). The average root-mean-square (r.m.s.) deviation after superimposition of the two subunits is 0.33 Å for

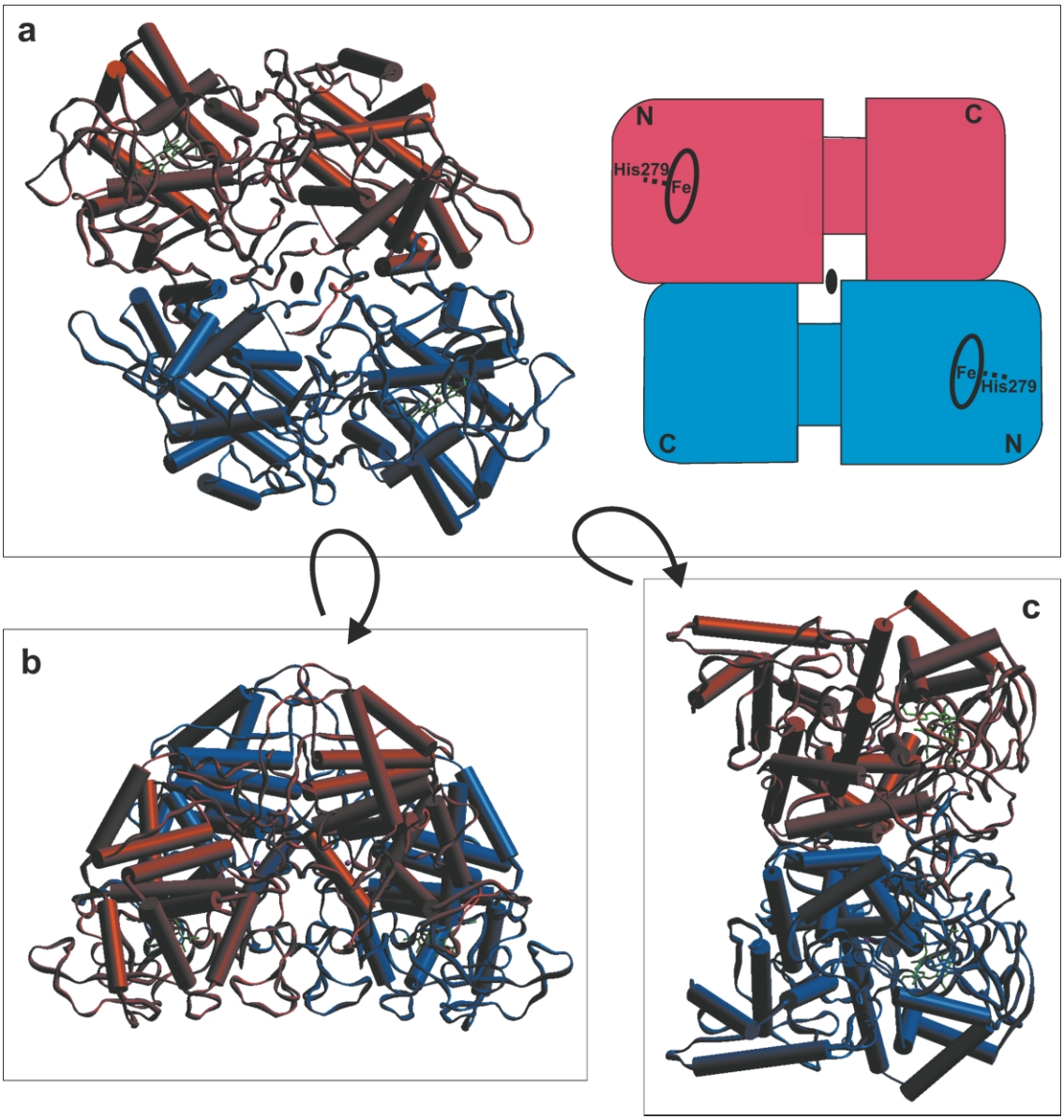

Figure 1. Three views of BpKatG rotated 90° relative to each other. The view in (a) is oriented looking down the axis of 2-fold symmetry (indicated by the oblong dot). The schematic on the right illustrates the relationship and interactions of the N and C-terminal domains in each subunit. (b) The molecule is rotated forward 90° so that the reader is viewing the molecule from the top in (a). (c) The molecule is rotated 90° to the right so that the reader is viewing the molecule from the left end in (a). The 20 helical regions are represented by tubes.

the 714 C^{α} atoms and 0.76 Å for all atoms, indicating only minor differences between the two subunits. Also, a testament to the quality of the data was the clarity, in the electron density, of a number of errors in a 12 amino acid residue region of the predicted sequence. An insertion and a deletion error were subsequently identified in the DNA sequence, which was corrected in GenBank (accession number AY040244).

The two crystallographically independent protein subunits found in the BpKatG crystal structure are related by accurate non-crystallographic 2-fold symmetry (179.7° after superimposition of the two subunits). The axis is perpendicular to the crystallographic **c** axis at 30° from the **a** axis. The dimer has a rather rectangular shape of about 85 Å × 95 Å × 105 Å in size with the dimer 2-fold axis oriented perpendicular to the largest face (Figure 1). In turn, individual subunits can be centered in parallelograms of about 45 Å × 50 Å × 95 Å with the two subunits in the dimer oriented anti-parallel to each other with a left-handed twist of about 30°. The two subunits in the dimer present extensive surface contacts of 7000 $Å^2$, but without any significant interdigitation. The subunit organization, with well defined globular N (to residue 438) and C-terminal regions arranged along the longest subunit axis, together with the anti-parallel

disposition of subunits in the dimer results in the intersubunit interactions being dominated by crossover interactions between the larger N-terminal domain of one subunit and the smaller C-terminal domain of the other, with a contact area of 2 × 2450 Å^2. However, interactions between N-terminal regions are present, with a contact area of 2 × 1250 Å^2 and could play a dominant role in dimerization.[13] Residue Cys27, not visible in the electron density, has been shown to participate in a conserved disulfide bond between subunits in KatG of *M. tuberculosis*, although data indicate that this bond is not a prerequisite for the formation of dimeric KatG.[13]

The structure is similar in many respects to that of the *H. marismortui* catalase-peroxidase,[5] to the extent that the average r.m.s. deviation for 685 C$^\alpha$ atoms, after superimposition of the two subunits and elimination of 29 non-corresponding residues, is 1.05 Å. Consideration of just the 133 highly conserved residues in the ten α-helical regions of the N-terminal region of the sequence (to residue 426), revealed an r.m.s. deviation between HMCPx and BpKatG of just 0.43 Å. The relative disposition of the two globular domains of the subunits, the N and C-terminal domains, appears to be extremely well preserved, as expected from the large contact area between the two domains of about 3300 Å^2, which is difficult to reconcile with the concept of a flexible hinge joining the two domains.[5]

Ramachandran plots (not shown) confirm that all residues in both subunits fall within the energetically favorable regions,[14] but a number of other unusual features were noted. Three *cis* proline residues (135, 228 and 509) are present, and six residues with double conformations (Ser50, His55, Thr119, Glu198, Arg426 and Gln711) were identified in both subunits. Extra density suggestive of modification, most likely the result of oxidation, was evident on the side-chains of Trp111, Trp139 and Met266, also in both subunits (see below). The unusual covalent adduct involving the side-chains of Trp111, Tyr238 and Met264, found in HMCP, was identified in BpKatG in close proximity to a modification on the heme (also see below). Finally, the single cysteine residue in the model of both subunits, Cys556, exhibited lower electron density than expected, which could be due to irradiation effects.[15,16]

About one-third of the residues of BpKatG exist in 20 α-helical regions and a small amount of β-sheet, with the remainder existing in regions interspersed among the helical coils (Figure 1). The individual subunits are organized in two structurally related domains, identified originally on the basis of sequence similarity.[11] The N-terminal domain and the C-terminal domain of BpKatG can be superimposed on one another, revealing a striking conservation of α-helical segments with ten N-terminal helices coinciding with ten C-terminal helices (Figure 1). The r.m.s deviation of 133 C$^\alpha$ atoms in the ten superimposed helical segments is 2.19 Å. This conserved motif of α-helical segments is found in the more distantly related (approximately 25% sequence identity) family of plant peroxidases. For example, superimposition of the N-terminal segment of BpKatG onto cytochrome *c* peroxidase (CCP), ascorbate peroxidase (APX) (both class I peroxidases) and horseradish peroxidase (a class III peroxidase) revealed r.m.s. deviations of 0.97 Å, 1.22 Å and 2.03 Å, respectively, for the 133 C$^\alpha$ atoms in the ten α-helical segments. In turn, superimposition of the C-terminal region of BpKatG onto the same proteins gave r.m.s. deviations of 3.62 Å, 3.75 Å and 4.06 Å, respectively. Clearly, demands on the heme-containing N-terminal domain of KatG have resulted in a greater conservation of structure relative to the peroxidases than in the hemeless C-terminal domain.

The heme environment is virtually identical with that in HMCP and similar to that of the plant peroxidases (Figure 2). On the distal side of the heme, the active-site triad of Arg108, Trp111 and His112 is typical of all catalase-peroxidases and of class I peroxidases such as yeast cytochrome *c* peroxidase and ascorbate peroxidase. In class 3 peroxidases, such as horseradish peroxidase, the Trp is replaced by a Phe, a change that greatly reduces catalatic activity in the catalase-peroxidases.[2–4] On the proximal side, His279 is the fifth ligand to the heme iron atom as in the peroxidases, and it is in close association with the fully conserved Asp389, which interacts with the indole N atom from the proximal Trp330. Carbonyl oxygen atoms from residues 279 and 281, not participating in hydrogen bonds, are pointing towards the proximal Trp330 side-chain, with a shortest distance of 3.01 Å between the C$^\beta$ from Trp330 and the main-chain oxygen atom from residue 279. The same type of interaction is present in APX and in CCP, where it appears to be particularly strong. Comparison of other residues surrounding the heme group reveals greater similarity to CCP with decreasing similarity in APX and HRP. This might be taken to imply that the compound I radical of catalase-peroxidases will dissociate from the heme to a nearby amino acid side-chain, as happens in CCP. However, existence of a porphyrin cation radical has been demonstrated for both EcKatG[2] and MtKatG,[17] indicating that a definition of the factors governing the migration of the radical remains elusive. The environment of the heme propionate groups is very similar between APX and BpKatG, with the only significant difference being the replacement of residue Arg172 in APX by Thr323 in BpKatG (Figure 3). Water molecule W4, the solvent molecule with the lowest temperature factor (5.1 Å^2), presents a tetrahedral bonding geometry with hydrogen bonds to the carboxylate groups of the two heme propionate groups and to the essential distal residue Arg108 (Figure 2(a)). It is worth mentioning that main-chain oxygen atom from residue Lys283 is pointing towards the carbon atom in the carboxylate group of the propionate group on ring IV, at a distance of

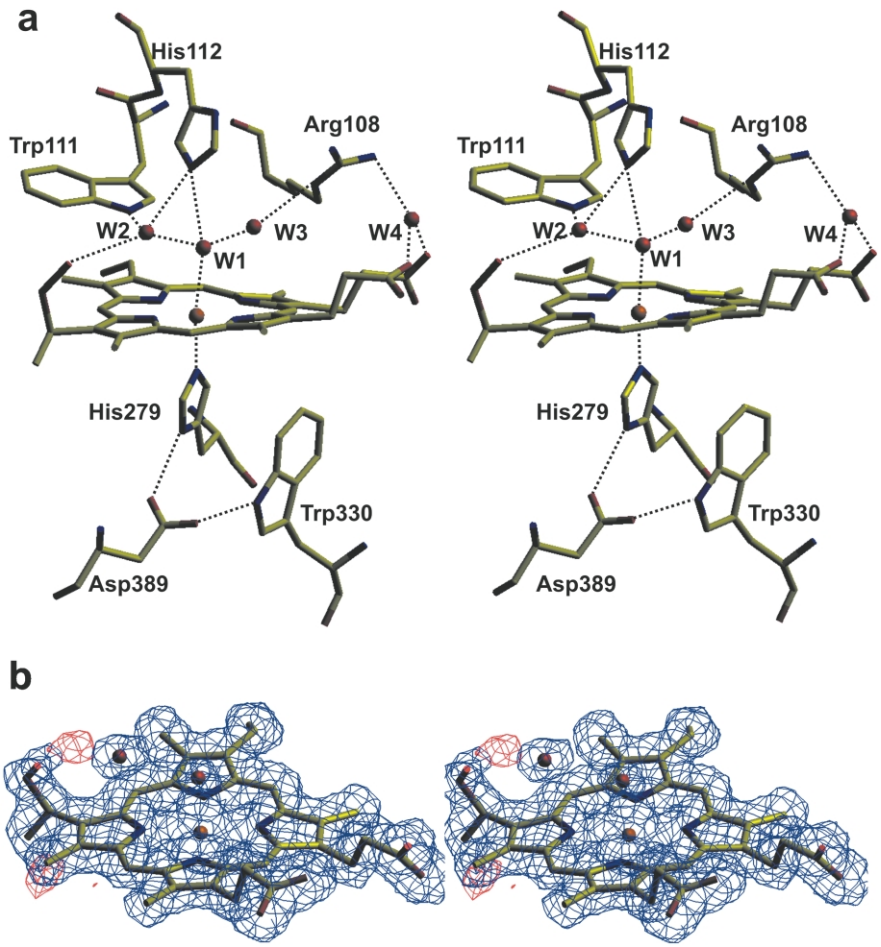

Figure 2. (a) Stereo view of residues in the vicinity of the heme. On the distal side, residues Arg108, Trp111 and His112 are shown, along with four water molecules labeled W1–W4. The adduct between Trp111 and Tyr238 is not shown here. On the proximal side, His279 Trp330 and Asp389 are shown. Interactions are indicated by the broken lines. (b) Stereo view of the $2F_o - F_c$ electron density map modeled at 1σ (in blue) and $F_o - F_c$ electron density map modeled at 3σ (in red) with the model of the heme superimposed. The postulated perhydroxy modification on the vinyl group of ring I is included.

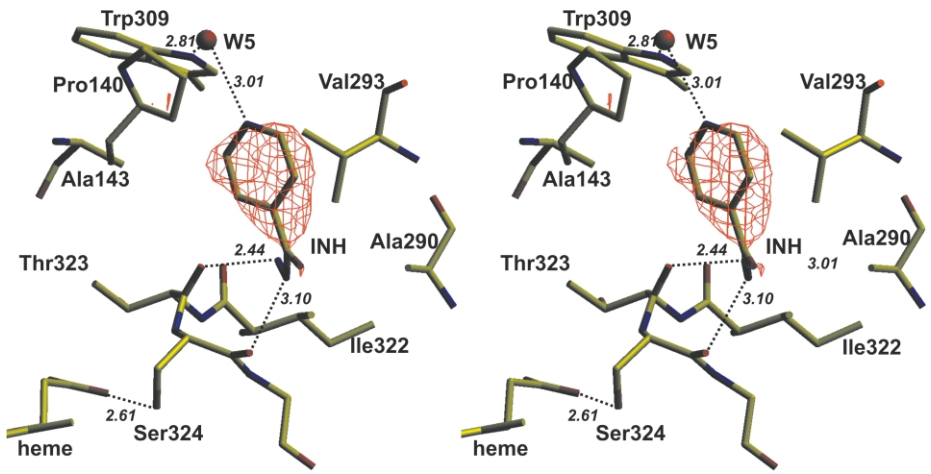

Figure 3. Stereo view of the environment surrounding the region of undefined electron density in the main channel leading to the heme. The $F_o - F_c$ electron density map modeled at 3σ is shown in red with a model of isoniazid (INH) superimposed. The individual nitrogen atoms of the –NH–NH2 group of INH are shown interacting with the main-chain carbonyl oxygen atoms of Thr323 and Ser324, The side-chain –OH of Ser324 is shown hydrogen bonded to the carbonyl oxygen atomof the heme propionate group. The ring nitrogen atom of INH is shown hydrogen bonding with water molecule W5, which also forms a hydrogen bond with the indole nitrogen atom of Trp309.

3.1 Å, making this an unlikely path for electron transfer.

Heme covalent modification

The electron density maps reveal an unambiguous addition of at least a two atom substituent to the vinyl group on ring I of the heme (Figure 2(b)). The nearby water molecule suggests that the modification can form hydrogen bonds, implying a polar nature. Hydrogen peroxide, as a substrate for the enzyme, has ready access to and will bind in the active site, making it a likely source for the modification *via* its addition across the vinyl group double bond. Including –OOH on the vinyl group in the model results in only a small amount of residual density in the $F_o - F_c$ map. The nature of such a modification satisfies the need for hydrogen bonding to the nearby water molecules and makes possible a facile removal or reversal of the modification. Indeed, it has not been possible to identify the modification by mass spectrometry, consistent with its expected labile nature, and only subtle changes in the 600–750 nm region of the absorption spectrum were evident.

The heme modification was not observed in HMCP, nor is it present in the closely related plant peroxidases. In all plant peroxidases, the residue equivalent to Tyr238 of BpKatG is a proline, and it is oriented to be in van der Waals contact with the vinyl group of ring I of the heme. This would sterically preclude modification of the heme on the vinyl group. A necessary prelude to the addition of –OOH to the heme of catalase-peroxidases would be exposure to hydrogen peroxide, and BpKatG was prepared from *E. coli* cells grown in aerated shake flasks for 22 hours, providing a long exposure to a highly oxygenated atmosphere and ample opportunity for the enzyme to encounter hydrogen peroxide. The highly oxidative environment experienced by BpKatG is further demonstrated by the oxidation of the $C^{\delta 1}$ of the indole rings of Trp111 and Trp139. Excess density associated with this carbon atom was evident in the $F_o - F_c$ map, and oxidation of the indole ring at this location is not unexpected.[18]

The orientation of the –OOH substituent predicted in the model allows interaction with water molecule W2 in the heme distal pocket, thereby providing indirect contact with both the indole N atom of Trp111 and the imidazole ring of His112, two of the active-site residues required for reduction of compound I in the catalatic reaction. The orientation of the –OOH would allow its participation in catalysis, both during the formation of the Trp-Tyr adduct and in the reduction of compounds I and II, possibly helping to bind or orient the substrate hydrogen peroxide in conjunction with the indole NH group of Trp111. Involvement of the heme modification in the formation of compound I is less likely because of the greater distance to the side-chain of Arg108, which is required for the reaction.

Location of a metal ion

Unlike HMCP, which contained 16 chloride and six sodium or potassium ions, only one metal ion per subunit has been localized in the structure of BpKatG. Tentatively identified as sodium on the basis of its refinement properties and pentavalent, square pyramidal coordination structure with distances of about 2.35 Å (Figure 4). Surprisingly, this single cation is not related to any of the ions in HMCP, but is coordinated to the carbonyl oxygen atoms of residues 122, 124 and 494 as well as two water molecules. Water molecule W6 interacts with Glu128 and Glu198. Water molecule W7 interacts with the side-chain of Asp427 in which the carbonyl group is rotated about 60° relative to its orientation in HMCP. The cation and its counterion Asp427 side-chain are readily accessible to the exterior of the protein through a large, funnel-shaped channel that is unique to the catalase peroxidases (Figure 5). Along this channel there are a number of residues showing multiple conformations, in particular His55 and Glu198, further supporting the idea of an inlet or exit path for ions. The position of this putative sodium ion is about 7 Å from the corresponding positions in peanut, manganese or lignin peroxidases where a distal calcium ion had been reported.[19] In barley peroxidase, replacement of the sodium ion by calcium appears related to the activation of the enzyme.[20]

Trp111-Tyr238-Met264 adduct

The Trp111-Tyr238-Met264 adduct was first identified in the structure of HMCP and the electron density maps very clearly suggest its presence in BpKatG (Figure 6), supporting speculation that it may be a general feature of catalase peroxidases. The bond lengths determined in BpKatG of 1.69 Å between $C^{\eta 2}$ of Trp111 and $C^{\epsilon 1}$ of Tyr238, and 1.78 Å between $C^{\varepsilon 2}$ of Tyr238 and S^{δ} of Met264, are somewhat long in comparison to most covalent bonds. This apparent ambiguity about the nature of the bonds is reinforced by the evident intermediacy between sp^2 and sp^3 character implied by the bond angles on the Tyr and Trp rings (Figure 6(b)). Despite this uncertainty, the existence of the adduct is supported by a recent matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) analysis of tryptic peptides (data not shown), and by the existence of the Tyr-Trp structure in the antibiotics chlorpeptin and kistamicins.[21,22] The mechanism leading to the formation of the adduct is fascinating to contemplate and a study of BpKatG variants should help to shed light on it.

The catalase-peroxidases bind hydrogen peroxide and use it as a substrate, providing a ready explanation for how the adduct might form. What is not as clear is whether the adduct has a function and provides some advantage to the enzyme. Other unusual adducts have been observed in

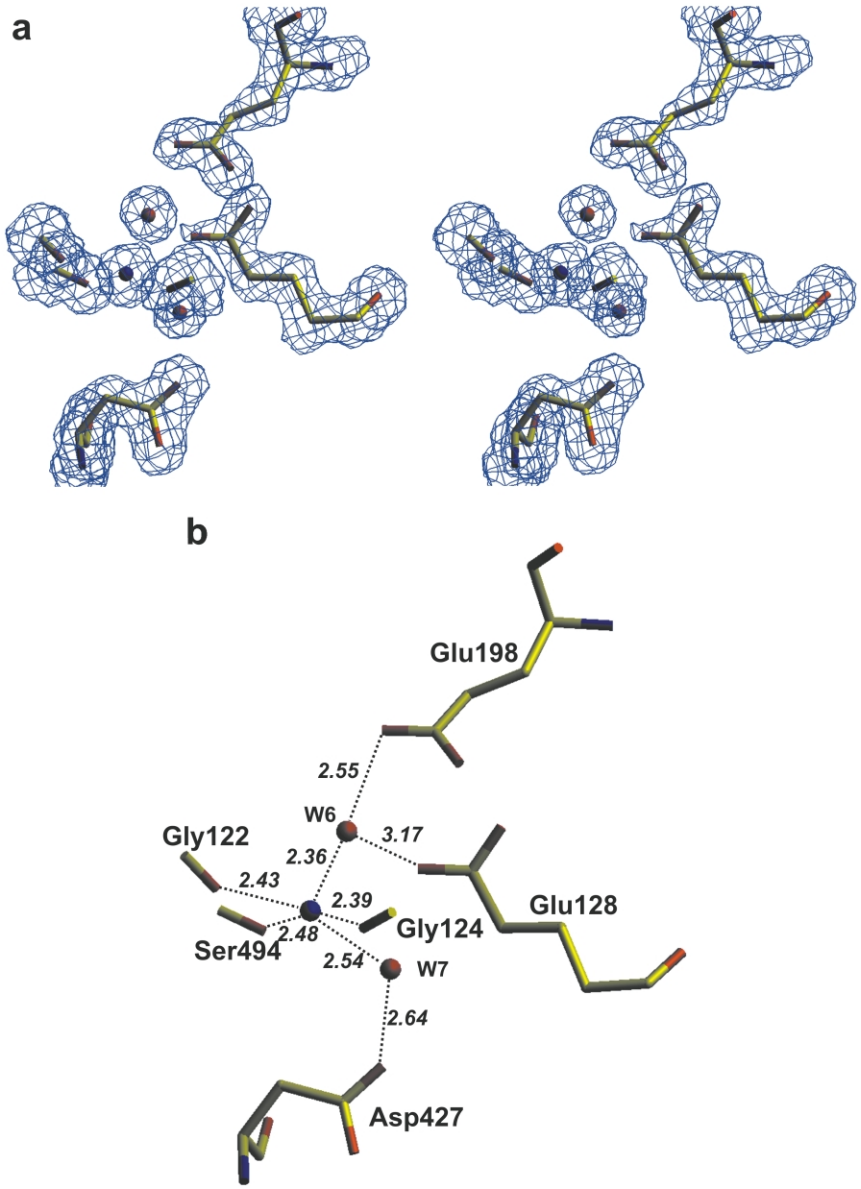

Figure 4. (a) Stereo view of the $2F_o - F_c$ electron density map modeled at 1σ in the vicinity of the metal ion with the model for the main-chain carbonyl groups of Gly122, Gly124 and Ser494, and water molecules W6 and W7 shown as coordinating groups. (b) Model of the residues in the environment of the metal ion with the interactions indicated by broken lines and associated distances between atoms indicated in italics.

peroxidases and in monofunctional catalases, including a hydroxy group on the C^{β} of Trp171 in lignin peroxidase,[19] a methionine sulfoxide residue in the catalase from *Proteus mirabilis*,[23] an oxidized heme d in catalases from *E. coli* and *Penicillium vitale*,[24] and a covalent bond between the N^{δ} of His392 and C^{β} of Tyr415 of *E. coli* HPII.[25] One rationale proposed for some of these modifications is that they stabilize the enzyme by reducing the likelihood of other, more damaging oxidations. The Trp-Tyr-Met adduct may impart a similar protection in that it will prevent further modification of the oxidation-susceptible methionine residue. The adduct may also contribute to the rigidity of what appears, from the clear definition of even surface-located side-chains, to be a very rigid enzyme, although the functional advantage of this is not clear. One other possibility is that it forms a route for electron transfer from the cleft region to the heme (see below).

A second catalytic center?

The side-chain of Arg426 exists in two orientations, of which the predominant (>70%) differs from the orientation in HMCP (Figure 7). Contributing to the complexity of this region are the two conformations of the side-chain of Thr119, of which the minor (<30%) is similar to the conformation in HMCP, suggesting coordination between the conformations of the two side-chains. Changes relative to HMCP (the single ion, the rotated Asp427 carbonyl group, and the displaced Thr119 and Arg426 side-chains) are in close proximity to one another, are associated directly or indirectly through hydrogen bonds or ionic interactions and represent the only changes in structure relative to HMCP in this region of the protein. This correlation suggests strongly that the changes are functionally related, and that the region of the protein may have an, as yet, undefined function, possibly

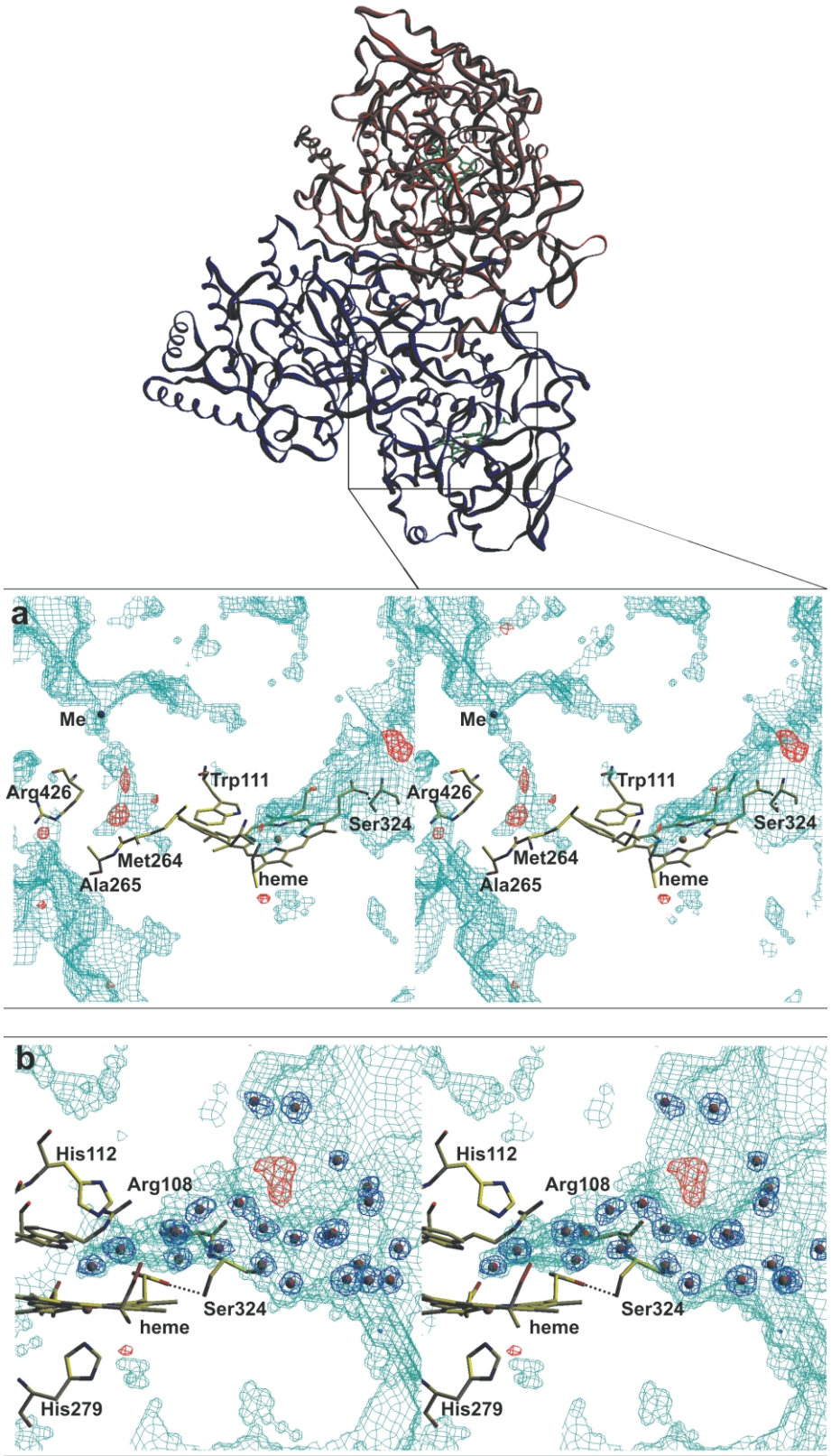

Figure 5. (a) Stereo view of the channel structure in the vicinity of the heme. The cross-section of the enzyme is oriented to include the main channel and region of undefined electron density ($F_o - F_c$ modeled at 3σ and shown in red), the modified heme, the Trp111-Tyr238-Met264 adduct, Arg426 adjacent to the large cleft, and the metal ion. The second funnel-shaped channel adjacent to the metal ion leads very close to the cavity created by the movement of Arg426. Some undefined electron density is visible in the $F_o - F_c$ map in this cavity. The region of the enzyme molecule that is included in (a) is indicated on the ribbon diagram of BpKatG shown at the top. The surface feature map generated by the program VOIDOO is presented in green. (b) Stereo view of the main channel leading to the heme. The

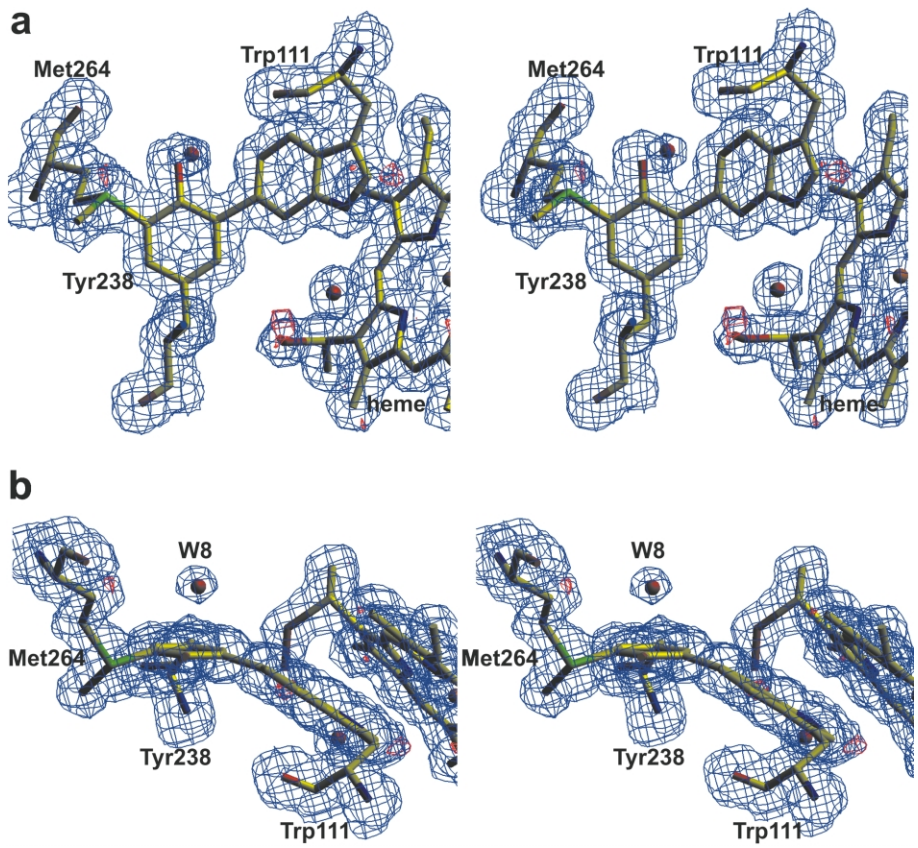

Figure 6. Two stereo views of the adduct formed among the side-chains of Trp111, Tyr238 and Met268. The $2F_o - F_c$ electron density map at 1σ is shown in blue and the model of the residues is superimposed. The view in (b) is rotated approximately 90° from that in (a), so that the reader is viewing the model towards the tyrosine ring OH. The intermediate sp2–sp3 nature of the bonds of the adduct on the phenyl ring of Tyr238 is evident in the deviation from planarity of the substituents on the phenyl ring in (b). The F_o–F_c electron density map at 3σ is shown in red. Note the extra density on Trp111.

as a second catalytic center besides the heme. This possibility is reinforced by the fact there is a region of undefined electron density located in the cavity vacated by the Arg426 side-chain in direct contact with the oxygen atom of the side-chain from Tyr238, the central residue of the covalent adduct.

Access channels and potential binding sites

Despite the apparent structural similarity to plant peroxidases, the larger subunit size of BpKatG (more than twice as large) results in the active site of BpKatG, including the resident heme, being buried more deeply within the subunit. The most obvious access route to the distal side of the heme, the active site of the enzyme for reaction with H_2O_2, is provided by a channel positioned similarly to, but longer than, the access route in peroxidases. The channel in BpKatG has a pronounced funnel shape and is narrowest near residues Ser324 and Asp141, about 14 Å from the heme iron atom (Figure 5). In peroxidases, the channel is not as constricted, and the peroxidatic substrate benzhydroxamic acid[26] binds to HRP in what is the equivalent of the constricted region of BpKatG closer to the heme. Substrate hydrogen peroxide entering the distal side cavity of BpKatG through the constricted portion of the channel would immediately come into contact with the active-site residues Arg109, Trp111 and His112 (Figure 5(b)) for generation of compound I in both the catalatic and peroxidatic modes of reaction, or for reduction of compound I in the catalatic reaction. Indeed, a continuum of water molecules is evident in this channel (Figure 5(b)). The mechanism by which organic substrates serve as electron donors for the peroxidatic reduction of compound I remains poorly defined.

Most peroxidases have a second access route, approximately in the plane of the heme, leading to the distal heme cavity, but the equivalent route in BpKatG is blocked by loops in the larger protein.

$2F_o - F_c$ electron density map of the water molecules in the channel (modeled at 1σ in blue) and the $F_o - F_c$ electron density map showing the region of undefined density (modeled at 3σ in red) are shown. The interaction between the side-chain of Ser324 and the heme propionate group is indicated by the broken line. The surface feature map indicated in green was generated in the program VOIDOO.

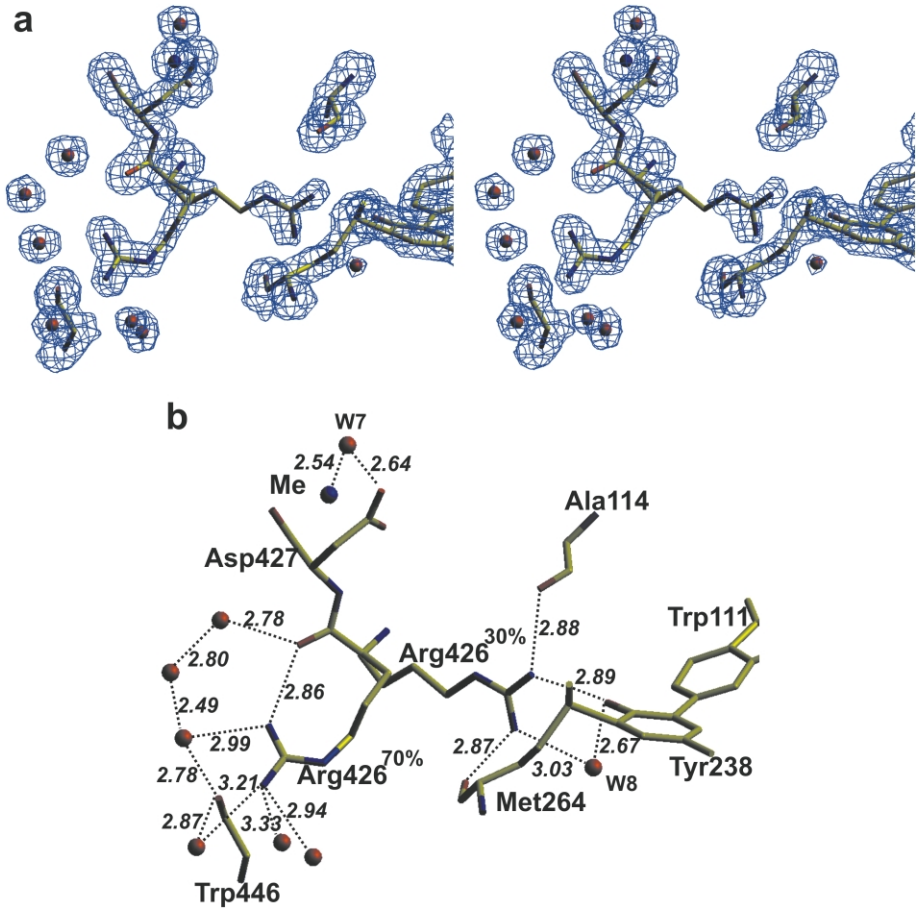

Figure 7. (a) Stereo view of the $2F_o - F_c$ electron density map modeled at 1σ in the vicinity of Arg426 with the model for both partial orientations of the Arg426 side-chain shown. The approximate proportions of the two orientations are indicated by the 30% and 70% designations. The main-chain atoms of Trp446 and other residues interacting with the Arg426 are included. (b) Model of the residues in the environment of Arg426 with the interactions indicated by broken lines and associated distances between atoms indicated in italics.

However, there does appear to be at least one other access channel providing direct access to the core of the protein in the vicinity of the single cation and to a region that encompasses two other structural features with possible functional significance. The first is a dramatic cleft in the side of the subunit formed between the two domains of the subunit that wraps around the protein. At one end of the cleft is located a well-defined, U-shaped region lined with polar residues (Figure 5(a)). Anionic residues, including the side-chains of Glu270, Asp587 and Glu589, and the carbonyl oxygen atoms of Ala262 and Ser265 surround a localized pocket of cationic groups including the side-chains of Lys422, Arg426 and Arg497. Significantly, the side-chains of Arg426 and Thr119 in their predominant conformations are located on the surface at the bottom of the U. They reduce the depth of the cleft slightly and allow the Arg426 side-chain to contribute to the cationic center on an otherwise predominantly anionic surface. Movement of the Arg426 side-chain to its minor conformation would increase the depth of the cleft and reduce the positively charged component on the surface. The associated rotation of the Thr119 side-chain would change the hydrogen bonding environment of the cleft by moving the side-chain OH group to a more external location. Such a striking and well-defined cavity, combined with the potential for functional changes through the simple movement of two side-chains, begs the suggestion that this is the binding site for a substrate. Unfortunately, the identity of the *in vivo* peroxidatic substrate of BpKatG remains unknown, but, if the cleft is a binding site, its elongated nature may imply a substrate with an extended, possibly even polymeric character.

A necessary extension of this model is that if a substrate did bind at this site, it would be necessary to transfer electrons from it to the heme for the reduction of compound I or II, as part of the peroxidatic reaction. A relatively short path for such electron tunneling is immediately obvious, beginning with the main-chain carbonyl group of Ala265 on the surface of the cleft adjacent to Met264. As part of the Met264-Tyr238-Trp111 adduct, the MetS is most likely carrying a positive charge and would provide a draw for electrons from the cleft. In turn, the adduct provides a direct pathway for electron transfer to the heme. Analysis

Table 2. Explanation of the phenotype of some mutations in KatG of *M. tuberculosis* on the basis of their location in the BpKatG structure

Mutation	Peroxidatic activity[a]	Residue	Effect of mutation in BpKatG
A. *Category 1: mutations affecting the INH reaction specifically*			
Ser315Thr	0.6	Ser324	Either the hydrogen bond with the propionate group of the heme is broken or significant distortion in the side-chain is required to maintain hydrogen bonding and this would change the binding site for the hydrazine group of INH.
B. *Category 2: mutations affecting peroxidatic activity generally*			
Asn138Ser	0.1	Asn142	Binds to imidazole group of active-site His112 and change may distort spatial organization of active-site residues.
Leu148Arg	0.1	Leu152	Introduction of charged guanidinium group into a polar region would require significant distortion of the main chain of the protein.
His270Gln	0.1	His279	The proximal side fifth ligand of the heme iron is removed, reducing heme binding
Thr275Pro	0.3	Thr284	Significant distortion in the main chain of the protein on the proximal side of the heme would be required to accommodate the change.
Trp321Gly	0.3	Trp330	The indole ring is in close proximity to the proximal side His and removal may result in distortion of the main-chain atoms reducing heme binding and activity.
Asp381Gly	0.0	Asp389	The aspartate side-chain interacts with the proximal side fifth ligand of the heme and its removal causes disruption in heme binding.
C. *Group 3: mutations with an uncertain mechanism*			
Leu587Pro	ND[b]	Leu594	The location is on the surface but far removed from the active site or INH binding site. Protein folding or stability may be affected

[a] Selected MtKatG variants and their peroxidatic activities taken from Rouse *et al.*[29]
[b] ND-not determined.

of the cleft may eventually provide insight into the *in vivo* substrate for KatG proteins.

In its minor conformation, the Arg426 side-chain is associated with the phenolic group on Tyr238 (Figure 7), similar to the predominant situation in HmCP, where the Arg409 side-chain is associated with Tyr218. The cavity created by the movement of the side-chain to its predominant (>70%) conformation is located very close to the cleft region, and the slight movement of one or two side-chains would provide access from the cleft to the cavity. Perhaps more importantly, the other end of the cavity approaches the cation, which has direct access to the exterior through the second funnel-shaped channel (Figure 5(a)). A region of electron density that cannot be explained by partial occupancy of the Arg426 side-chain is present in the cavity, suggesting the presence of another bound molecule. It is not clear if this is an alternate substrate-binding site, but the cavity would provide easy access for hydrogen peroxide needed for the oxidation of Met264 in the formation of the covalent linkage.

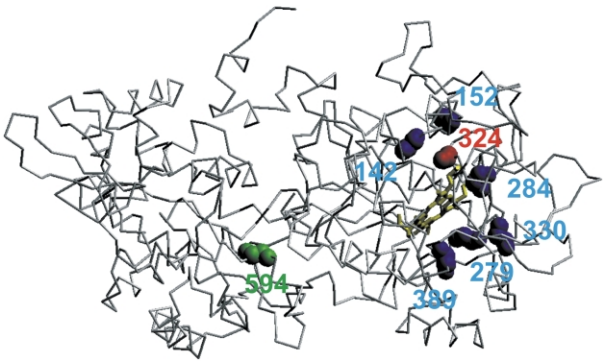

Figure 8. Location of mutations described in Table 2 located on a single subunit of BpKatG presented in the same orientation as the lower subunit shown in Figure 1(a). Only the main-chain atoms of the residues are shown and are represented as spheres. The residue in category 1 is colored red; the residues in category 2 are colored blue; and the residue in category 3 is colored green.

Analysis of the potential isoniazid binding site

A region of undefined electron density is located just before the constricted region in the main channel leading to the distal heme cavity of BpKatG (Figures 3 and 5). The location of the density is over 10 Å further away from the heme than is the benzhydroxamic acid bound in HRP, but is in close proximity to Ser324, the equivalent of Ser315 in MtKatG, which is thought to be involved in INH binding because changing it interferes with INH activation. This extra density was found both in the structure obtained after a short soaking with INH and in the structure obtained without soaking (Table 1). However, no INH had been introduced to the enzyme subsequent to purification, and only phosphate buffer was used during the purification, making exposure to other pyridine-like molecules unlikely. Therefore, it is concluded that the undefined density is the result of a metabolic constituent of *E. coli*, that may closely resemble INH. One such molecule is pyridoxol

or vitamin B6, a common growth-medium component, and its oxidation would lead to pyridoxal required for the synthesis of pyridoxal phosphate. Another closely related common metabolite is nicotinamide, although why it would require oxidation is not clear. The potential for involvement in modification of common metabolites provides a role for the catalase-peroxidases other than being simply protective enzymes removing hydrogen peroxide. More importantly, it provides an explanation for why the enzyme has a binding site for isoniazid, which is not a normal bacterial metabolite.

Definition of the binding of isoniazid is one of the main goals of elucidating the structure of KatG and, despite the distance from the heme, the region around Ser324 is a strong candidate for INH binding. To investigate this possibility further, models of INH and pyridoxol were superimposed into the region of undefined density, and the environment was analyzed for the likelihood of their binding (Figure 3). The surrounding region is composed predominantly of hydrophobic amino acid residues including Pro140, Ala143, Ala290, Val293, Trp309 and Ile322, all of which might interact with the region of density suggesting the presence of a relatively hydrophobic molecule. Only two potential ionic or hydrogen bonding opportunities are presented at opposite ends of the electron-dense region. The hydrogen bond of indole N of Trp309 with water molecule W5 could potentially interact with the N atom of the pyridine ring of INH, pyridoxol or nicotinamide. At the opposite end, the carbonyl oxygen atoms of Thr323 and Ser324 are situated such that an interaction with the hydrazine portion of INH is possible. The oxidizable $-CH_2OH$ of pyridoxol would be oriented in close proximity to the same carbonyl oxygen atoms. The interaction of the nicotinamide $-NH_2$ group with the carbonyl group of Ile322 is predicted but the center to be oxidized is not evident, unless it is a very unusual reduced form of the ring.

The region in the main channel leading to the heme therefore presents a remarkably good candidate site for the binding of both INH and related pyridine derivatives. A possible mechanism for the oxidation of INH might involve distortion in the N–CO of INH bond caused by interaction with the Thr323-Ser324 carbonyl oxygen atoms. The side-chain of Ser324 forms a hydrogen bond with the carboxylate group of the heme propionate side-chain, and this direct association will result in a pull of electrons away from the serine residue towards the electron-deficient heme of compound I and compound II. This will result in polarization of the N–CO bond of INH, most likely leading to formation of the pyridine carbonyl and hydrazine radicals.[27] The pyridine carbonyl radical would be stabilized through resonance across the aromatic ring and delocalization of electrons from the pyridine nitrogen would break the hydrogen bond with water molecule W5, which might be necessary to allow reorientation of the molecule for further reaction with NADH to form the InhA inhibitor.[28] The hydrazine radical might have an electron stripped for passage to the heme, generating a positively charged $NH_2–NH^+$, which in turn would react rapidly with water to form $NH_2–NHOH$. Speculatively, if a molecule such as pyridoxol is the substrate, it might bind, oriented similarly to what has been proposed for INH, resulting in the $-CH_2OH$ *para* to the ring nitrogen atom being in close contact with the carbonyl oxygen atom of Thr322 ready for oxidation. As for INH, the electron-deficient heme of compounds I and II would serve to polarize electrons to promote the oxidation. Because Mn^{2+} was not introduced into the system and because KatG can activate INH independent of added Mn ion,[28] the role of Mn in this mechanism is not considered here.

This model provides an insight into the possible multiple roles of Ser315 of MtKatG in the oxidation of INH, and explains how its change to Thr would prevent reaction with INH. The first role lies in the main-chain interaction with the hydrazine portion of INH and direct participation in the catalytic reaction. The second role lies in providing a direct route for electron transfer from the INH to the radical of either compound I or compound II on the heme ring. Changing Ser to Thr would result in unfavorable interactions between the Thr side-chain and the heme propionate group in all conformers except one that could not form hydrogen bonds, thereby breaking the electron conduit. The other alternative of main-chain atom movement to allow hydrogen bonding would distort the catalytic site, preventing reaction.

All other residues in the pocket surrounding the INH-like electron density are fully conserved between BpKatG and MtKatG. Significantly, HMCP and the other catalase-peroxidases of haloarchaebacterial origin have a one residue insertion of Asp269 compared to BpKatG, located in a loop adjacent to the pocket. The insertion forces the ring of the adjacent Pro270 into a position that would interfere with the region of INH-like electron density. The implication is that HMCP should not bind INH in this region, and this might explain why a similar region of electron density was not observed in the HMCP electron density maps.

On the basis of the structure of BpKatG, the mechanisms by which the various clinically identified mutations in MtKatG[1,29] may impart INH resistance can be divided into three categories (Table 2). The first category includes only the Ser315Thr variant (equivalent to Ser324 in BpKatG), which has a minor effect on peroxidatic activity but a significant effect on INH activation, suggesting that INH binding and/or transfer of electrons to the heme is affected. The second category includes six variants with reduced peroxidatic activities, and correspondingly reduced abilities to activate INH. Included are Asn138Ser, Leu148Arg, His270Gln, His275Pro, Trp321Gly and Asp381Gly (equivalent to Asn142, Leu152, His279, His284, Trp330 and Asp389, respectively, in

BpKatG). The third category contains only one variant, Leu587Pro (equivalent to Leu594), which seems to be impart INH-resistance through enzyme instability, most likely the result of defective folding. Other clinical isolates of MtKatG, initially thought to impart INH resistance, including Ser140Asn, Ala350Thr, Arg463Leu and Leu587Met are not included in the Table, because the variants retain native peroxidatic activities and native abilities to activate INH.[1,29] Also not included in the list are the active-site variants Arg104Leu and His108Gln, which directly modify catalytic residues. The locations of the residues in BpKatG equivalent to the residues included in Table 2 are shown in Figure 8. The single residue in the first category (colored red) is found in the channel leading to the heme; the six residues in the second category (colored blue) are found in the general vicinity of the heme; and the single residue in the third category (colored green) is in the C-terminal domain.

These comparisons are based on the presumption of extensive similarity between the *M. tuberculosis* and *B. pseudomallei* enzymes and, indeed, the two enzymes are very similar. The sequences are 64.6% identical over the complete sequence of 749 residues, and, more importantly, they are 75.7% identical over 272 residues (residues 88–359) in the catalytic core of the enzyme. The discussions are also based on the assumption that BpKatG utilizes isoniazid as a substrate, and radical generation by BpKatG is indeed at a rate equivalent to that of *E. coli* HPI, about one-fifth of that of MtKatG[30] (data not shown)

The design of derivatives of INH that would be more effective and might possibly curtail the developing widespread resistance to INH in different strains of *M. tuberculosis* was one hoped-for objective that would result from the definition of the structure of KatG. Unfortunately, the structure suggests that such a goal will be elusive. The variability in the INH-resistance phenotypes is a result of mutations spread widely throughout the enzyme with no single focus or mechanism. Furthermore, the structural environment of the potential INH-binding site does not present obvious motifs that might be exploited for the binding of INH derivatives. The binding site is composed largely of hydrophobic residues surrounding a relatively small volume that is apparently optimized for a pyridine ring with a limited number of substituents, including the carbonyl hydrazide group *para* to the pyridine nitrogen (Figure 3(b)). One note of optimism is the fact that other different pyridine derivatives seem to bind to the site.

Conclusions

The crystal structure of the catalase-peroxidase of *B. pseudomallei*, determined at 1.7 Å resolution, presents a number of structural features that can be interpreted as implying the existence of, as yet undetermined, catalytic functions of the catalase-peroxidases. The immediate environment of the heme-containing active site is structurally similar to that of plant peroxidases and to the catalase-peroxidase from *H. marismortui*, confirming its already ascribed functions as a catalase and a peroxidase. Indeed, the potential binding site for isoniazid is found within what is considered to be the usual substrate access channel of peroxidases. Despite its binding to catalase-peroxidases and the interest this has generated, isoniazid is not a normal substrate for the enzyme, but analysis of its binding site, a small hydrophobic pocket with limited functional capability, suggests that the natural substrate cannot be too different in size or shape.

The presence of a modification on the heme, likely a perhydroxylation, of a single sodium ion in each subunit, of multiple conformations of side-chains and of a new access channel to a cavity in the subunit interior, all within the same region of the subunit, imply the existence of a second, functionally important part of the enzyme. Immediately adjacent to this region are a large cleft on the enzyme surface, suggestive of a binding site for an extended substrate, and the unusual adduct of the side-chains of Trp111, Tyr238 and Met264, presenting a possible route for electron tunneling to the heme from substrates bound either in the cavity or in the cleft on the surface. Further work is required to define the actual *in vivo* peroxidatic substrates of the catalase-peroxidases, and the structure of BpKatG will provide direction to that study.

Materials and Methods

Crystallization and structure determination

The BpKatG protein analyzed in this work was purified from the catalase-deficient *E. coli* strain UM262 as described.[9] Crystals were obtained at 20 °C by the vapor-diffusion, hanging-drop method with 2 μl of a 22 mg/ml of protein solution and 1 μl of the reservoir solution containing 16–20% (w/v) polyethylene glycol (PEG 4K), 20% (v/v) 2-methyl-2,4-pentanediol (MPD) and 0.1 M sodium citrate pH 5.6.[9] Diffraction data were obtained from crystals cooled with a nitrogen cryostream using the same reservoir solution as cryobuffer at 20% PEG-4K. However, the best data set was obtained from a crystal where 100 mM INH had been added to the cryobuffer (Table 1). Crystals were primitive orthorhombic space group $P2_12_12_1$ with one dimeric molecule in the crystal asymmetric unit. The diffraction data set was processed using the program DENZO and scaled with program SCALEPACK.[31] A part (10%) of the measured reflections in every data set were reserved for R_{free} monitoring during automatic refinement (Table 1). All the structural analysis reported in this work, except where indicated explicitly, has been done on the structure derived from the crystals soaked briefly with INH. However, no significant differences have been found with respect to the structure obtained without being exposed to INH. Even the extra density found in the major channel leading to the heme is found in both structures. Therefore, the structure derived from the crystal

after a short soak in INH is considered to be the native BKG structure. Reference to the unsoaked data set has been added only as a control to the presence of INH.

Structure determination was carried out with the program AMoRe[32] and the CPx of *H. marismortu*[5] as searching model. Phases were improved and extended to 1.7 Å resolution with the program ARP-WARP.[33] The resulting map showed clear continuity over the complete length of BpKatG from Asn35 to Ala748 in both subunits. The quality of the data was confirmed by the clarity in the electron density of a number of errors in a 12 amino acid residue region of the predicted sequence. Refinement was started with programs in the CNS suite[34] and completed using the program REFMAC[35] with solvent molecules modeled with the program WATPEAK[36] and manually with the graphics program O.[37] Solvent molecules were introduced only when they corresponded to the strongest peaks in the difference Fourier maps that could make at least one hydrogen bond with atoms already in the model. In the final rounds of refinement, the two subunits were treated independently with the bulk solvent correction applied and the whole resolution range available used for each variant (Table 1). The analysis of solvent accessibility and molecular cavities was carried out with program VOIDOO[38] using a reduced atomic radius for polar atoms in accounting for possible hydrogen bonds.[39] All the Figures were prepared using SETOR.[40]

Protein Data Bank accession number

Structure factors and coordinates have been submitted to the Protein Data Bank under accession number 1MWV.

Acknowledgements

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Oxidant-inducible resistance to hydrogen peroxide killing in *Agrobacterium tumefaciens* requires the global peroxide sensor-regulator OxyR and KatA

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Abstract

Induced adaptive and cross-protective responses to peroxide stress are important strategies used by bacteria to survive stressful environments. We have shown that exposure to low levels of peroxide (adaptive) and superoxide anions (cross-protection) induced high levels of resistance to peroxide killing in *Agrobacterium tumefaciens*. The mechanisms and genes involved in these processes have not been identified. Here, the roles played by peroxide (*oxyR*) and superoxide (*soxR*) global regulators and a catalase gene (*katA*) during these responses were investigated. H_2O_2-induced adaptive protection was completely abolished in both the *oxyR* and *katA* mutants. Superoxide generator (menadione)-induced cross-protection to H_2O_2 killing was observed in a *soxR* mutant, but not in either an *oxyR* or a *katA* mutant. In vivo analysis of the *katA* promoter, using a *katA::lacZ* transcriptional fusion, revealed that it could be induced by menadione in an *oxyR*-dependent manner. These results lead us to conclude that H_2O_2 and superoxide anions directly or indirectly oxidize OxyR and it is the resulting activation of *katA* expression that is responsible for the induced protection against lethal concentrations of H_2O_2.

Keywords: *Agrobacterium tumefaciens*; Adaptation; Catalase; H_2O_2; Menadione; *oxyR*; *soxR*

1. Introduction

Agrobacterium tumefaciens is a plant pathogenic soil bacterium that infects wound sites in many dicotyledonous plants causing the formation of crown gall tumors through the insertion of a segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of the infected cells [1]. This microorganism is also widely used for the introduction of foreign genes into plant cells and the generation of genetically engineered plants [2].

Aerobically growing bacteria always encounter reactive oxygen species (ROS) that are generated as by-products of oxygen-dependent metabolism [3]. In addition, rapid production and accumulation of ROS is an important part of the initial plant defense response against invading microorganisms [4]. ROS are highly toxic to bacterial cells and bacteria have evolved several enzymatic and non-enzymatic mechanisms to protect themselves against them. Physiological adaptation to is one of the important protective strategies used by bacteria when growing under oxidative stress conditions [5–9]. Exposure to sublethal concentrations of one oxidant can induce a protective response against subsequent exposure to lethal concentrations of either the same oxidant (adaptive protection) or unrelated agents (cross-protection) [5]. Oxidant-induced protective responses often result from the coordinated activation of gene(s) involved in both oxidant detoxification and damage repair [10]. Intensive studies, done in the en-

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teric bacterium *Escherichia coli*, show that key regulators of the adaptive response in this organism are the oxidant sensing transcriptional regulators SoxRS and OxyR, which regulate the inducible expression of antioxidant genes in response to superoxide anion and H_2O_2 exposure, respectively [11].

We have demonstrated the existence of H_2O_2-induced adaptive and menadione-induced cross-protection against H_2O_2 killing treatments in *A. tumefaciens* [12]. These inducible responses could play important roles in plant/microbe interactions since the initial active plant defense against microbes involves the increased production and accumulation of H_2O_2 and other ROS [4]. Here we report the characterization of the regulatory and structural gene responsible for these responses.

2. Materials and methods

2.1. Bacterial growth conditions

A. tumefaciens NTL4 [13] and mutant strains were grown aerobically in LB medium at 30°C with continuous shaking at 150 rpm. Overnight cultures were inoculated into fresh LB medium to give an OD_{600} of about 0.1. Exponential phase (OD_{600} about 0.6, after 4 h of growth) cells were used in all experiments, as indicated.

2.2. Molecular biology techniques

General molecular genetics techniques including plasmid preparation, restriction endonuclease digestion, ligation, transformation in *E. coli* and agarose electrophoresis were performed using standard protocols [14]. Plasmid purification for DNA sequencing was performed using a Qiaprep Spin Miniprep kit (Qiagen). DNA was sequenced using a BigDye terminator cycle sequencing kit (PE Biosystems) on an ABI 310 automated DNA sequencer (Applied Biosystems Inc.). *A. tumefaciens* was transformed by electroporation under conditions previously described [13].

2.3. Construction of a soxR mutant

Two primers designed from the putative *A. tumefaciens soxR* nucleotide sequence (AE009322), BT523-5′TGATACGCGTCTGGAGC 3′ and BT524-5′TCGAAGGCTGGCGCACC 3′, were used to amplify a 210-bp *soxR* fragment from *A. tumefaciens* NTL4 genomic DNA using the polymerase chain reaction (PCR). The PCR product was cloned into pGEM-T-easy (Promega) before determining its nucleotide sequence. Subsequently, a *Sal*I–*Sac*II fragment of the PCR clone was subcloned into pKNOCK-Gm [15], a non-replicative plasmid in *Agrobacterium*, cut with the same restriction enzymes. The resultant plasmid, pKNOCKsoxR, was then transferred to *A. tumefaciens* by conjugation. A *soxR* mutant was generated by a homologous recombination of the *soxR* fragment in pKNOCK-soxR and its counterpart on the NTL4 chromosome. Inactivation of *soxR* was confirmed by PCR using two specific primers: one located upstream of the insertion site and the other located on the pKNOCK-Gm vector sequence.

2.4. Determination of resistance levels to oxidants

The peroxide-induced adaptive and cross-protective experiments were performed by adding either 250 μM H_2O_2 or 200 μM menadione to exponential phase cultures. These cultures were grown for an additional 30 min before aliquots of cells were removed and treated with a range of lethal concentrations of H_2O_2 (20, 30, 40 mM) for 30 min. After treatment, cells were removed and washed once with fresh LB before appropriate dilutions were plated on LB agar. Colonies were counted after 48 h incubation at 30°C. Surviving fractions were defined as the number of colony forming units (cfu) recovered after the treatment divided by the cfu prior to treatment. All experiments were independently repeated three times and representative data are shown.

2.5. Superoxide dismutase (SOD) activity gels and assays

Xanthine–xanthine oxidase coupled reduction of cytochrome *c* was used to monitor total SOD activity [16]. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. To visualize SOD activity, non-denaturing electrophoresis was performed using a 10% polyacrylamide gel (pH 8.7) with a 5% stacking gel (pH 8.0) followed by staining with nitroblue tetrazolium/riboflavin photochemical stain as described by Beauchamp and Fridovich [17]. In the enzyme inhibition tests H_2O_2 or potassium cyanide were added to a riboflavin-TEMED solution at final concentrations of 5 and 2 mM, respectively. [Cu,Zn]-SOD is inactivated by cyanide and [Fe]-SOD by H_2O_2 while [Mn]-SOD is resistant to both.

3. Results and discussion

3.1. Induced adaptive protection against H_2O_2 killing requires functional oxyR and katA

In *A. tumefaciens,* exposure to sublethal concentrations of H_2O_2 is known to induce adaptive protection to a subsequent treatment with lethal concentrations of H_2O_2 (Fig. 1A) [12]. The regulatory gene involved in this process has not been identified. Thus, the role of OxyR, a global regulator of the peroxide stress response, in the process was investigated using the *oxyR* mutant strain PN03 [18]. PN03 was grown to exponential phase prior to pretreatment with 200 μM H_2O_2 for 30 min. The induced cultures

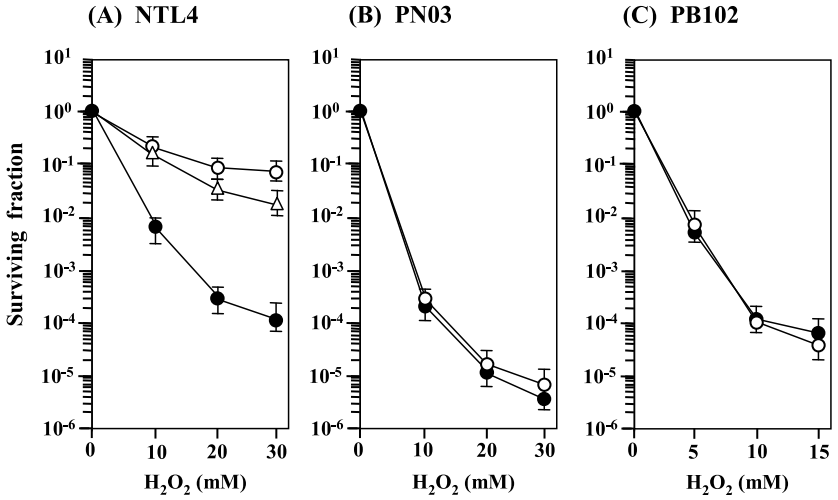

Fig. 1. Induced adaptive responses to H_2O_2 in various *A. tumefaciens* strains. The induced protection experiments were performed by adding 250 μM H_2O_2 to exponential phase cultures of *A. tumefaciens* NTL4 (A), PN03 (B), and PB102 (C). These cultures were grown for an additional 30 min before aliquots of cells were removed and treated with the indicated concentrations of H_2O_2 for 30 min. After treatment, cells were removed and washed once with fresh LB before appropriate dilutions were plated on LB agar. Colonies were counted after 48 h incubation at 30°C. Surviving fractions were defined as cfu recovered after the treatment divided by cfu prior to treatment. All experiments were independently repeated three times and representative data are shown. (●), uninduced; (○), H_2O_2-induced; and (△), menadione-induced (cultures were induced with 200 μM menadione).

were subsequently treated with lethal concentrations of H_2O_2 for 30 min. The results clearly showed that H_2O_2-induced adaptation, against subsequent killing by the oxidant, was completely abolished in PN03 (Fig. 1B) indicating that functional OxyR was required for the response. Similar observations regarding the essential role of OxyR in peroxide adaptive responses have been made in *E. coli* and *Xanthomonas campestris* [19–21]. The data suggest that the adaptive response is due to the oxidation of OxyR during the peroxide pretreatment that leads to up-regulation of the genes in the OxyR regulon. Thus, we extended the investigation to determine which genes in the OxyR regulon are required for the increased resistance to H_2O_2. We have previously shown that the expression of *katA*, encoding a bifunctional catalase–peroxidase HPI, could be induced by pretreatment of *A. tumefaciens* with sublethal concentrations of H_2O_2 in an OxyR-dependent manner [18]. In many bacteria, catalase levels correlate with the levels of H_2O_2 resistance [5,6,9]. Consequently, the role of *katA* in the H_2O_2 adaptive response was investigated using an *A. tumefaciens katA* mutant (PB102). The results show that inactivation of *katA* abolished the adaptive response (Fig. 1C). Although, there are many genes that are regulated by OxyR, the results suggest that up-regulation of *katA* alone is sufficient to confer protection against H_2O_2 killing in *A. tumefaciens*.

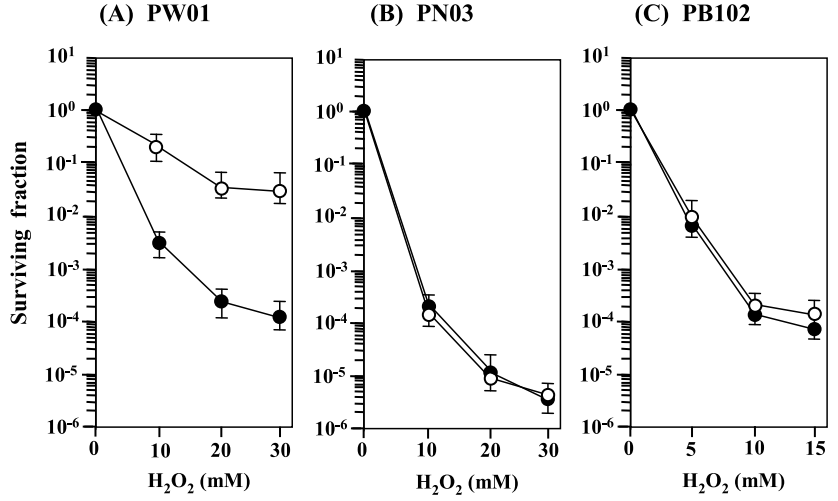

Fig. 2. Induced cross-protective responses to H_2O_2 in various *A. tumefaciens* strains. The experiment was performed as described in the legend to Fig. 1 except 200 μM menadione was used for the induction instead of 250 μM H_2O_2. *A. tumefaciens* PW01 (A), PN03 (B) and PB102 (C) were used in the experiments. (●), uninduced; (○), menadione-induced.

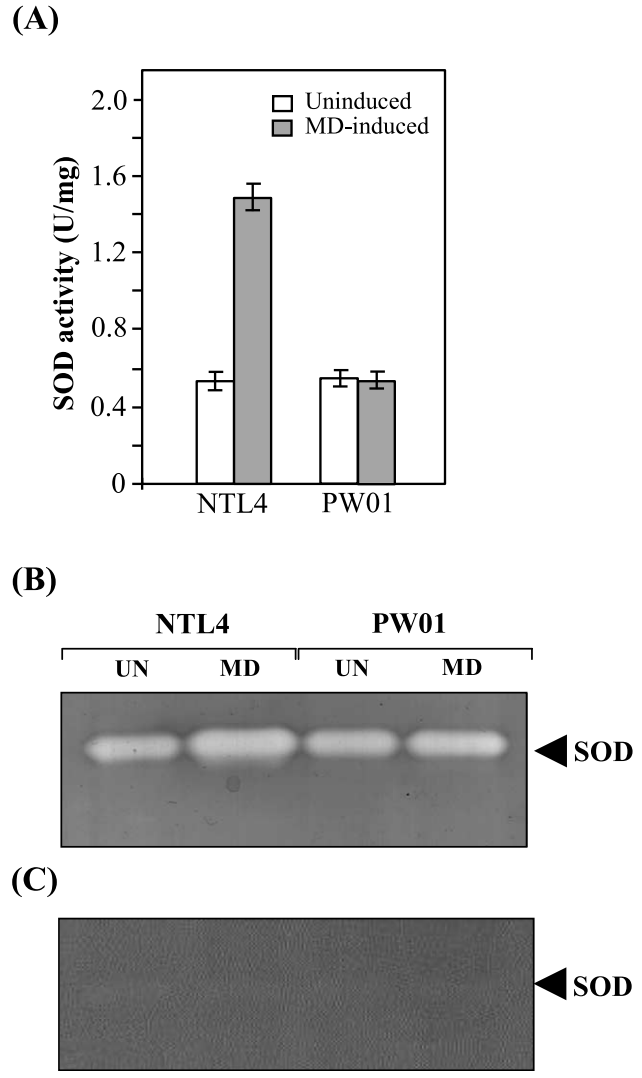

Fig. 3. Induction of SOD by menadione pretreatment in *A. tumefaciens*. A: Total SOD activity in cleared lysates prepared from exponential phase cultures of *A. tumefaciens* NTL4 and PW01 grown under uninduced and menadione (200 μM)-induced conditions. B: SOD activity stain of cleared lysates (50 μg total protein) of uninduced (UN) and menadione-induced (MD) cultures of NTL4 and PW01 separated on a non-denaturing polyacrylamide gel. SOD activity staining was performed as described in Section 2. C: SOD activity stain of a non-denaturing polyacrylamide gel, identical to that described in (B) that was treated with H_2O_2 prior to staining.

3.2. *Menadione-induced cross-protection against H_2O_2 killing is* soxR*-independent*

Treatment of *A. tumefaciens* NTL4 with 200 μM of the superoxide generator, menadione, induced cross-protection to a subsequent challenge with lethal concentrations of H_2O_2 (Fig. 1A) [12]. In *E. coli*, superoxide anions mediate changes in global gene expression via the superoxide sensing transcriptional regulators SoxR and SoxS [11,22]. Analysis of the *A. tumefaciens* genome [23] using the BLASTP program [24] revealed the presence of an open reading frame (ORF) (Atu3915) that encodes a 151-amino-acid polypeptide with 55% sequence identity to *E. coli* SoxR. The *A. tumefaciens* SoxR homolog also contains a conserved CX_2CXCX_5C domain that is involved in the formation of the Fe–S cluster (data not shown). We inactivated the gene resulting in the *A. tumefaciens soxR* mutant strain designated PW01. PW01 was used to determine the role of SoxR in the menadione-induced cross-protection to H_2O_2. The results in Fig. 2A clearly show that the ability of menadione treatment to induce cross-protection against subsequent exposure to H_2O_2 was retained in PW01, indicating that *soxR* was not responsible for mediating the response. The ability of menadione pretreatment to induce the elevated expression of genes in the *soxR* regulon was also tested by measuring SOD activity in cleared lysates prepared from cultures of strains NTL4 and PW01 grown under uninduced and menadione (200 μM)-induced conditions. The results clearly showed that pretreatment of NTL4 with menadione resulted in a 2.7-fold increase in total SOD activity and this induction was abolished in the *soxR* mutant PW01 (Fig. 3A). This evidence suggested that induction of genes in the *soxRS* regulon was not sufficient to protect *A. tumefaciens* from H_2O_2 toxicity.

Analysis of the genome also revealed that *A. tumefaciens* possesses three putative ORFs (namely Atu0876, Atu4583 and Atu4762) identified as SODs and all of these ORFs show a high degree of homology to the family of [Mn,Fe]-SOD [23]. However, only one band of SOD activity was observed by activity staining of polyacrylamide gels of lysates of *A. tumefaciens* NTL4 and PW01 grown under both uninduced and menadione-induced conditions (Fig. 3B). In order to distinguish which family of SOD isozymes was responsible for the activity detected in the gels, the experiments were repeated and the gels were soaked in either H_2O_2 or potassium cyanide prior to SOD activity staining. The results revealed that the inducible SOD band could be detected in the cyanide-treated gel (data not shown) but not in the H_2O_2-treated gel (Fig. 3C), indicating that a [Fe]-SOD was responsible for the observed SOD activity. The gene encoding this SOD is currently being characterized.

3.3. *Induction of* katA *promoter by menadione is mediated by OxyR*

We have previously shown that catalase levels correlate with the resistance levels to H_2O_2 [12]. The possibility that menadione-induced cross-protection to H_2O_2 killing is a result of the OxyR-dependent induction of *katA* expression was investigated. In vivo promoter analysis using pP_{katA} containing the *katA* promoter region transcriptionally fused to a promoter-less *lacZ* (*katA::lacZ*) was performed in NTL4 and PN03. The levels of β-galactosidase activity in bacterial cultures were determined under menadione-induced and uninduced conditions. The β-galactosidase activity from NTL4 bearing pP_{katA} was increased

about 1.7-fold when cells were challenged with menadione at a concentration of 200 μM (data not shown). A similar induction of β-galactosidase activity was not detected in PN03 harboring pP_{katA}. The ability of menadione to activate the expression of genes under the regulation of OxyR has previously been observed in several bacteria including *X. campestris* [25]. However, it is still unclear whether OxyR is activated through oxidation by superoxide anions directly or via H_2O_2 liberated as a consequence of dismutation of superoxide anions [26].

The finding that menadione induced *katA* expression suggests that this gene might play an important role in the observed menadione-induced cross-protection to H_2O_2. Induction experiments were then repeated in PN03 and PB102. The results illustrated that the ability of menadione to induce cross-protection against subsequent lethal treatment with H_2O_2 was lost in both mutant strains (Fig. 2B,C). The evidence presented here indicates that *katA* is essential for the menadione-induced cross-protective response against H_2O_2 toxicity in *A. tumefaciens* and that the cross-protective response is under the control of OxyR.

We conclude that the *soxR* homolog in *A. tumefaciens* is not involved in the menadione-induced cross-protection against H_2O_2 killing. In *A. tumefaciens*, both H_2O_2- and menadione-induced protection against H_2O_2 killing is the result of the upregulation of *katA* expression by the global peroxide sensor and transcriptional activator OxyR.

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SHORT COMMUNICATION

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Compensatory increase in *ahpC* gene expression and its role in protecting *Burkholderia pseudomallei* against reactive nitrogen intermediates

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Abstract In the human pathogen *Burkholderia pseudomallei, katG* encodes the antioxidant defense enzyme catalase-peroxidase. Interestingly, a *B. pseudomallei* mutant, disrupted in *katG*, is hyperresistant to organic hydroperoxide. This hyperresistance is due to the compensatory expression of the alkyl hydroperoxide reductase gene (*ahpC*) and depends on a global regulator OxyR. The KatG-deficient mutant is also highly resistant to reactive nitrogen intermediates (RNI). When overproduced, the *B. pseudomallei* AhpC protein, protected cells against killing by RNI. The levels of resistance to both organic peroxide and RNI returned to those of the wild-type when the *katG* mutant was complemented with *katG*. These studies establish the partially overlapping defensive activities of KatG and AhpC.

Keywords Melioidosis · Alkyl hydroperoxide reductase · Catalase-peroxidase

Introduction

Burkholderia pseudomallei is a human pathogen that can cause an acute, often fatal, septicemic melioidosis or it can remain dormant in the body for many years while retaining its ability to cause an acute septicemia at any time (Jones et al. 1996). The disease varies greatly in its clinical presentation, ranging from an asymptomatic state or a benign pneumonitis, to an acute or chronic pneumonia, or to a suppurative process and rapidly fatal illness. Currently, there is no effective vaccine for melioidosis and treatment is prolonged due to the natural resistance of the pathogen to many of the commonly used antibiotics. The ability of *B. pseudomallei* to cause a long-term latent infection suggests that it is capable of surviving in an intracellular environment. It was reported that *B. pseudomallei* has the capacity to invade cultured cell lines and survive inside phagocytic cells where it was found to be localized inside vacuoles in human monocyte-like U937 cells, a histiocytic lymphoma cell line with phagocytic properties (Jones et al. 1996). The two major microbicidal mechanisms for phagocyte cells are the production of reactive nitrogen intermediates (RNI), such as nitric oxide, and reactive oxygen intermediates (ROI), consisting of hydrogen peroxide, hydroxyl radical, superoxide anion, and singlet oxygen (Klebanoff and Shepard 1984; Hibbs et al. 1987). The contributions of both RNI and ROI to macrophage bactericidal activity against *B. pseudomallei* was examined and it was found that γ-interferon-induced microbicidal activity is mediated to a large extent by the RNI killing mechnism (Miyagi et al. 1997). Although there are many studies on oxidative stress response genes in bacteria, little is known about the role these genes play in oxidant defense in a life-threatening pathogen like *B. pseudomallei*. Previous studies in our laboratory indicate that OxyR, the peroxide stress global regulator, and KatG, catalase-peroxidase, play important roles in protecting *B. pseudomallei* against ROI toxicity (Loprasert et al. 2002, 2003). To date, the gene(s) that confer resistance to RNI in *B. pseudomallei* have not been identified. We compared the levels of resistance of *katG* and *oxyR* knockout strains to compounds that produce RNI. In this communication, we report that a *katG* knockout mutant compensates for the loss of catalase-peroxidase activity by up-regulation of alkyl hydroperoxide reductase (AhpC), which protects *B. pseudomallei* against killing by RNI.

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Materials and methods

Bacterial strains and growth conditions

The clinical isolate *B. psedomallei* P844 and its derivatives were grown in Luria-Bertani (LB) medium. The construction of

B. pseudomallei knockout mutants in *oxyR* (strain R957), *katG* (strain G221) and *oxyR katG* (strain RG27) and their corresponding complemented strains R957TnR, G221TnG, and RG27TnRG, has been described in previous studies (Loprasert et al. 2002, 2003). *Escherichia coli* TA4315 is an AhpC-deficient strain (Storz et al. 1989). All cultures were grown at 37 °C. Tetracycline (60 µg/ml), chloramphenicol (40 µg/ml), spectinomycin (100 µg/ml), and erythromycin (100 µg per ml) were used, when required.

Organic hydroperoxide and hydrogen peroxide sensitivity assays

In order to test the susceptibility of *B. pseudomallei* strains to *tert*-butyl hydroperoxide (*t*-BOOH) and H_2O_2, disk inhibition assays were carried out as previously described (Loprasert et al. 2002, 2003). Briefly, bacterial cells from an exponential-phase culture (10^8 cells) were added to 3 ml warm LB top agar. The mixture was poured onto LB agar and allowed to set. Paper discs (6 mm) containing 6 µl of either 250 mM *t*-BOOH or 1.0 M. H_2O_2 were placed on the surface of the plate. Zones of growth inhibition were measured after 24 h incubation at 37 °C.

Overexpression of *katG* in wild-type

A 3-kb *Sma*I–*Hin*dIII DNA fragment, containing *katG*, was ligated into *Hin*cII–*Hin*dIII-digested pBBR-Cm vector (the broad-host-range cloning vector pBBR1MCS) (Kovach et al. 1995) to generate pG. This was then conjugated into *B. pseudomallei* creating strain P844G (Loprasert et al. 2003).

RNA extraction and Northern blot hybridization

Extraction of total RNA, using the modified hot acid phenol method, and Northern blot analysis of mRNA were carried out as previously described (Loprasert et al. 2002). Mid-exponential-phase *B. pseudomallei* cultures of wild-type and *katG* mutants were harvested for total RNA isolation.

Cloning of the *ahpC* promoter region

The oligonucleotide primers C1 and C2, bracketing the promoter region of *ahpC*, were synthesized according to the genome sequence of *B. pseudomallei* (accessible from the Sanger Institute website: http://www.sanger.ac.uk). The C1 and C2 sequences are 5′ CTGCAGCCGAACTACAGCAGCGC 3′ and 5′ TGCACGAAGTCGCCGTTGTGG 3′, respectively. These primers were used to PCR amplify a 374-bp fragment, spanning the promoter region of *ahpC*, from the chromosome of *B. pseudomallei* P844 and its *katG* mutant (G221) using *Pfu* polymerase (Promega). The nucleotide sequences were subsequently determined.

Cloning of the *ahpC* structural gene

The oligonucleotide primers C3 and C4, corresponding to the N and C-terminus of *ahpC*, were used to PCR amplify a 560-bp fragment from the *B. pseudomallei* P844 chromosome that contains an *Nco*I site overlapping the *ahpC* start codon. The fragment was then sequenced to confirm the presence of the introduced restriction site as well as to check for mutations in *ahpC*. The C3 and C4 sequences are 5′ GTGACCATGGCGATCATC 3′ and 5′ TCAGATCTTGCCGATCAGGTC 3′, respectively. The PCR product was digested with *Nco*I and ligated into *Nco*I–*Eco*RV-digested pET2 (Novagen) resulting in pCET. An *Xba*I–*Sma*I *ahpC* fragment from pCET was ligated into *Xba*I–*Eco*ICRI-digested pBBR-Cm creating plasmid pC that was then conjugated to *B. pseudomallei*.

Western blot analyses

Cell lysates were prepared from fresh cell pellets using bacterial protein extraction reagent (Pierce). Protein concentration was measured by the dye binding method (Bradford 1976). AhpC was detected by Western blotting as follows: 15 µg protein was separated on SDS-PAGE gels and electrotransferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat milk and reacted with anti-*E. coli* AhpC antibody as previously described (Mongkolsuk et al. 1997). The relative amount of AhpC in each lane was determined using a Bio-Rad GS-700 densitometer.

Sensitivity to RNI

The susceptibility of *B. pseudomallei* strains to RNI was tested as previously described (Chen et al. 1998). Overnight cultures in LB medium were used to inoculate acidified M9 minimal medium (Difco), adjusted to pH 5.0 with 1 M. HCl and supplemented with 0.5% casamino acids, to an OD_{600} of 0.1. Sodium nitrite was added to a final concentration of 0.5 mM and the OD_{600} was measured after incubation for 7 h at 37 °C with shaking. The percentage survival was calculated by comparing the absorbance of treated cultures with comparable untreated cultures.

Results

Compensatory expression of *ahpC*

Surprisingly, the *katG*-deficient mutant (strain G221) displayed a smaller zone of inhibition (i.e. higher level of resistance) in the presence of the organic hydroperoxide *t*-BOOH than that of the wild-type parental strain P844 (Fig. 1). This resistant phenotype was lost when a copy of *katG* was transposed into the chromosome of strain G221, yielding strain G221TnG. *B. pseudomallei* wild-type harboring the *katG* plasmid (strain P844G) did not show any altered inhibition zone relative to strain P844. Only strain G221 exhibited increased resistance to *t*-BOOH killing. This observation, combined with the fact that the *katG oxyR* double mutant (RG27) showed an unaltered resistance relative to the parental strain P844, suggested that

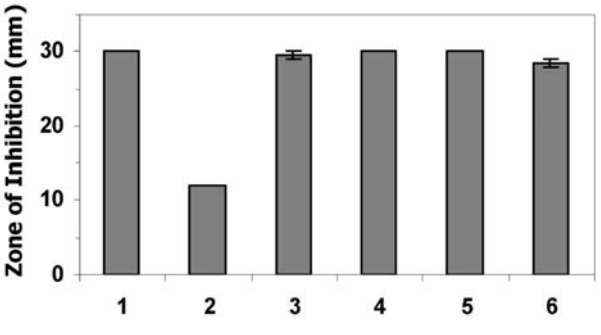

Fig.1 Determination of the levels of resistance to *tert*-butyl hydroperoxide killing displayed by the *Burkholderia pseudomallei* parent strain P844 (*lane 1*), *katG* mutant G221(*lane 2*), *katG oxyR* mutant RG27 (*lane 4*), and their complemented strains G221TnG (*lane 3*) and RG27TnRG (*lane 5*). P844G (l*ane 6*) is the parental strain harboring *katG* plasmid. Resistance levels were determined by disk inhibition assay as described in Materials and methods. Each value shown is the mean of three separate experiments

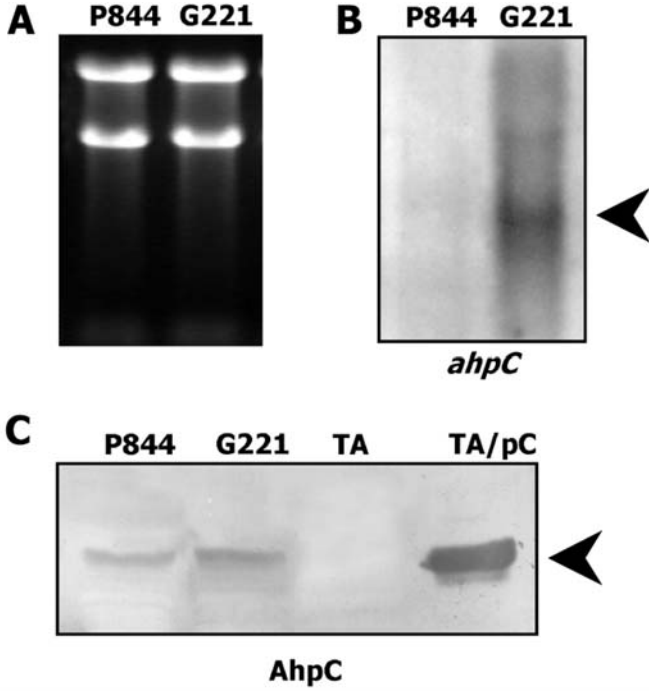

Fig. 2A–C Northern and Western blot analyses of *ahpC* mRNA and AhpC protein from *B. pseudomallei* P844, *katG* mutant G221, and an *Escherichia coli* mutant deficient in AhpC. **A, B** Total RNA samples were extracted from uninduced cell cultures. Each lane contained 20 µg RNA. **A** Ethidium-bromide-stained gel. **B** Hybridization signals obtained when the Northern blot of the gel in **A** was hybridized to a radioactively labeled *ahpC* probe. *Arrowhead* mRNA transcript. **C** Protein lysates (15 µg each) from *B. pseudomallei* P844, *katG* mutant G221, the *E. coli* AhpC deficient mutant TA4315 (TA), and TA4315 harboring pC (TA/pC) were separated by SDS-PAGE, blotted, and reacted with anti-*E. coli* AhpC antibody as described in Material and methods. *Arrow* AhpC protein band

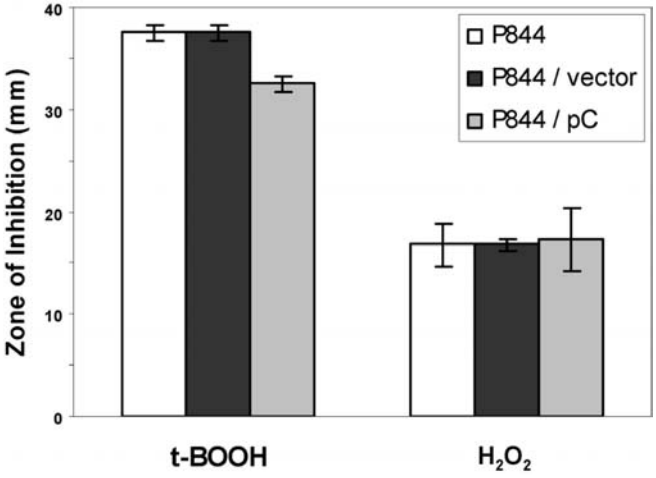

Fig. 3 Effect of increased expression of *ahpC* on the sensitivity of *B. pseudomallei* to *tert*-butyl hydroperoxide (*t*-BOOH) and H_2O_2. The resistance levels displayed by the *B. pseudomallei* parent strain P844, strain P844 with plasmid vector pBBR-Cm (strain P844/vector), and strain P844 with *ahpC* plasmid pC (strain P844/pC) were determined by disk inhibition assay. Each value shown is the mean of values obtained in three separate experiments. *Error bars* Standard error of the mean

the increased resistance to organic hydroperoxide in strain G221 might be mediated by the transcription factor OxyR. Moreover, it was reasoned that altered expression of *ahpC* was a likely cause of the increased organic hydroperoxide resistance in strain G221 since *E. coli ahpC* protects cells from organic hydroperoxide and its expression is regulated by OxyR (Tartaglia et al. 1989). Therefore, it was of interest to examine whether the disruption of *katG* induces a compensatory increase in the expression of *ahpC*. Northern blotting experiments demonstrated that the *katG*-deficient mutant strain G221 indeed had an increased level of *ahpC* mRNA, compared to the expression level in the parent strain P844 (Fig. 2B). The level of AhpC in G221 was two-fold higher than in the parent strain as assayed by Western blot analysis (Fig. 2C). In *Mycobacterium tuberculosis*, an isoniazid-resistant *katG* mutant was found to compensate for the loss of KatG by the acquisition of a promoter mutation upstream of *ahpC* that resulted in hyperexpression of *ahpC* (Sherman et al. 1996). In order to check for a promoter mutation, the 5′ region of *ahpC* was sequenced in both the *katG* mutant and the parent *B. pseudomallei* strains. No base alterations were found (data not shown).

Protection against organic hydroperoxide by AhpC

In order to test our hypothesis that increased resistance to organic hydroperoxide is due to increased *ahpC* expression, *ahpC* was amplified from the *B. pseudomallei* chromosome and cloned into plasmid pBBR-Cm creating plasmid pC. *ahpC* was then cloned into plasmid vector pBBR-Cm such that transcription of *ahpC* was driven by the Cm resistance cassette promoter. To verify that plasmid pC actually overproduces AhpC, Western blot analysis was carried out on an AhpC-deficient strain (*E. coli* TA4315) carrying pC. This was done to eliminate the endogenous AhpC band in *B. pseudomallei*. Plasmid pC was transformed into *E. coli* TA4315 and the level of AhpC expression was examined. AhpC protein was indeed overproduced as shown in Fig. 2C, lane TA/pC, whereas no AhpC band was detected in the host strain TA4315 (lane TA). The *B. pseudomallei* wild-type strain harboring pC (strain P844/pC), and the wild-type strain carrying the plasmid vector pBBR-Cm with no insert (strain P844/vector) were tested for their sensitivity to ROI using disk inhibition assays. Strain P844/pC showed increased resistance to *t*-BOOH killing compared to both the wild-type strain and strain P844/vector (Fig. 3). It is also worth noting that overproduction of AhpC in *B. pseudomallei* P844/pC did not confer resistance to H_2O_2 (Fig. 3). This result is similar to that observed in *Xanthomonas campestris*, in which overproduction of AhpC did not alter the sensitivity to H_2O_2 (Loprasert et al. 1997).

Protection against RNI by AhpC

It was of interest to know whether the up-regulation of *ahpC* in the *B. pseudomallei katG* mutant enhances its

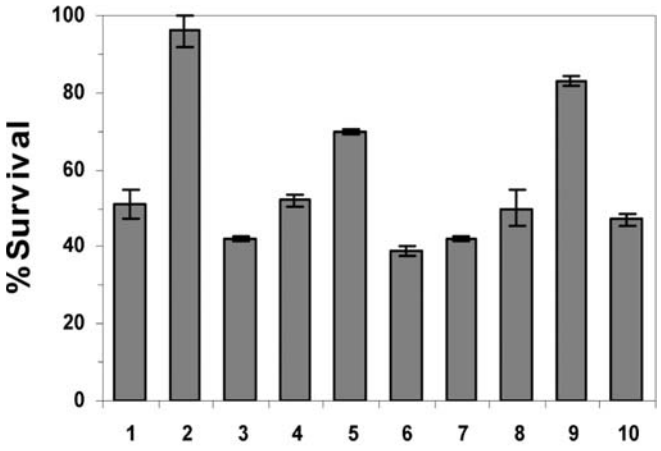

Fig. 4 Survival of *B. pseudomallei* wild-type and derivatives exposed to reactive nitrogen intermediates (RNI). *B. pseudomallei* parent strain P844 (*lane 1*), strain P844 incubated with nitrite, pH 7.0 (*lane 2*), *oxyR* mutant R957 (*lane 3*), complemented *oxyR* mutant R957TnR (*lane 4*), *katG* mutant G221 (*lane 5*), complemented *katG* mutant G221TnG (*lane 6*), *oxyR katG* double mutant RG27 (*lane 7*), complemented *oxyR katG* mutant RG27TnRG (*lane 8*), wild-type *ahpC* overexpressing strain P844/pC (*lane 9*), vector control strain P844/pBBR-Cm (*lane 10*). Conditions used in *lanes 1–10* (except in *lane 2*) were acidified nitrite, pH 5.0, as described in Material and methods. The results shown represent the average values of at least three independent experiments. *Error bars* Standard error of the mean

survival in a hostile environment similar to that within a macrophage. The *katG* mutant G221, together with *oxyR* mutant R957 and *katG oxyR* double mutant RG27, were tested for their sensitivity to RNI. In this experiment, mildly acidified nitrite (pH 5.0), which is similar to the environment encountered in phagolysosomes (Ehrt et al. 1997), was used. Under this condition, a small portion of nitrite is protonated to generate HNO_2, which can then undergo dismutation to nitric oxide (NO), dinitrogen trioxide (N_2O_3), and dinitrogen tetraoxide (N_2O_4) (Ehrt et al. 1997). When the *B. pseudomallei* wild-type and mutant strains were exposed to acidified sodium nitrite (0.5 mM, pH 5.0) for 7 h, the *oxyR* and *oxyR katG* double mutants were found to be more sensitive than the wild-type strain to RNI. Surprisingly, the *katG* knockout mutant G221 showed increased resistance to RNI relative to wild-type (70% survival versus 51%, respectively, Fig. 4, lanes 5 and 1). This increased resistance was not observed in the *katG oxyR* mutant RG27, suggesting that OxyR is required in the induction process. The higher resistance to RNI in G221 was confirmed to be due to the lack of KatG since the percent survival fell below the wild-type level in the complemented strain G221TnG (39% survival for strain G221TnG, Fig. 4, lane 6). The fact that *katG* mutant G221 is more resistant to RNI and overexpresses *ahpC* mRNA led us to hypothesize that AhpC protects *B. pseudomallei* against RNI killing. Strain P844/pC is highly resistant to RNI, with 83% survival, indicating that AhpC directly protects *B. pseudomallei* from RNI toxicity (strain P844/pC, Fig. 4, lane 9). As a control, *B. pseudomallei* was exposed to sodium nitrite at pH 7.0 and exhibited 100% survival (Fig. 4, lane 2).

Discussion

Reactive nitrogen intermediates together with ROI play various physiological roles in living cells, e.g. the activation/inactivation of transcription factors, enzymes, and ion channels; they are also able to mutate DNA and to induce apoptosis (Stamler 1995; Keefer and Wink 1996). Infected hosts use both RNI and ROI to counter infection. For these reasons, it seems likely that an intracellular pathogen like *B. pseudomallei* expresses genes that confer resistance not just to ROI, but also to RNI. The results presented here are direct evidence that *B. pseudomallei katG* mutants compensate for the loss of KatG by an *oxyR*-dependent increase in the expression of *ahpC* and that this process protects *B. pseudomallei* against RNI. While the mechanism of *ahpC* induction is unknown, the up-regulation of *ahpC* could be due to the absence of an antioxidant catalase-peroxidase that results in a more oxidized cellular state, which in turn activates OxyR. No mutations were found in the promoter region of *ahpC* that would explain the compensatory increase in *ahpC* expression as was observed in a *katG* mutant of *M. tuberculosis* (Sherman et al. 1996). This is reasonable since *M. tuberculosis* has no OxyR due to frameshifts and deletions within *oxyR* (Sherman et al. 1995). In order to survive during infection, *Mycobacterium* has acquired promoter mutations that result in hyperexpression of *ahpC* (Sherman et al. 1996). The protective affect of AhpC against RNI may be due to an intrinsic peroxynitritase activity. AhpC from *Salmonella typhimurium* has been suggested to have peroxynitritase activity (Bryk et al. 2000). Additionally, a strain of *M. tuberculosis* deficient in AhpC was found to be sensitive to peroxynitrite (Master et al. 2002), and AhpC from *M. tuberculosis* has been indirectly implicated in RNI resistance when expressed in heterologous systems such as *Salmonella* (Chen et al. 1998). This is the first report describing compensatory *ahpC* expression in a *B. pseudomallei katG* mutant and the role of AhpC in resistance to RNI. This finding is clinically important for developing drugs against an intracellular pathogen that has to encounter and combat a nitrosative stress environment.

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Regulation of the *katG-dpsA* operon and the importance of KatG in survival of *Burkholderia pseudomallei* exposed to oxidative stress

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Abstract Homologues of the catalase-peroxidase gene *katG* and the gene for the non-specific DNA binding protein *dpsA* were identified downstream of *oxyR* in *Burkholderia pseudomallei*. Northern experiments revealed that both *katG* and *dpsA* are co-transcribed during oxidative stress. Under conditions where the *katG* promoter is not highly induced, *dpsA* is transcribed from a second promoter located within the *katG-dpsA* intergenic region. A *katG* insertion mutant was found to be hypersensitive to various oxidants. Analysis of *katG* expression in the *oxyR* mutant indicates that OxyR is a dual function regulator that represses the expression of *katG* during normal growth and activates *katG* during exposure to oxidative stress. Both reduced and oxidized OxyR were shown to bind to the *katG* promoter.

Key words: Melioidosis; Oxidative stress; Catalase-peroxidase; Non-specific DNA binding protein; OxyR; Gene regulation

1. Introduction

Burkholderia pseudomallei is a facultative bacterial intracellular pathogen that can cause melioidosis, a potentially fatal infection with septicemic, subacute, and chronic forms. *B. pseudomallei* can survive and multiply in human phagocytes in vitro, moreover, it can persist in a dormant stage in macrophages for months or years [1]. Currently, the mechanism by which this organism survives in human phagocytes is not known. Addition of superoxide dismutase and catalase enzymes significantly inhibits macrophage bactericidal activity against *B. pseudomallei* in vitro suggesting the importance of antioxidant enzymes for the intracellular survival of this pathogen [2]. KatG is a bifunctional enzyme having both catalase and peroxidase activities which protect *Mycobacterium tuberculosis* from killing by oxidative stress [3].

Melioidosis is a life-threatening disease but little is known about the genes involved in the oxidative stress response in its causative agent, *B. pseudomallei*. We began an investigation of the *B. pseudomallei oxyR* homologue and demonstrated that *oxyR* mutants are hypersensitive to oxidative stress, auto-aggregate, more readily form biofilms, and have decreased extracellular protease activity [4]. In this report, we cloned the catalase-peroxidase gene (*katG*) together with the non-specific DNA binding protein gene (*dpsA*). We monitored their expression and constructed a knockout mutant in order to determine the role *katG* plays in protecting *B. pseudomallei* against oxidative killing. Moreover, we demonstrated that OxyR can both repress and activate *katG* expression in vivo and bind to the *katG* promoter region in vitro.

2. Materials and methods

2.1. Media and growth conditions

B. pseudomallei P844 is a clinical isolate [5]. *B. pseudomallei* P844 and *Escherichia coli* were routinely maintained in Luria–Bertani (LB) medium [6]. *Pseudomonas* agar base supplemented with SR103E (cetrimide, fucidin, and cephaloridine) from Oxoid was used after conjugation as a selective medium to inhibit growth of *E. coli*. All cultures were grown at 37°C. Tetracycline (60 μg/ml), chloramphenicol (40 μg/ml), erythromycin (100 μg/ml), and trimethoprim (100 μg/ml) were added to media when required.

2.2. Cloning of full-length katG and dpsA

A 3.5-kb *Eco*RI-*Sal*I fragment of *B. pseudomallei* P844 chromosomal DNA, containing *recG*, *oxyR* and part of *katG*, was previously cloned to generate plasmid pR35 [4]. In order to clone the C-terminal part of *katG*, an *Xma*I genomic blot of *B. pseudomallei* P844 DNA was hybridized with a 700-bp *Xho*I probe from pR35, containing part of *katG*. A 6.0-kb hybridizing band was gel-purified and cloned into pBluescript KS. Restriction analysis and DNA sequencing verified that the newly cloned 6.0-kb *Xma*I fragment contained all of *katG*.

2.3. Northern analysis of katG and dpsA

Extraction of total RNA, using the modified hot acid phenol method, and Northern blot analysis were carried out as previously described [7]. Oxidants, *tert*-butylhydroperoxide (t-BOOH), H_2O_2 and menadione were added to mid-log-phase *B. pseudomallei* cultures to a final concentration of 0.5 mM. Induced and uninduced cultures were grown for 15 min before harvest for total RNA isolation.

2.4. Construction of chromosomal katG::lacZ transcriptional fusion strains

The 1064-bp *Eco*RV-*Bgl*II fragment containing the 5′ end of *katG* and upstream sequence was subcloned into pUC18 to generate pUC18*Sfi*I *lacZ*, subsequently the *Sfi*I fragment containing the *katG* promoter and *lacZ* reporter was excised and ligated into the modified minitransposon pUT-*dfr* [4] to create pUT-TnpG which was then conjugally transferred into *B. pseudomallei* P844 and stable trimethoprim-resistant transconjugants selected. Integration of the *katG::lacZ* transcriptional fusion into the chromosome of *B. pseudomallei* P844 and the *oxyR* knockout mutant R957 [4] created strains P844TnpG and R957TnpG, respectively.

2.5. Assays for the induction of the katG promoter by oxidative stress

Overnight cultures of *B. pseudomallei* P844TnpG and R957TnpG

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Abbreviations: t-BOOH, *tert*-butylhydroperoxide; NEM, *N*-ethylmaleimide; HOCl, hypochlorite

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were subcultured ($OD_{600} \approx 0.1$) in LB at 37°C. Mid-exponential-phase (~2 h) cells were induced with either 0.5 mM menadione, 1 mM t-BOOH, 2 mM H_2O_2 or 800 mM NaCl for 1 h before harvest. Cell lysates were prepared using bacterial protein extraction reagent (Pierce) and assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactoside as the substrate as previously described [8].

2.6. *Construction of* katG *and* katG oxyR *mutants*

A *katG* knockout mutant was created by insertion of a tetracycline resistance plasmid into *katG*. Briefly, a 240-bp *Mlu*I-*Hin*cII fragment from pR35 was subcloned into pSPORT 1 (Life Technologies), excised with *Sca*I and *Eco*RI, then ligated into suicide vector pKNOCK-TC [9] to create pG221, which was mobilized from *E. coli* S17-1 λ*pir* into *B. pseudomallei* P844 by conjugation. The mutant designated G221 was confirmed as carrying a single insertion of pKNOCK-TC within *katG*. A Tc^r Cm^r *katG oxyR* double mutant, RG27, was created by chromosomal mobilization between G221 (*katG*) and R957 (*oxyR*). Both G221 and RG27 were shown by Southern analysis to have the desired gene disruptions.

2.7. *Complementation of* katG*,* oxyR*, and* katG oxyR *mutants by modified Tn5*

The 3.0-kb *Bam*HI-*Hin*dIII fragment, containing the *katG* upstream region along with *katG*, was cloned into pUT-*dfr* [4]. The minitransposon pUT-*dfr-katG* was then conjugally transferred into *B. pseudomallei* G221 and the stable trimethoprim- and tetracycline-resistant strain G221TnG was selected. Similarly, the 4.0-kb *Eco*RI fragment containing both *oxyR* and *katG* (pUT-*dfr-oxyR-katG*) was mobilized into RG27 by conjugation. The complemented strain, RG27TnRG, was selected on media containing trimethoprim, tetracycline and chloramphenicol. The *oxyR* mutant strain R957 was complemented by the minitransposon carrying *oxyR* and an erythromycin resistance gene (*ery*) from pIC20HEry [10].

2.8. *Purification of OxyR protein*

Primer R1 (5′-CGCCGGTCGACATTCACCGTCG-3′) and primer R2 (5′-CTTGCCATGGCGCTTACC-3′), which covered the 5′ and 3′ regions, respectively, were used to PCR amplify the *oxyR* gene from a plasmid pR35 template. The 700-bp PCR product was digested with *Sal*I and *Nco*I and ligated to pET2-blue (Novagen) digested with *Xho*I and *Nco*I to generate pET-*oxyR*. For purification of His-tagged OxyR, *E. coli* DE3 (Novagen) harboring pET-*oxyR* was grown in CG medium (Bio101) to log phase and induced with 1 mM IPTG for 1 h at 37°C. Cells were washed once with Tris buffer, pH 7.8 and resuspended in binding buffer (20 mM Tris, pH 8.0, 500 mM NaCl and 0.1 mM EDTA) plus 0.1% Triton X-100 before being sonicated on ice. A clear lysate was obtained after centrifugation at $10\,000 \times g$ for 10 min. The extract was loaded onto a Talon metal affinity column (Clontech). Elution of the bound OxyR by imidazole was carried out according to the manufacturer's procedure. The homogeneity of purified OxyR was determined by SDS–PAGE. Protein concentration was measured using the Bradford dye binding assay [11].

2.9. *Gel shift assay*

The assay was performed as previously described [12]. Briefly, the purified OxyR was dialyzed against TEG buffer. A 540-bp *Bam*HI-*Hin*cII fragment spanning the promoter region of *katG* was end-labeled using [α-^{32}P]dCTP and Klenow fragment. Gel shift reactions were performed by adding 3 fmol of labeled probe to TM buffer. To assay binding under reducing conditions, 200 mM dithiothreitol (DTT) was added to binding reactions.

2.10. *Oxidative stress sensitivity assays*

To test the susceptibility of *B. pseudomallei* strains to oxidative stress agents, disk inhibition assays were performed as previously described [4]. Thus, 6 μl of 0.5 M H_2O_2, 1 M menadione, 0.1 M *N*-ethylmaleimide (NEM), and 18 μl of 6% sodium hypochlorite (NaOCl) were used and the zones of growth inhibition around the disks were measured after 24 h of incubation.

2.11. *Nucleotide sequence accession number*

The GenBank accession number for *oxyR-katG-dpsA* genes is AY040244.

3. Results and discussion

3.1. *Cloning and sequence analysis of* katG *and* dpsA

We cloned a 6.0-kb *Xma*I fragment of *B. pseudomallei* P844 chromosomal DNA. DNA sequencing of the clone revealed that it overlapped the previous clone and contained two open reading frames oriented in the same direction. The first encoded the entire catalase-peroxidase enzyme (KatG) and the second encoded a protein of 162 amino acids with strong homology to the non-specific DNA binding protein (DpsA).

The deduced amino acid sequence of *B. pseudomallei* KatG (748 residues) shares very high levels of identity with eight other KatG sequences in the databases when compared and aligned using the CLUSTAL W program [13] (data not shown). The amino acid identities between *B. pseudomallei* KatG and its homologues in the databases are: 72% for *Mesorhizobium loti* (NP 107344), 68% for *Caulobacter virioides* (O31066), 66% for *Geobacillus stearothermophilus* (P14412), 64% for *Mycobacterium tuberculosis* (Q08129), 61% for *Legionella pneumophila* (Q9WXB9), *E. coli* (P13029) and *Vibrio cholerae* (AAF94714), and 58% for *Yersinia pestis* (NP 406785). The distal histidine refers to the residue forming the peroxide binding site on the distal side of the heme. The proximal residue is the histidine that binds to the proximal side of the heme. The distal and proximal histidine residues and sequences surrounding them are present in the *B. pseudomallei* clone and are highly homologous to those from other bacterial catalase-peroxidases [14]. Previously, the *B. pseudomallei* P844 catalase-peroxidase enzyme was purified, crystallized and analyzed by X-ray diffraction [15]. The enzyme is a homotetramer of 81.6-kDa subunits and contains heme *b*. The complete structure elucidation at high resolution of 1.7 Å of this enzyme is now in progress.

When the deduced amino acid sequence of DpsA (with a calculated molecular mass of 18 kDa) was analyzed and aligned with other Dps homologues, it revealed high homology (data not shown). The amino acid identities found are 74% for *Pseudomonas aeruginosa* (D83524), 58% for *Synechocystis* PCC6803 (S77503), 50% for *Xylella fastidiosa* (B82689), 47% for *Caulobacter crescentus* (AAK24837), and 30% for *Bacteroides fragilis* (AAG02618) and MrgA of *Bacillus subtilis* (G69660). DNA binding regions I and II, previously reported in Dps homologues from other bacteria [16], are conserved in *B. pseudomallei* DpsA.

3.2. *Regulation of* katG *and* dpsA *transcription*

To investigate the regulation of the expression of *katG* and *dpsA*, total RNA extracted from mid-exponential-phase cells, exposed to different oxidative stress conditions, was probed with radiolabeled *katG* and *dpsA* gene internal DNA fragments. The results are shown in Fig. 1A,B,D along with a map showing the genetic organization of both genes, including *oxyR*, together with the transcripts encoding each gene (Fig. 1C). Northern blot hybridization analysis revealed that expression of *katG* and *dpsA* mRNA was regulated at the transcriptional level. Under uninduced conditions, a transcript of approximately 2.9 kb was detected using a probe specific for *katG* (Fig. 1A), while a *dpsA*-specific probe detected a message of approximately 0.6 kb (Fig. 1B). By contrast, during growth under induced conditions in the presence of the oxidants t-BOOH, menadione, and H_2O_2, a common 3.5-kb transcript was detected encoding both *katG* and *dpsA* in addition to the

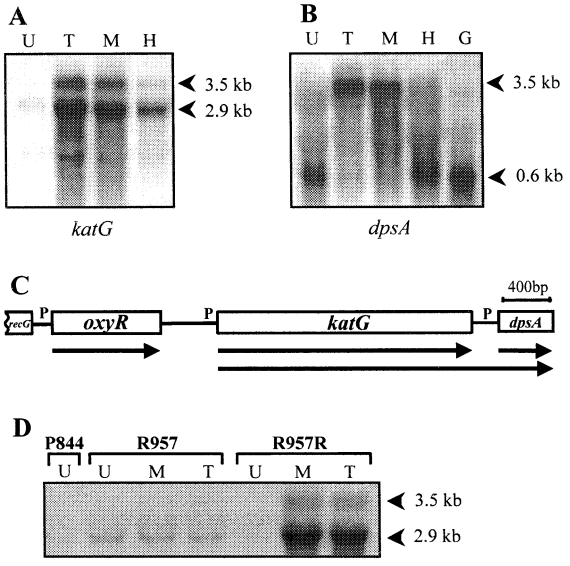

Fig. 1. Expression of *katG* and *dpsA* mRNA in response to oxidative stress and a diagram of their genetic organization. A: Northern analysis of *katG* mRNA from uninduced *B. pseudomallei* P844 cells (U), and from cells induced with t-BOOH (T), menadione (M), and H_2O_2 (H). B: Northern blot analysis of *dpsA* mRNA from *B. pseudomallei* P844 cells grown under the same conditions. Lane G contains RNA from the *katG* mutant, G221. Arrowheads indicate hybridizing mRNAs and their sizes (kb) are indicated. C: Diagram of the genetic organization of *oxyR*, *katG*, and *dpsA*. The arrows indicate the direction and extent of transcription. P indicates promoter regions. D: Northern analysis of *katG* mRNA from *B. pseudomallei* parent P844, *oxyR* mutant R957, and the complemented strain R957R. Total RNA was prepared from uninduced cells (U), and cells induced with menadione (M) and t-BOOH (T). Arrowheads indicate mRNA transcripts.

2.9-kb *katG* transcript (Fig. 1A,B, lanes T, M, H). Interestingly, under induced conditions, the 0.6-kb *dpsA* transcript was present in significant levels only during growth in the presence of H_2O_2 where it appeared to be induced only slightly (Fig. 1B, lanes T, M, H). H_2O_2 was also found to be a less effective inducer of *oxyR* expression in our previous study [4] suggesting that *B. pseudomallei* P844 may have high endogenous catalase activity that rapidly detoxifies H_2O_2. The fact that *dpsA* is transcribed on a 0.6-kb mRNA suggested that this message arose either from processing of the 3.5-kb *katG-dpsA* transcript or from initiation from a promoter situated in or near the *katG-dpsA* intergenic region. In order to differentiate between these two possibilities, Northern blot analysis was performed on total RNA extracted from the *katG* knockout mutant G221 grown in the presence of menadione. Only the 0.6-kb *dpsA* transcript was detected (Fig. 1B, lane G). Taken together, the Northern hybridization data indicate that the 3.5-kb *katG-dpsA* and 2.9-kb *katG* transcripts initiate from a promoter 5′ to *katG* while the 0.6-kb *dpsA* transcript is initiated from a separate promoter immediately upstream of *dpsA*.

Moreover, transcription initiation at the *dpsA* promoter appears to be inhibited by transcriptional read-through from the upstream *katG* promoter. This is suggested by the observation that the 0.6-kb *dpsA* transcript was absent when the upstream *katG* promoter was strongly induced (i.e. in the presence of t-BOOH or menadione, Fig. 1B, lanes T, M) and present only when the *katG* promoter was inactive (Fig. 1B, lanes U, G) or weakly induced by H_2O_2 (Fig. 1B, lane H). Co-transcription of *katG* and *dpsA* allows for a prompt response to oxidative stress. While KatG inactivates peroxides and hydroxyl radicals, Dps binds to the chromosome where it has been shown, in *E. coli*, to protect against the mutagenic effects of oxidative damage to the DNA [17]. KatG of *E. coli* is known to be regulated by OxyR during exponential-phase growth [18]. To test if *katG* of *B. pseudomallei* is regulated by OxyR, Northern blots of total RNA from the *oxyR* knockout mutant, R957 [4], was analyzed for *katG* mRNA during growth in the presence and absence of oxidants (Fig. 1D). The 3.5-kb mRNA (*katG-dpsA*) was not detected in R957, and the 2.9-kb mRNA (*katG*) was only very weakly expressed even in the presence of oxidants. The normal levels of the *katG* transcripts were restored and fully oxidant-inducible when a copy of *oxyR* was re-introduced into the mutant strain (Fig. 1D, R957R) indicating that the activation of *katG* transcription in response to oxidative stresses requires OxyR. The four putative OxyR binding tetranucleotide sequences (**ATTG**×7 **CCCG**×6 **ATTG**×7 **CAAG**×CTGCCA) and a putative −35 promoter element (underlined) were found 202 nucleotides upstream of the translation start site of *katG*. The residues in bold type within the putative OxyR binding site match the consensus sequence derived for *E. coli* OxyR-regulated promoters (ATAG×7 CTAT×7 ATAG×7 CTAT) [19]. Primer extension and DNase I footprinting experiments are needed to verify if these putative binding sequences are within the *katG* promoter.

3.3. katG *promoter responses to various stresses*

In order to further investigate the regulation of *katG* transcription response to both oxidative and osmotic stresses, β-galactosidase expression was monitored in strain P844TnpG, containing a chromosomal *katG-lacZ* transcriptional promoter fusion. The expression of *katG* in P844TnpG was induced eightfold, sixfold, and threefold after menadione, t-BOOH, and salt exposure, respectively (Fig. 2). The induction by oxidants was abolished in the *oxyR* knockout mutant R957TnpG. This is consistent with the Northern blot analysis result indicating that a functional OxyR is required to activate *katG* expression. It is worth noting that salt-induced *katG* promoter activity is OxyR-independent. In fact, *katG* expression is double in an *oxyR* mutant grown in the presence of high NaCl. In the case of *E. coli*, *katG*, in addition to being regulated by OxyR, is also a component of the *rpoS* regulon which has been shown to be activated by osmotic stress [20]. It is possible that *katG* in *B. pseudomallei* is also under control of both OxyR and RpoS thus explaining its activation by

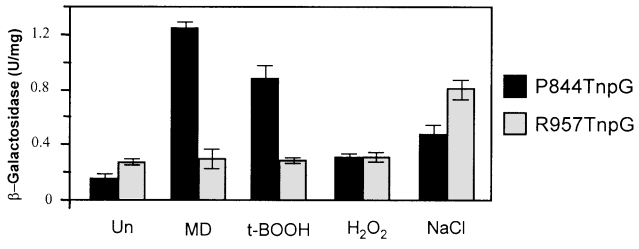

Fig. 2. Expression of the *katG* promoter in response to various stresses. β-Galactosidase activities in crude extracts of the *katG-lacZ* fusion parent (P844TnpG), and *oxyR* mutant (R957TnpG) prepared from uninduced cells (Un) and cells induced with menadione (MD), t-BOOH, H_2O_2, and NaCl. Each value shown is the mean of three separate experiments and error bars indicate S.E.M.

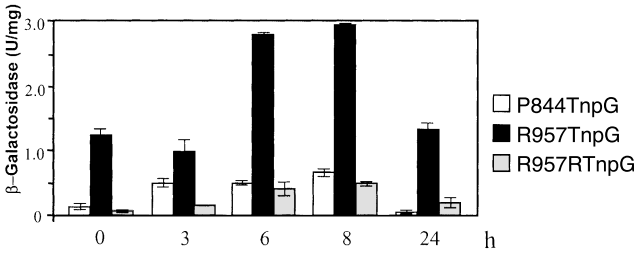

Fig. 3. Expression of *katG* promoter during growth. β-Galactosidase activities in crude extracts of a *katG-lacZ* fusion in parent (P844TnpG), *oxyR* mutant (R957TnpG), and the complemented strain (R957RTnpG). The enzyme activities were monitored and expressed as the mean of three separate experiments and error bars indicate S.E.M.

osmotic stress. Moreover, we observed that the *oxyR* mutant R957 exhibited a higher *katG* promoter activity during osmotic stress (this experiment, Fig. 2, Un) and higher basal *katG* mRNA expression during growth under uninduced conditions (Northern analysis, Fig. 1D) compared to the wild-type strain suggesting that OxyR represses *katG* transcription in the absence of oxidative stress.

3.4. katG *expression in wild-type and* oxyR *mutant during growth phase*

In order to determine whether OxyR functions as a repressor of *katG* transcription, β-galactosidase activities were monitored in wild-type (P844TnpG) and *oxyR* mutant (R957TnpG) backgrounds for 24 h (Fig. 3). Over the course of the experiment *katG* promoter activity was consistently higher in an *oxyR* mutant background (R957TnpG) compared to a wild-type background. Complementation of the *oxyR* mutant R957TnpG with a plasmid-borne *oxyR* in strain R957RTnpG reduced *katG* promoter activity to the level in the wild-type background. This finding suggests that OxyR, in addition to being an activator of *katG* when cells encounter oxidative threats, also acts as a repressor during the normal uninduced conditions. In *B. pseudomallei* P844, *katG* promoter activity reaches its highest level after 8 h of growth and then declines upon entry into stationary phase. This pattern is similar to that found in *E. coli* containing a *katG::lacZ* fusion. In *B. pseudomallei oxyR*, the expression of *katG* is higher than that of the wild-type in all phases of growth. This is in contrast to the observation in an *E. coli oxyR* mutant that *katG* expression is greatly reduced [21]. The results suggest that, unlike *E. coli* OxyR, *B. pseudomallei* OxyR represses *katG* expression during normal growth.

3.5. *Binding of both oxidized and reduced OxyR to* katG *promoter*

It has been shown in *E. coli* that during normal growth the bacterial cytosol is in a reduced state and that OxyR exists in a reduced form [22]. In the case of *Xanthomonas*, reduced OxyR was demonstrated to bind to and repress the *ahpC* promoter whereas oxidized OxyR activates *ahpC* expression [12]. We reported here that OxyR in untreated *B. pseudomallei* cells represses *katG* expression in vivo. This repression is reasonable and beneficial to keep the expression of *katG* low when cells are not exposed to an oxidative threat. We examined if purified OxyR protein can specifically bind to *katG* promoter. *B. pseudomallei* OxyR was purified to greater than 90% purity as judged by SDS–PAGE. OxyR was able to bind to the *katG* promoter under both oxidized and reduced conditions (Fig. 4). The differences in the pattern of binding of oxidized and reduced OxyR in the mobility shift may reflect dissimilar binding sites and affinities for the two forms. These data support our in vivo experiment that indicated that OxyR, in the absence of oxidative stress, represses *katG* expression. To our knowledge, repression of *katG* expression by OxyR has never been demonstrated in any bacterial system.

3.6. *Sensitivity of* katG *and* katG oxyR *mutants to various stresses*

The sensitivity of the *B. pseudomallei* P844 *katG* mutant G221 and the *katG oxyR* double mutant RG27 to several oxidants was measured. Both G221 and RG27 had increased sensitivity to H_2O_2, menadione, NEM, and hypochlorite (HOCl) killing compared to wild-type (P844) with RG27 having the highest sensitivity to these oxidants (Fig. 5). The altered sensitivities were restored to wild-type levels in the complemented strains G221TnG and RG27TnRG. KatG is a bifunctional enzyme having both catalase and peroxidase activities, therefore it was expected that the *katG* mutants would exhibit increased sensitivity to H_2O_2. Menadione, apart from being an intracellular superoxide generator, also causes a decreased total glutathione level, an increase in oxidized glutathione, and a decrease in the ratio of reduced to oxidized glutathione, thus shifting the cells to a more oxidized state and stimulating transcription of OxyR-inducible genes [23]. The thiol-alkylating agent NEM was reported to cause oxidative stress in human keratinocytes and, like menadione, rapidly depletes reduced glutathione [24]. Catalase protects *Xanthomonas* against electrophile toxicity of NEM [25]. HOCl exposure can generate hydroxyl radicals via a Fenton-type reaction and exponential-phase induction of the *oxyR* regulon protects *E. coli* cells against HOCl toxicity [26]. The fact that the lack of KatG expression causes *B. pseudomallei* to be more vulnerable to killing by different kinds of oxidants reflects the important roles *katG* plays in oxidative stress defense. Since catalase (KatA) of *Campylobacter jejuni* was found to be essential for its survival in macrophages [27], it would be interesting and of clinical importance to test if our mutants have an altered pattern of intramacrophage survival.

In conclusion, we described the genetic organization of *oxyR*, *katG*, and *dpsA* and their genetic regulation. Under uninduced conditions, both *katG* and *dpsA* are transcribed

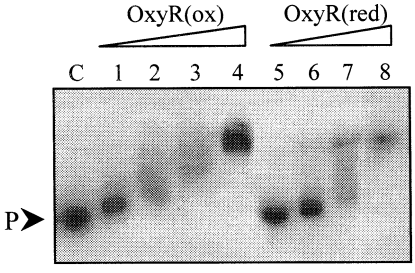

Fig. 4. Gel mobility shift assays of OxyR binding to the *katG* promoter region. To generate reduced conditions, 200 mM DTT was added to the binding reactions. ox and red indicate oxidized and reduced OxyR respectively. The following amounts of OxyR were added to the reactions: 75 ng (lanes 1 and 5), 150 ng (lanes 2 and 6), 250 ng (lanes 3 and 7) and 400 ng (lanes 4 and 8). P represents unbound probe. A labeled 540-bp DNA fragment containing the *katG* promoter was used as the probe.

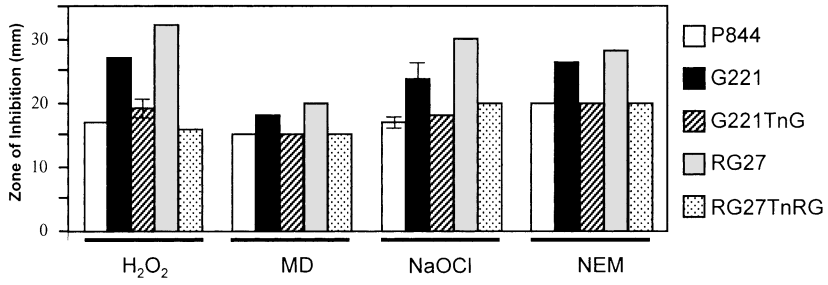

Fig. 5. Determination of levels of resistance to oxidative stress killing displayed by the *B. pseudomallei* parent strain P844, *katG* mutant G221, *katG oxyR* mutant RG27, and their complemented strains G221TnG and RG27TnRG. Determination of the levels of resistance to H_2O_2, menadione (MD), NEM, and NaOCl was performed as described in Section 2. Each value shown is the mean of three separate experiments and error bars indicate S.E.M.

monocistronically but when cells are exposed to oxidative stress both genes are activated and co-transcribed. OxyR is a dual function transcription regulator that represses *katG* expression under normal reduced conditions, but activates *katG* when cells encounter oxidative threats. KatG plays a significant protective role against various types of oxidants such as hydrogen peroxide, menadione, NaOCl, and NEM.

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The *oxyR* from *Agrobacterium tumefaciens*: evaluation of its role in the regulation of catalase and peroxide responses

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Abstract

The gene for *Agrobacterium tumefaciens* OxyR, a peroxide sensor and transcriptional regulator, was characterized. Phylogenetic analysis of bacterial OxyR showed that the protein could be divided into four clades. The *A. tumefaciens* OxyR grouped in clade III that consists primarily of OxyRs of Alphaproteobacteria displayed the highest homology to OxyR from *Rhizobium leguminosarum*. *oxyR* is located next to, and is divergently transcribed from, a bifunctional catalase-peroxidase gene (*katA*). An *A. tumefaciens oxyR* mutant was constructed and shown to be hyper-sensitive to H_2O_2, but not to the superoxide generator, menadione, or an organic hydroperoxide. Exposure of *A. tumefaciens* to H_2O_2 resulted in induction of the catalase-peroxidase enzyme. This induction was abolished in the *oxyR* mutant. In vivo analysis of a *katA::lacZ* promoter fusion confirmed the results of enzyme assays and indicated that induction of the *katA* promoter by H_2O_2 was dependent on functional OxyR. We also examined the regulation of *oxyR* in *A. tumefaciens*. Exposure to H_2O_2 did not induce expression of the gene but simply changed OxyR from a reduced to an oxidized form. The in vivo *oxyR* promoter analysis showed that the promoter was auto-regulated and that transcription was not induced by H_2O_2.

Keywords: *Agrobacterium tumefaciens*; Adaptation; Catalase; H_2O_2; Organic peroxide; *oxyR*

Agrobacterium tumefaciens is a soil-borne plant pathogenic bacterium causing crown gall tumors in many dicotyledonous plants. The bacteria are also widely used as a tool to generate genetically engineered plants [1]. During interaction with plants and aerobic respiration, *Agrobacterium* is exposed to reactive oxygen species that have to be rapidly detoxified if the bacterium is to survive.

Bacteria have evolved multiple systems to protect themselves from ROS, some of which are regulated by OxyR, a global regulator for the peroxide stress response. The protein is a bifunctional protein that acts both as a peroxide sensor and a transcriptional regulator in response to peroxide stress [2,3]. In *Escherichia coli* and other bacteria, OxyR regulates many genes involved in detoxification (*katG*, *ahpC*, and *ahpF*,) and protection (*dps*, *gor*, *grxA*, and *trxC*). OxyR is a tetrameric protein that can be reversibly oxidized, resulting in the formation of disulfide linkages between Cys-199 and Cys-208 in the presence of H_2O_2 [3]. The reduced and oxidized forms bind differently to the regulated promoter but only the oxidized form activates gene expression [3,4]. Inactivation of *oxyR* in many bacteria often results in increased sensitivity to peroxides and other oxidants [5–9].

Although *Agrobacterium* is extensively used in plant gene manipulation, our knowledge concerning its ability to survive under oxidative stress conditions is still limited. Recently, the essential role of *A. tumefaciens* catalase (KatA) as a virulence factor involved in tumorigenesis on its host plant has been reported [10,11]. In this communication, we report characterization of *oxyR* and the physiological analysis of a constructed *oxyR* insertion mutant. The regulation of *katA* by OxyR and auto-regulation of *oxyR* were demonstrated.

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Materials and methods

Bacterial growth conditions. Agrobacterium tumefaciens NTL4 [12] and the mutant strains were grown aerobically in LB medium at 30 °C with continuous shaking at 150 rpm. To ensure synchronous growth, overnight cultures were inoculated into fresh LB medium to give an OD_{600} of about 0.1. Exponential phase (OD_{600} about 0.6, after 4 h of growth) cells were used in all experiments, as indicated. For Northern blot analysis and enzymatic assays, the exponential phase cultures were induced with sublethal concentrations of H_2O_2 (250 μM), menadione (200 μM), or tBOOH (250 μM) for 15 and 30 min, respectively, before the cells were harvested.

Molecular biology techniques. General molecular genetics techniques including genomic DNA preparation, plasmid preparation, RNA preparation, restriction endonuclease digestion, ligation, transformation in *E. coli*, agarose, and polyacrylamide gel electrophoresis, as well as Northern blot analysis were performed using standard protocols [13]. The labeling of DNA probes with [α-^{32}P]dCTP was performed using a DNA labeling bead (Amersham Pharmacia Biotech). Plasmid purification for DNA sequencing was prepared using Qigen Meniprep. DNA was sequenced in both orientations by the primer walking technique using a BigDye terminator cycle sequencing kit (PE Biosystems) on an ABI 310 automated DNA sequencer. Routinely, *A. tumefaciens* was transformed by electroporation under conditions previously described [12].

Construction of an oxyR mutant. Two primers designed from the sequence of a putative *oxyR* gene, identified from the *A. tumefaciens* C58 genome sequence [14], BT521-5′ATCAGCACGCGAGGCGGC3′ and BT522-5′GGTGACGCAGAAGCTCAT3′ (Fig. 1), were used to amplify a 200-bp *oxyR* fragment using *A. tumefaciens* NTL4 genomic DNA as templates. The PCR product was cloned into pGEM-T-easy (Promega) and its nucleotide sequence was determined. Subsequently, the *Sac*II–*Sa*II fragment of the PCR clone was subcloned into pKNOCK-Gm [15], a non-replicative plasmid in *Agrobacterium*, cut with the same restriction enzymes. The resultant plasmid, pKNOCK-oxyR, was then transferred to *A. tumefaciens* by conjugation. Recombination of the cloned *oxyR* fragment in the suicide plasmid with the homologous counterpart on *A. tumefaciens* chromosome resulted in the disruption of *oxyR* gene. The putative mutants were selected for a Gm^R and Ap^R phenotype and screened by PCR with gene specific primers and Southern blot hybridization.

Cloning of oxyR. The full-length *oxyR* gene was amplified from *A. tumefaciens* genomic DNA with two primers, BT582-5′CGAAGCCA TTACGGCGCGA3′ and BT583-5′TAAAGGCTGCGATATGCTG3′ (Fig. 1). The 0.9 kb PCR product was cloned into pDrive cloning vector (Qiagen) before determining its nucleotide sequence and subcloning into the broad host range plasmid pBBR1MCS-4 [16] to form plasmid pOxyR.

Cloning of katA and oxyR promoter fragment. The putative *katA* and *oxyR* promoter region was amplified using primers BT584-5′GC CAGCGCATCGAAATAAC3′ and BT585-5′CCGATTTGCCGGAG GCCGA3′ (Fig. 1). The 330 bp product was cloned into pDrive (Qiagen). After checking its nucleotide sequence, the DNA fragment was digested with *Eco*RI and cloned into the promoter probe vector, pUFR027*lacZ*, a derivative of pUFR027 [17], cut with the same enzyme. Since the *katA* and *oxyR* promoters overlap and function divergently from one another, we used the same DNA fragment (but in a different orientation) to construct both promoter fusions and the resultant plasmids were named pP_{katA} and pP_{oxyR}, respectively. The orientation of each promoter was checked by PCR using two specific primers, one located in the DNA fragment and the other within the vector sequence.

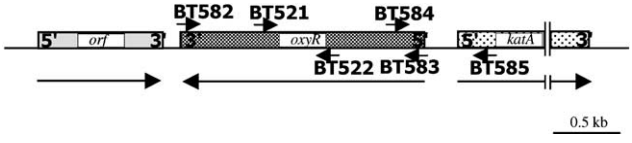

Fig. 1. Gene and transcriptional organization of the *oxyR* locus. The physical map of *A. tumefaciens oxyR*, small arrows indicate the positions of primers and the long arrows indicate the direction of transcription.

Alignments and phylogenetic analyses. Amino acid sequences of OxyR proteins were retrieved from public sequence databases using the BLAST program [18]. The alignments were performed by using the multiple alignment feature of CLUSTAL W version 1.7 [19] with maximal fixed-gap and gap extension penalties. A phylogenetic tree was constructed by the neighbor-joining method using the TREE program from the phylogenetic analysis page of D.L. Robertson, E. Beaudoing, and J.M. Claverie (at http://igsserver.cnrss-mrs.fr/anrs/ phylogenetic). The analysis results were displayed using the program PHYLODENDRON, version 0.8d (D.G. Gilbert, Department of Biology, University of Indiana, USA at http://iubio.bio.indiana.edu).

Determination of oxidant resistance by inhibition zone measurement. Analysis of the killing effects of various reagents on *A. tumefaciens* strains was done by using an inhibition zone assay [5]. Briefly, 1 ml log phase cells were mixed with 10.0 ml molten top agar (LB containing 0.7% agar) pre-warmed at 50 °C and overlaid onto LB plates (14-cm-diameter petri dishes containing 40 ml LB agar). The plates were left at room temperature for 15 min to let the top agar solidify. Sterile 6 mm-diameter discs (prepared from Whatman filter paper no. 3) soaked with either 5 μl of 1.0 M H_2O_2, 1.0 M *tert*-butyl hydroperoxide (tBOOH), 1.0 M menadione (MD) or 200 mM *N*-ethyl maleimide (NEM) were placed on the cell lawn and zones of growth inhibition were measured after 24 h of incubation at 30 °C.

Enzyme activity assays. Preparation of crude bacterial lysates and protein assays were performed as previously described [5]. Briefly, 20 ml cultures were pelleted and washed once with 50 mM sodium phosphate buffer, pH 7.0 (PB). Bacterial suspensions in PB containing 1 mM PMSF, a protease inhibitor, were lysed by brief sonication followed by centrifugation at 10,000*g* for 10 min. Clear lysates were used for enzyme assays and total protein determination. β-Galactosidase was assayed as described earlier and expressed in Miller units [20]. Superoxide dismutase (SOD), glutathione reductase, glucose-6-phosphate dehydrogenase, and catalase activity was monitored as described previously [21]. One unit of the antioxidant enzymes was defined as the amount of enzyme capable of catalyzing the turnover of 1 μmol of substrate per minute under the assay conditions.

Results and discussion

Genome organization of A. tumefaciens oxyR

Analysis of the genome sequence of *A. tumefaciens* C58 [14] using the BLAST program [18] revealed an open reading frame encoding a predicted protein of 302-amino acids (AAL45435, Fig. 1), showing a high score of identity to known OxyR proteins from *Rhizobium leguminosarum* (57%), *Streptomyces coelicolor* (39%), *E. coli* OxyR (38%), *Xanthomonas campestris* (37%), and *Burkholderia pseudomallei* (34%). A phylogenetic tree, constructed using OxyR sequences from various bacteria, showed that OxyR could be classified into 4 clades (Fig. 2). Clade I consisted of OxyR from Actinobacteria such as *Mycobacterium* spp., *S. coelicolor*, and *Corynebacterium glutamicum*, clade II was composed of OxyRs mostly from Alphaproteobacteria including *Caulobacter crescentus* and the Rhizobiaceae such as *R. leguminosarum*,

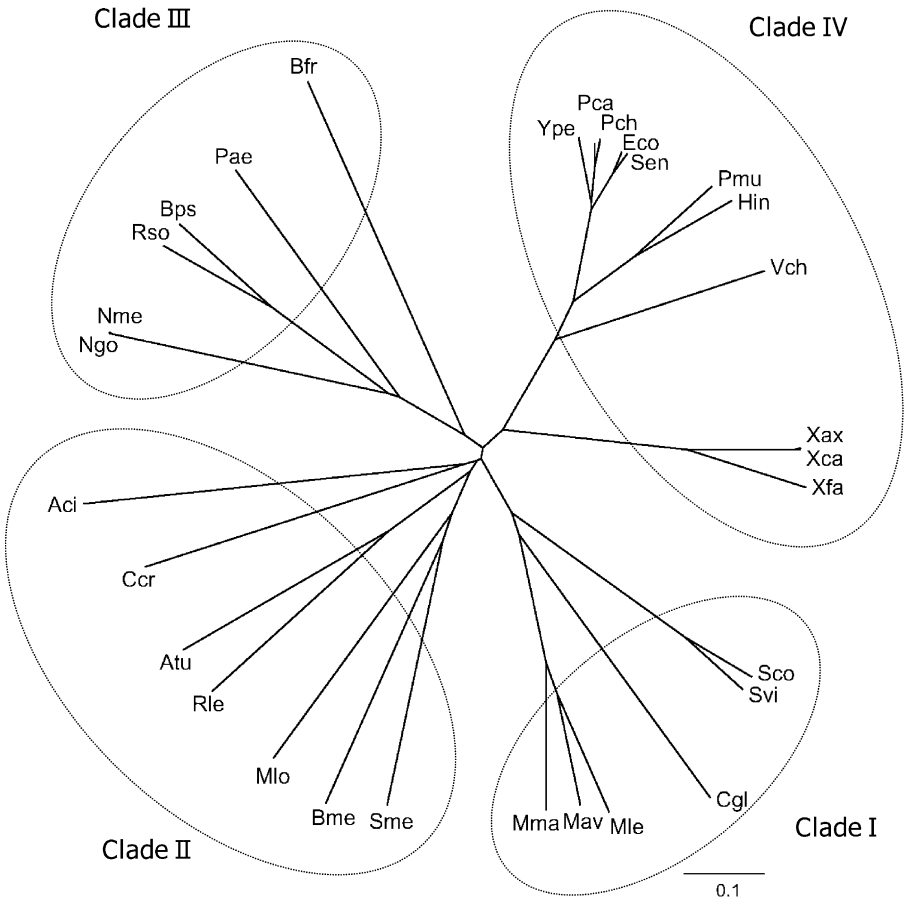

Fig. 2. Phylogenetic tree constructed from OxyR sequences from various microorganisms. Aci, *Acinetobacter* sp. CAA86928; Atu, *Agrobacterium tumefaciens* AAK88806; Bfr, *Bacteroides fragilis* AAG02620; Bme, *Brucella melitensis* biovar Abortus AAD00508; Bps, *Burkholderia pseudomallei* AAK72465; Ccr, *Caulobacter crescentus* AAK25659; Cgl, *Corynebacterium glutamicum* CAD32032; Eco, *Escherichia coli* X52666; Hin, *Haemophilus influe* nzae NP_438728; Mav, *Mycobacterium avium* AAA79918; Mle, *Mycobacterium leprae* P52678, Mlo, *Mesorhizobium loti* BAB53129; Mma, *Mycobacterium marinum,* AAC61302; Ngo, *Neisseria gonorrhoeae* AF514857; Nme, *Neisseria meningitidis* AAF40630; Sen, *Salmonella enterica* subsp. enterica NP_457935; Pae, *Pseudomonas aeruginosa* AE004946; Pca, *Pectobacterium carotovorum* AAC72241; Pch, *Pectobacterium chrysanthemi* Q9X725; Pmu, *Pasteurella multocida* NP_246285; Rle, *Rhizobium leguminosarum* bv. phaseoli CAD27227; Rso, *Ralstonia solanacearum* NP_520811; Sco, *Streptomyces coelicolor* NP_629185; Sme, *Sinorhizobium meliloti* NP_384869; Svi, *Streptomyces viridosporus* AAD25084; Vch, *Vibrio cholerae* AAF95777; Xax, *Xanthomonas axonopodis* pv. *citri* AAM35793; Xca, *Xanthomonas campestris* pv. *phaseoli* AAC45427; Xfa, *Xylella fastidiosa* A82669; and Ype, *Yersinia pestis* CAC93381.

A. tumefaciens, *Mesorhizobium loti*, and *Sinorhizobium meliloti*. An unusual exception is that *Acinetobacter* sp., a member of the Betaproteobacteria, was classified in clade II. Most of the members of clade III are from the Betaproteobacteria such as *Neisseria* spp., *Ralstonia solanacearum*, and *B. pseudomallei*. OxyRs from the Gammaproteobacteria, *Pseudomonas aeruginosa*, and from the anaerobic bacterium *Bacteroides fragilis* were also classified in clade III. Clade IV contained OxyR from the Gammaproteobacteria including several genera in the family Enterobacteriaceae, Pastuellaceae, and from *Xanthomonas*, *Xylella*, and *Vibrio* sp. Amino acid sequences of all OxyRs showed absolute conservation at two cysteine residues, namely Cys-199 and Cys-208, which are thought to be involved in activation of the protein by oxidation [3].

Analysis of the sequence surrounding *Agrobacterium oxyR* indicated that the gene was located next to *katA* [11] encoding a bifunctional catalase-peroxidase (Fig. 1). *katA* has been shown to be important in tumorigenesis of *A. tumefaciens* inside the host plant [11]. *oxyR* and *katA* were transcribed divergently from one another and separated by 208 nucleotides (Fig.1). Downstream of *oxyR* was an unidentified *orf* of 316 codons in length.

Examination of the genes surrounding *oxyR* in different bacteria indicated that the majority of genes located adjacent to *oxyR* are involved in oxidative stress protection, such as *ahpC* [6,22], *kat* [8,23], *dps* [24], and the regulatory RNA *oxyS* [25], and are regulated by OxyR. The position of *A. tumefaciens oxyR* is similar to that of *B. pseudomallei*, in that *oxyR* is located next to *katG* encoding a bifunctional catalase-peroxidase [9].

However, unlike *B. pseudomallei katG*, *A. tumefaciens katA* is transcribed in the opposite direction to *oxyR* [9].

Construction and characterization of the oxyR mutant

An *oxyR* mutant (designed PN03) was constructed using insertional inactivation of the gene performed as described in Materials and methods. In many bacteria, inactivation of *oxyR* has resulted in pleiotropic changes in growth and oxidative stress responses [5,6,8,9]. We examined the growth rate of the PN03 and the parental strain in either a complex or a minimal media and found no difference in the two strains. Furthermore, the PN03 did not show any defect in aerobic plating efficiency (data not shown). Next, the levels of resistance against various oxidants in the PN03 and the parental strain were determined using the inhibition zone method. For the parental strain, the zones of growth inhibition for H_2O_2 (1.0 M), menadione (1.0 M), tBOOH (1.0 M), and NEM (200 mM) were 13, 18, 22, and 28 mm, respectively, compared to zones of 23, 17, 23, and 28 mm, respectively, for the PN03 (Fig. 3). The PN03 showed increased sensitivity to H_2O_2 but not to other oxidants, including the superoxide generator, menadione, and the thiol-depleting agent, NEM. Expression of the functional *oxyR* in the PN03 was able to complement the H_2O_2 hypersensitive phenotype, since the extent of the zone of growth inhibition to H_2O_2 of the complemented strain (PN03/pOxyR) was similar to that of the parental strain. Also, high level expression of *oxyR* from the expression vector did not confer increased resistance to H_2O_2 (Fig. 3).

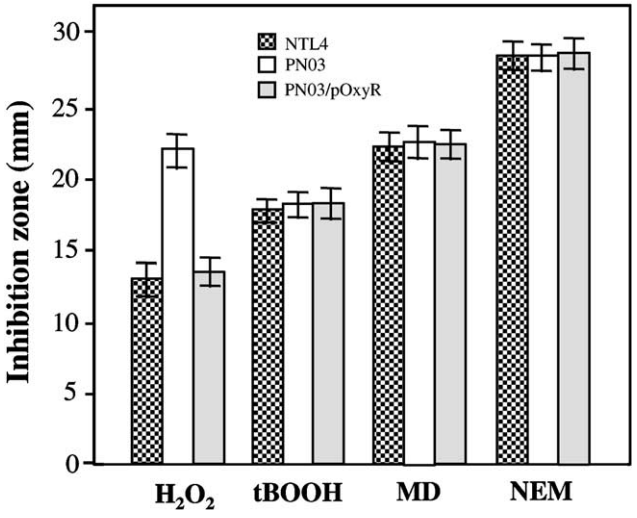

Fig. 3. Determination of the levels of resistance to killing concentrations of oxidants in the *A. tumefaciens* PN03, PN03 harboring pOxyR, and the parental strain NTL4. The resistance levels to oxidants of the *A. tumefaciens* PN03, the *oxyR* mutant complemented with *oxyR* on the expression vector (PN03/pOxyR), and the NTL4 were measured by zones of growth inhibition around paper discs soaked with H_2O_2, tBOOH, menadione (MD), and NEM as described in Materials and methods. Values are means and SD of four replicates.

By contrast to *E. coli*, *Xanthomonas*, and other bacterial *oxyR* mutants, the *A. tumefaciens* PN03 showed no significant increase in sensitivity to organic peroxide (tBOOH) killing. A possible explanation for this is that analysis of *A. tumefaciens* genome sequence [14] did not show any ORF with a high degree of sequence identity to alkyl hydroperoxide reductase (AhpC), the well-characterized organic peroxide protective enzyme under the regulation of OxyR (data not shown). This suggested that *Agrobacterium* uses other organic hydroperoxide protective systems that are not under OxyR regulation.

We further examined the effect of *oxyR* inactivation on the levels of enzymes involved in oxidative stress protection. The superoxide dismutase (SOD, *sod*), glucose-6-phosphate dehydrogenase (G6PD, *zwf*), and glutathione reductase (GR, *gor*) activities were measured in the PN03 and parental strains. As shown in Table 1, the levels of SOD, G6PD, and GR were not significantly different. Additionally, high expression of *oxyR* from pOxyR did not affect the levels of these antioxidant enzymes. These results indicate that *oxyR* is not involved in the regulation of *sod*, *zwf*, and *gor* expression. The findings are consistent with the phenotypic analysis that showed no significant alteration in resistance levels to the superoxide generator, NEM, in the PN03.

Regulation of catalase-peroxidase by oxyR

The fact that the PN03 was more sensitive to H_2O_2 than its parental strain suggested that *oxyR* might be involved in controlling the expression of *kat*. Experiments were performed to determine the effect of H_2O_2 pre-treatments on the total catalase levels in the *A. tumefaciens* NTL4 and the PN03. As it was previously observed, pretreatment of *A. tumefaciens* NTL4 with H_2O_2 induced high catalase activity (Table 1). This induction was not observed in the PN03 (Table 1). This result is consistent with the notion that OxyR is acting as a peroxide sensor and a transcriptional activator of catalase in *A. tumefaciens*. This feature of OxyR is highly conserved in many bacteria [5,7–9], with only one reported exception in *Streptomyces coelicolor* [6].

Analysis of the *A. tumefaciens* genome sequence using the BLAST program [18] revealed two putative open reading frames identified as encoding a monofunctional catalase CatE (ALL46177) and a bifunctional catalase-peroxidase KatA (AAL45436) that is located next to and divergently transcribed from *oxyR*. CatE shows high degree of identity to atypical catalase including a σ^S-regulated *E. coli* KatE (48%) [26]. *A. tumefaciens* KatA whose expression could be induced by plant tissue sections and by acidic pH has been cloned and characterized [10,11]. We hypothesized that the increased level

Table 1
Determination of antioxidant enzymes in *A. tumefaciens* NTL4 and the PN03

Strains/conditions	Enzyme activity				
	Catalase (U/mg)	Peroxidase (mU/mg)	SOD (U/mg)	G6PD (mU/mg)	GR (mU/mg)
A. tumefaciens NTL4					
Uninduced	7.1 ± 2.1	5.7 ± 1.8	0.26 ± 0.1	55.0 ± 5.5	10.5 ± 2.0
H_2O_2-induced (250 μM)	22.6 ± 5.5	10.5 ± 2.7	0.27 ± 0.05	60.5 ± 6.5	9.4 ± 2.5
tBOOH-induced (250 μM)	7.4 ± 2.5	5.9 ± 2.3	0.27 ± 0.05	62.0 ± 6.5	10.0 ± 1.5
A. tumefaciens PN03					
Uninduced	7.0 ± 1.5	3.9 ± 1.5	0.25 ± 0.03	60.0 ± 8.5	10.5 ± 1.5
H_2O_2-induced (250 μM)	6.7 ± 1.5	3.4 ± 1.0	0.24 ± 0.05	55.0 ± 7.5	10.2 ± 2.0
tBOOH-induced (250 μM)	7.1 ± 1.5	4.0 ± 1.2	0.25 ± 0.50	63.0 ± 6.5	11.0 ± 2.5

of catalase induced by H_2O_2 was due to elevated levels of KatA. To prove this hypothesis, total peroxidase activity in crude lysates of *A. tumefaciens* NTL4 and the PN03 strains, induced with H_2O_2, was monitored. As expected, the pattern of peroxidase induction in both strains was identical to that for catalase (Table 1). These data suggest that increased levels of catalase and peroxidase, induced by H_2O_2 pretreatment, are due to increased expression of *katA* and this induction is mediated by OxyR.

The total catalase activities from uninduced *A. tumefaciens* NTL4 and the PN03 (Table 1) were not significantly different. However, measurement of basal peroxidase levels in the mutant was approximately 30% lower than that of the parental strain. These findings suggest that *katA* expression was actually reduced in the *oxyR* mutant. Furthermore, they implied that there was a compensatory increase in catalase activity in response to the decrease in KatA levels that is most likely due to increased expression of CatE. We do not know the nature of the regulatory process governing this compensatory catalase response. A similar compensatory increase in the activity of KatE resulting from lower levels of a KatA isozyme has been observed in *Xanthomonas* [27].

In vivo promoter analysis of katA

The putative *katA* promoter region was transcriptionally fused to a promoter-less *lacZ* in a low-copy-number plasmid vector, pUF027*lacZ* [17], to give pP_{katA}. The recombinant plasmid was transferred into both *A. tumefaciens* NTL4 and the PN03 and the levels of β-galactosidase were determined under uninduced and H_2O_2 induced conditions. The results are shown in Fig. 4A. The β-galactosidase activity from *A. tumefaciens* bearing pP_{katA} was increased about 2-fold when cells were pre-challenged with H_2O_2. This induction did not occur in the PN03 *oxyR* mutant harboring pP_{katA}. The findings affirmed that *katA* expression is regulated by OxyR. Additionally, basal β-galactosidase activity from the *A. tumefaciens* PN03 *oxyR* mutant was lower than

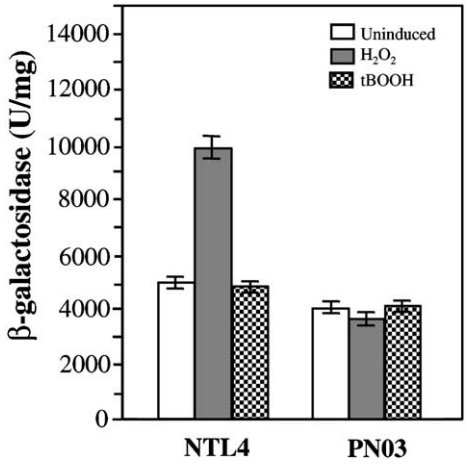

Fig. 4. In vivo *katA* promoter characterization. β-Galactosidase activity of *A. tumefaciens* NTL4 and the PN03 harboring pP_{katA}. Cells were harvested from cultures that were either uninduced or induced with either H_2O_2 or tBOOH. Values are means and SD of four replicates.

that of the *A. tumefaciens* NTL4 parental strain, supporting the conclusion that inactivation of *oxyR* reduces *katA* expression (Fig. 4).

Expression analysis of oxyR

Upon exposure to H_2O_2, OxyR is converted from a reduced to an oxidized form in *E.coli* and many other bacteria [4,28]. Also, during exposure to H_2O_2 there is no accompanying change in the OxyR concentration. This had been generally accepted until we reported in *Xanthomonas* that, upon exposure to oxidants, OxyR not only changes form from reduced to oxidized but also increased in concentration [29]. Similar observations have been made in *B. pseudomallei* and *S. coelicolor* [6,9]. Thus, we determined the level of *oxyR* expression in response to oxidative stress using Northern blot analysis. Total RNA prepared from exponential phase cells induced with H_2O_2, menadione, or tBOOH was separated and immobilized on a nylon membrane. The blot was probed with ^{32}P-labeled *oxyR* probe revealing a

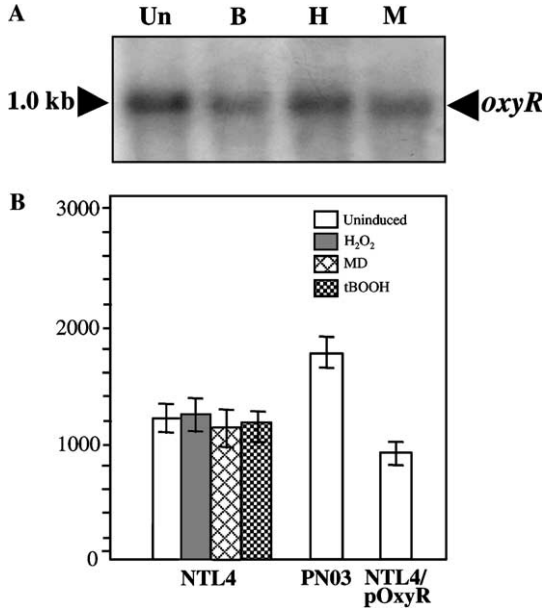

Fig. 5. In vivo regulation of the *oxyR* promoter. In (A) Northern blot analysis of *oxyR* in response to oxidant pre-treatments. Total RNA was prepared from *A. tumefaciens* induced with tBOOH (B), H_2O_2 (H), menadione (M), and an uninduced culture (Un) was separated and probed with *katA* as described in Materials and methods. In (B) β-galactosidase activity of *A. tumefaciens* NTL4, the PN03, and NTL4 containing the *oxyR* expression plasmid pOxyR (NTL4/pOxyR), all of which contain the *oxyR* promoter fusion plasmid pP_{oxyR}. Cells were cultured in uninduced, induced with H_2O_2, menadione (MD), or tBOOH. Values are means and SD of four replicates.

single hybridizing mRNA of about 1.0 kb in length, suggesting that *A. tumefaciens oxyR* was transcribed as a monocistronic mRNA (Fig. 5A). Quantitation of mRNA levels revealed that exposure of *A. tumefaciens* to H_2O_2, menadione, or organic hydroperoxides did not increase expression of *oxyR* (Fig. 5A). Thus, *oxyR* is constitutively expressed. This favors the idea that in *A. tumefaciens*, exposure to H_2O_2 results in OxyR simply changing from its reduced to oxidized form, with no change in its concentration. This finding also explained the observation that increased OxyR concentrations in cells harboring pOxyR did not show significantly increased levels of oxidative stress resistance (Fig. 3). There is no correlation between the concentration of OxyR and the oxidant resistance levels.

In vivo promoter analysis of oxyR

OxyR belongs to the LysR family of transcriptional regulators [4]. One of the common features of genes in this family is that they are all autoregulated. OxyR can act either as a transcriptional activator or repressor depending on its oxidation state and the target promoter [28,30]. The in vivo regulation of the *oxyR* promoter was investigated using the *oxyR* transcriptional promoter fusion plasmid pP_{oxyR}. The levels of β-galactosidase activity were determined in *A. tumefaciens* NTL4, the PN03 harboring pP_{oxyR}, the PN03 harboring pP_{oxyR} plus either pBBR1MSC-4 (vector control), and or pOxyR (carrying a functional *oxyR*) were determined. The results in Fig. 5B clearly showed that the lack of functional *oxyR* (in the PN03) resulted in increased *oxyR* transcription while high OxyR levels (in *A. tumefaciens* NTL4 harboring pOxyR) repressed it. These data suggest that OxyR is autoregulating its own expression. We also investigated the effect of oxidant pre-treatment on the transcription level of the *oxyR* promoter in *A. tumefaciens* NTL4 bearing pP_{oxyR}. The results showed that exposure of cells to inducing concentration of H_2O_2, menadione or tBOOH did not induce high expression of *oxyR* (Fig. 5B). These data are in good agreement with the Northern blot analysis in Fig. 5A. Interestingly, the *oxyR* promoter activity, at physiological uninduced conditions, was about 4 times lesser than that of the *katA* promoter (Figs. 4 and 5B), suggesting that *oxyR* was transcribed at a comparatively low level.

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Induction of peroxide and superoxide protective enzymes and physiological cross-protection against peroxide killing by a superoxide generator in *Vibrio harveyi*

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Abstract

Vibrio harveyi is a causative agent of destructive luminous vibriosis in farmed black tiger prawn (*Penaeus monodon*). *V. harveyi* peroxide and superoxide stress responses toward elevated levels of a superoxide generated by menadione were investigated. Exposure of *V. harveyi* to sub-lethal concentrations of menadione induced high expression of genes in both the OxyR regulon (e.g., a monofunctional catalase or KatA and an alkyl hydroperoxide reductase subunit C or AhpC), and the SoxRS regulon (e.g., a superoxide dismutase (SOD) and a glucose-6-phosphate dehydrogenase). *V. harveyi* expressed two detectable, differentially regulated SOD isozymes, [Mn]-SOD and [Fe]-SOD. [Fe]-SOD was expressed constitutively throughout the growth phase while [Mn]-SOD was expressed at the stationary phase and could be induced by a superoxide generator. Physiologically, pre-treatment of *V. harveyi* with menadione induced cross-protection against subsequent exposure to killing concentrations of H_2O_2. This induced cross-protection required newly synthesized proteins. However, the treatment did not induce significant protection against exposures to killing concentrations of menadione itself or cross-protect against an organic hydroperoxide (*tert*-butyl hydroperoxide). Unexpectedly, growing *V. harveyi* in high-salinity media induced protection against menadione killing. This protection was independent of SOD induction. Stationary-phase cells were more resistant to menadione killing than exponential-phase cells. The induction of oxidative stress protective enzymes and stress-altered physiological responses could play a role in the survival of this bacterium in the host marine crustaceans.

Keywords: Catalase; Menadione; Superoxide dismutase; Superoxide; Oxidative response; *Vibrio harveyi*

1. Introduction

Vibrio harveyi is a luminous bacterium commonly found as part of the normal flora in light organs of certain marine cephalopods and in the intestine of tropical marine animals. It is also recognized throughout Asia and Australia as a destructive pathogen of cultured marine animals, and particularly in the farmed black tiger prawn *Penaeus monodon* [1–3].

Reactive oxygen species (ROS) are important components of the defense response generated by crustacean hemocytes against invading microorganisms [4,5]. At the same time, environmental degradation and contamination of natural waters with herbicides and chemical residues that are strong oxidants could modulate the bacterial oxidative stress response making bacteria more resistant to such stress. Thus, it is possible that environmental exposure to strong oxidants could alter disease progression and outcomes in crustaceans. Most ROS including superoxide anions, peroxides and hydroxyl radicals are highly toxic and can cause damage to macromolecules. In order to colonize and proliferate in a host, bacteria must protect themselves from such ROS by detoxification and they have multiple, appropriate defense responses that can be adjusted according to nature and amount of ROS. For example, *Escherichia coli* has independent multigene responses to at least two types of oxidative stress. Excess H_2O_2 triggers the expression of genes in the OxyR regulon, including those for catalase and alkyl hydroperoxide

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reductase. Excess superoxide radicals trigger the SoxRS regulon that coordinates the transcriptional induction of many promoters including those for a manganese containing superoxide dismutase ([Mn]-SOD) gene and a glucose-6-phosphate dehydrogenase (G6PD) gene [6,7]. SODs are essential enzymes for aerobic organisms. They are important for the maintenance of cells and DNA integrity during aerobic metabolism since they eliminate superoxide radicals that induce lipid damage and oxidative inactivation of essential enzymes. More importantly, SOD prevents the formation of the highly toxic and mutagenic hydroxyl radicals, the product of superoxide radicals, reduced iron and H_2O_2 [8].

Defense against ROS is an important mechanism for bacteria to survive in stressful environments. We have previously shown that prior exposure of *V. harveyi* to sublethal concentrations of peroxide resulted in a several fold increase in expression of OxyR-regulated catalase and alkyl hydroperoxide reductase genes [9]. Here, we describe the effects of a superoxide generator menadione on induction of peroxide scavenging and superoxide protective enzymes and on menadione-induced cross-protection to peroxide killing.

2. Materials and methods

2.1. Bacterial growth conditions

A *V. harveyi* strain pathogenic to shrimp [3] was grown aerobically in LBS medium (Luria–Bertani supplemented with 2%, w/v NaCl) at 30°C. To ensure uniform growth, overnight cultures were inoculated into fresh LBS medium to give an OD_{600} of 0.1. By subsequent monitoring of bacterial growth spectrophotometrically at OD_{600}, exponential-phase cells (OD_{600} 0.8, were obtained at approximately 3 h) and stationary-phase cells (OD_{600} 5.5, at approximately 18 h cultivation) were used in experiments.

2.2. Quantitative determination of resistance levels to oxidants

Induced adaptive and cross-protection response experiments were performed by adding sub-lethal concentrations of menadione (400 μM), H_2O_2 (250 μM) or *tert*-butyl hydroperoxide (tBOOH; 200 μM) to exponential-phase cultures. The cultures were then allowed to grow for an additional 30 min before aliquots of cells were treated with killing concentrations of menadione (200, 300 and 400 mM), H_2O_2 (100, 200, and 300 mM) or tBOOH (100, 150, and 200 mM). After 30 min treatment, samples were pelleted and washed once with fresh medium before appropriate dilutions were plated on LBS agar plates. Cells that survived various treatments were counted after 24 h incubation at 30°C. Surviving fractions are defined as the number of colony forming units (cfu) obtained after the treatment divided by cfu prior to treatment. All experiments were repeated at least three times and representative data are shown.

2.3. Catalase activity gels and assays

Crude bacterial lysates were prepared by resuspending *V. harveyi* cell pellets in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride and exposing them to intermittent sonication on ice until the suspensions become clear. The lysates were then centrifuged at $10\,000 \times g$ for 10 min before being used for enzyme assays and activity gel staining. Total catalase activity was spectrophotometrically measured by the disappearance of H_2O_2 at A_{240} [10]. Catalase activity was visualized on 7.5% non-denaturing polyacrylamide gels prepared as previously described [9].

2.4. SOD activity gels and assays

Xanthine–xanthine oxidase coupled reduction of cytochrome *c* was used to monitor total SOD activity [11]. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%. To visualize SOD activity, non-denaturing electrophoresis was performed using a 10% polyacrylamide gel (pH 8.7) with a 5% stacking gel (pH 8.0) followed by staining with nitroblue tetrazolium/riboflavin photochemical stain as described by Beauchamp and Fridovich [12]. In the enzyme inhibition tests H_2O_2 or potassium cyanide were added to a riboflavin–TEMED solution at final concentrations of 5 and 2 mM, respectively. [Cu,Zn]-SOD is inactivated by cyanide and [Fe]-SOD by H_2O_2 while [Mn]-SOD is resistant to both [13].

2.5. Western immunoblot analysis

For Western immunoblot analysis of AhpC levels, conditions of SDS–PAGE, blotting to PVDF membranes and immunodetection against anti-*Escherichia coli* AhpC polyclonal antibody were performed as previously described [9]. Antibody reactions were developed using an alkaline phosphatase antibody detection kit from Promega (Madison, WI, USA).

2.6. Nucleic acid manipulations

All nucleic acid manipulations were performed according to standard molecular biology techniques [14] or to manufacturers' recommendations. The labelling of the DNA probes with [α-^{32}P]dCTP was performed using a DNA labelling bead (Amersham Pharmacia Biotech). A DNA fragment of *soxR* coding region from *V. harveyi* was amplified by polymerase chain reactions (PCR). Two degenerated oligonucleotide primers [BT133 5′-GTTTC(AT)GC(AT)CT(AT)CA(CT)TT(CT)TA(CT)GA-

3′ and BT134 5′-CA(AG)CCACA(AG)CC(AG)AT(AG)-CA(GT)CC-3′] were designed based on two conserved regions, VSALHFY and GCIGCGC, of the SoxR (accession number AAF95998) obtained from the genome sequence of *Vibrio cholerae* [15]. A primer pair, genomic DNA of *V. harveyi*, a PCR reaction mix and 2 U of *Tag* polymerase were mixed and amplified for 30 cycles under the following conditions: denaturation at 96°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The 300-bp PCR product was cloned into pGem-T-easy and its nucleotide sequences were determined. Alignment of amino acid sequences was performed using CLUSTAL W program [16].

3. Results and discussion

3.1. Analysis of SOD isozymes during different phases of growth

Analysis of genomic DNA sequences from the GenBank database of a closely related bacterium, *V. cholerae* [15] using BLAST program [17] showed three possible open reading frames with homology to [Mn]-SOD (accession no. AAF95835), [Fe]-SOD (accession no. AAF95193) and [Cu,Zn]-SOD (accession no. AAF94737). This suggested that *V. harveyi* might also possess more than one SOD isozymes. Thus, experiments were done to test for different SOD isozymes using cell lysates prepared at various growth phases of *V. harveyi*. Lysate proteins were separated on native PAGE and stained for SOD activity as described in Section 2. The results showed that exponential-phase cells produced only one detectable SOD isozyme and that stationary-phase cells produced an additional SOD (Fig. 1A). However, the additional isozyme in stationary-phase cells was present in minor quantity as indicated by staining intensity. It was possible to distinguish amongst the SOD isozymes using specific enzyme inhibitors. The results revealed that the major SOD band was abolished by H_2O_2 treatment, identifying it as an [Fe]-SOD (data not shown). By contrast, activity of the minor band present at stationary phase could be detected in the presence of either H_2O_2 or cyanide treatments, identifying it as an [Mn]-SOD (data not shown). Induction of [Mn]-SOD by cells entering the stationary phase has been previously reported for bacteria [18]. Additionally, a open reading frame resemble to [Cu,Zn]-SOD was identified in *V. cholerae* genome sequence, we did not detect this isozyme in all growth phases of *V. harveyi* (data not shown). The expression of this isozyme might be too low to be detected by activity gel staining.

3.2. Induction of [Mn]-SOD and G6PD by menadione pre-treatment

Menadione (2-methyl-1,4-naphthoquinone) is an agent

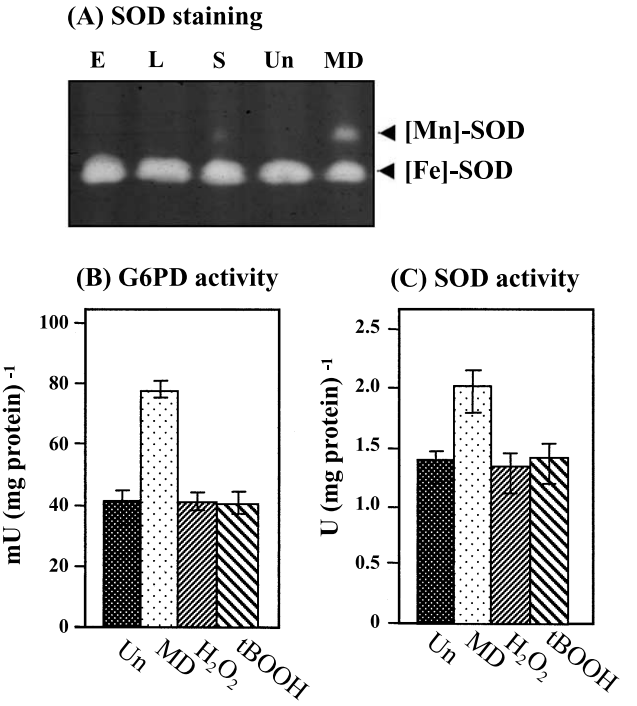

Fig. 1. Visualization of SOD isozymes and induction of SOD and G6PD in *V. harveyi* by pre-treatment with menadione, and the existence of soxR. A: SOD-activity staining was performed as described in Section 2 to visualize SOD isozymes in various growth phases; early exponential (E), late-exponential (L) and stationary phases (S), and in uninduced (Un) and menadione-induced (MD) cultures. G6PD activity (B), and total SOD activity (C) in lysates prepared from *V. harveyi* culture induced with menadione (MD), H_2O_2, tBOOH and uninduced control (Un).

that mediates transfer of electrons from NADH or NADPH to O_2, generating a flux of superoxide anion in a redox cycling process [19]. We examined the effects of sub-lethal concentrations (determined from maximal inhibitory concentrations) of menadione and peroxide pre-treatments on levels of two enzymes, SOD and G6PD that are known to be regulated by superoxide anion in other bacteria. Total G6PD and SOD activity was measured in lysates prepared from cells untreated and pre-treated with 400 μM menadione, 250 μM H_2O_2 and 200 μM tBOOH. G6PD levels increased 1.8-fold in response to menadione pre-treatment, while pre-treatments with peroxides (both H_2O_2 and tBOOH) had no effect (Fig. 1B). Similarly, SOD activities increased 1.4-fold with menadione pre-treatment (Fig. 1C). H_2O_2 and tBOOH pre-treatment have no effect on total SOD activity (Fig. 1C).

Additional experiments were done to determine which SOD isozyme was induced by the menadione treatment. By activity staining of a native gel (Fig. 1A), the intensity of the [Fe]-SOD band from menadione-induced and -uninduced cultures was not significantly different. However, the [Mn]-SOD band was detected only in menadione-induced cultures and this was correlated with the increase in total SOD activity (Fig. 1C). The results suggested that

menadione-induced expression of [Mn]-SOD most likely occurred via the production of superoxide anion.

Thus, induction of G6PD and SOD activities seem to be specific to the menadione pre-treatment as has been observed in other bacteria. For example, superoxide induction in *E. coli* is regulated by a superoxide sensor and transcription repressor called SoxR. SoxR is, in turn, regulated by expression of an AraC family transcription regulator called SoxS [7]. Genetic analysis has shown that SoxR/S regulate both [Mn]-SOD and *zwf* (coding for G6PD) genes. It is likely that superoxide anion-induced elevated levels of [Mn]-SOD and G6PD might be mediated by the SoxR system. Although the *soxR* gene has not been isolated in *V. harveyi* yet, analysis of the genome sequences of *V. cholerae* [15] by BLAST program [17] revealed an open reading frame with coding potential for a SoxR homolog (accession no. AAF95998). Therefore, two degenerated primers designed from two conserved regions of *V. cholerae* SoxR homolog (Fig. 2A) were used to amplify a part of *soxR* gene using *V. harveyi* as a DNA template. The 300-bp PCR product was then cloned into pGem-T-easy (Promega) prior to determination of its nucleotide sequence (Fig. 2B). Analysis of this nucleotide sequence

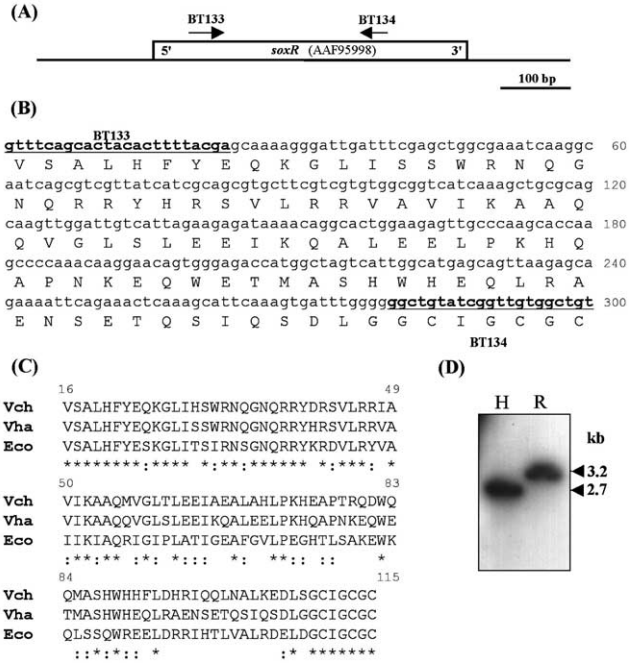

Fig. 2. The existence of *soxR* homolog in *V. harveyi*. A: The physical map of *soxR* from *V. cholerae*. Arrows indicate the positions used to design the degenerated oligonucleotide primers. B: The nucleotide and putative amino acid sequences of *soxR* homolog from *V. harveyi*. Bold and underlined sequences represent primers. C: Multiple alignment of *V. harveyi* SoxR (Vha) with SoxR from *V. cholerae* (Vch, accession number AAF95998) and *E. coli* (Eco, accession number CAA42161). *, identical amino acid in all SoxR. :, identical amino acid between SoxR from *V. cholerae* and *V. harveyi*. Numbers represent the amino acid position of *V. cholerae* SoxR. D: Southern blot analysis of *V. harveyi* genomic DNA digested with *Hin*dIII (H) and *Eco*RI (R), and hybridized with ^{32}P-labelled *soxR* homolog probe.

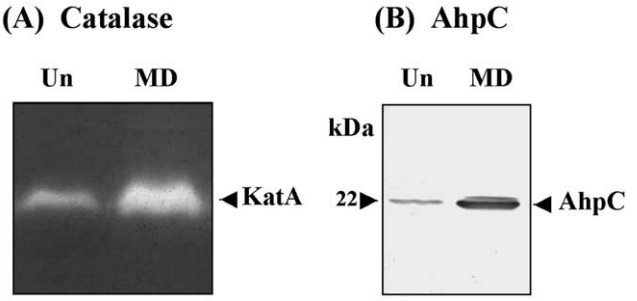

Fig. 3. Induced peroxide scavenging enzymes in *V. harveyi* by pre-treatment with menadione. Crude lysate (80 μg protein) prepared from the exponential phase of uninduced (Un) and menadione-induced (MD) cultures were separated by PAGE and stained for catalase activity (A) or processed Western immunoblot for detection of AhpC (B) as described in Section 2.

with BLASTX program [17] against the GenBank database suggested that this DNA fragment encoded the SoxR homolog. The putative SoxR from *V. harveyi* showed a high score of identity to SoxR from *V. cholerae* (70%) and *E. coli* (55%) as shown by an alignment of the amino acid sequences in Fig. 2C. This putative *soxR* was then radioactively labeled and used as DNA probe in Southern blot hybridization experiments against *V. harveyi* genomic DNA digested with *Hin*dIII and *Eco*RI. The results showed strong hybridized signals at the size of 2.7 and 3.2 kb, respectively (Fig. 2D). The data suggest the existence of *soxR* in *V. harveyi*.

3.3. *Induction of peroxide scavenging enzymes in* V. harveyi *pre-treated with menadione*

Different bacteria have evolved different responses to oxidative stress. Pre-treatment of *V. harveyi* with a sublethal concentration of H_2O_2 or organic hydroperoxide could induce the expression of catalase (*katA*) and alkyl hydroperoxide reductase catalytic subunit C (*ahpC*) [9]. In vivo, menadione gives rise to production of superoxide anion that can be dismutated or chemically broken down to H_2O_2 [19]. The effect of menadione pre-treatment on levels of AhpC and catalase was investigated in *V. harveyi* cultures pre-treated with 400 μM menadione for 30 min before harvesting. The levels of AhpC and catalase were measured by enzyme assay and by Western immunoblot analysis coupled with activity staining. The results (Fig. 3A,B) showed that pre-exposure of *V. harveyi* to menadione resulted in a several fold increase in the levels of both AhpC and KatA. Densitometer analysis of AhpC in Western immunoblots indicated a five-fold increase in the protein level. Total catalase activity increased three-fold from 39.5 ± 5.6 U to 126.9 ± 10.1 U (mg protein)$^{-1}$. These results suggested that superoxide anions activated the expression of *ahpC* and *katA* genes. We have shown that H_2O_2 is a potent inducer of catalase and AhpC in *V. harveyi* [9]. Thus, induction of these enzymes by superoxide anions would most likely occur via the pro-

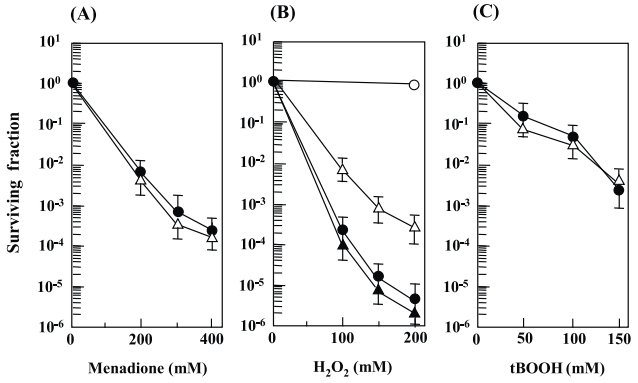

Fig. 4. Induced protective responses in *V. harveyi*. Cultivation conditions, induction and killing were as described in Section 2. A: Menadione survival curves for uninduced (●) and menadione-induced cultures (△); B: H_2O_2 survival curves for cultures uninduced (●), menadione-induced (△) and menadione induced in the presence of a protein synthesis inhibitor (▲) at various concentrations of H_2O_2. ○ represents the survival of untreated cultures in the present of protein synthesis inhibitor alone. C: tBOOH survival curves for uninduced (●) and menadione-induced (△) cultures. Values presented are means and standard deviations of three replicates.

duction of peroxide as the result of superoxide anion breakdown. Elevated H_2O_2 would then induce the expression of catalase and AhpC. Alternatively, superoxide anions may directly act as an inducer.

3.4. The effects of menadione-induced adaptive and cross-protective responses against peroxide killing

Pre-exposure to a low concentration of an agent often confers resistance to sub-sequent challenge with a lethal concentration of the same agent (i.e. an adaptive response). Thus, we measured the physiological effects of menadione pre-treatment on superoxide and peroxide killing. *V. harveyi* cultures pre-treated with menadione and subsequently challenged with a lethal concentration (Fig. 4A) showed no adaptive protection even though menadione induced high levels of G6PD, [Mn]-SOD and the peroxide scavenging enzymes, catalase and AhpC.

In addition to adaptive response, ROS can sometimes induce protection to an unrelated agent. This cross-protection response has been observed in many bacteria including *V. harveyi*. Since menadione induced many peroxide scavenging enzymes, its ability to induce protection against H_2O_2 and an organic peroxide was also investigated. *V. harveyi* cultures pre-treated with 400 μM menadione prior to being challenged with lethal doses of H_2O_2 or tBOOH showed over 100-fold more resistance to H_2O_2 killing than uninduced cells (Fig. 4B). By contrast, there was no cross-protection against tBOOH killing (Fig. 4C). Addition of the protein synthesis inhibitor, chloramphenicol, completely abolished cross-protective response to H_2O_2 killing, indicating that it required newly synthesized protein(s) (Fig. 4B).

Superoxide anion-induced cross-protection to H_2O_2 or tBOOH killing has been observed in other bacteria such as *Rhizobium leguminosarum* [20] and *Xanthomonas campestris* [21]. Although menadione was a potent inducer of AhpC, the catalytic subunit of alkyl hydroperoxide reductase, this induction did not result in protection against tBOOH for *V. harveyi*. Similarly, we have observed that *V. harveyi* pre-treated with H_2O_2 induced high levels of AhpC but do not confer protection against tBOOH killing [9]. These results suggest that other protective enzymes are required for protection against tBOOH toxicity in *V. harveyi*.

3.5. High-salinity protects V. harveyi from menadione killing

The absence of an adaptive response to menadione killing would seem to be a disadvantage to bacteria. However, in warm marine water, the physiological habitat of *V. harveyi*, bacteria are exposed to a high-salinity environment. We discovered that growing *V. harveyi* in LBS (Luria–Bertani broth supplemented with 2% NaCl) resulted more resistance to menadione killing than with growth in normal LB broth (data not shown). The effect of high salinity in growth media on menadione killing of *V. harveyi* was investigated in LB broth without NaCl and with 1, 2, 3, 4, and 5% sea salt (Sigma, St. Louis, MO, USA) supplementation. Full-strength marine water corresponds to approximately 3.5% salt. Exponential-phase cultures were subsequently treated with 200 mM menadione for 30 min. The results showed that high salinity made *V. harveyi* more resistant to menadione killing (Fig. 5A). Similar findings have been reported in other bacteria treated with the redox cycling agent paraquat [22]. It is known that *V. harveyi* causes the most serious outbreaks in farmed tiger prawns during summer months in Thailand when the salt concentration in coastal shrimp farms is relatively higher than in other seasons [3]. Since shrimps are euryhalic and adjust internal osmotic balance to match

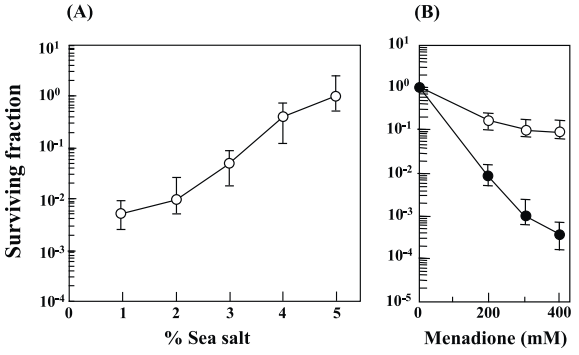

Fig. 5. The effect of sea salt and growth phases on menadione resistance. A: Exponential phase of *V. harveyi* growing in LB broth containing various concentrations of sea salt and treated with 200 mM menadione for 30 min. B: Exponential-phase (●) or stationary-phase (○) cultures of *V. harveyi* in LBS medium treated with the indicated concentrations of menadione. Values presented are means and standard deviations of three replicates.

that of the environment, it is possible that high salt concentration protects *V. harveyi* from superoxide anions generated by host prawn hemocytes as a part of their defense mechanism.

3.6. Stationary-phase cells were more resistant to menadione killing

Stationary phase *V. harveyi* are more resistant to lethal doses of H_2O_2 and the thiol-depleting agent (iodoacetamide) than exponential-phase cells [9]. By contrast, stationary phase cells are highly susceptible to tBOOH killing [9]. Thus, the pattern of resistance or sensitivity to oxidative stress of stationary cells is likely dependent on the type of stress. Resistance to lethal concentrations of menadione was compared for stationary-phase cells and exponential-phase cells. The survival curves (Fig. 5B) showed that cells from stationary-phase cultures were 100-fold more resistant to menadione killing than exponential-phase cells. However, the intracellular level of SOD did not account for this stationary-phase resistance (data not shown). We have already shown that [Mn]-SOD induced by menadione pre-treatment (Fig. 1A) could not protect cells from lethal concentrations of menadione (Fig. 3A). On the other hand, multi-stresses resistance of stationary-phase cells is common in many bacteria [23–26] and expression of genes controlled by a stationary-phase-specific sigma factor RpoS (σ^{38}) is responsible for this increased resistance [24,25,27]. A recent study in *V. harveyi* revealed contradictory results that the *rpoS* null mutant did not show any alterations in the resistance of the cells to oxidative stress suggesting the existence of an alternative mechanisms responsible for the resistance to ROS stress [28].

4. Conclusion

Pre-exposure of *V. harveyi* to menadione induced elevated levels of both peroxide and superoxide detoxification enzymes. The induction resulted in cross-protection against subsequent treatment with a lethal concentration of H_2O_2. The results clearly showed an overlap between peroxide-inducible and superoxide-inducible operons. High salinity and the stationary-growth state also resulted in increased resistance to menadione killing. These responses may play important roles in protection of pathogenic *V. harveyi* against host hemocyte generated ROS.

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Atypical Adaptive and Cross-Protective Responses Against Peroxide Killing in a Bacterial Plant Pathogen, *Agrobacterium tumefaciens*

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Abstract. Physiological adaptive and cross-protection responses to oxidants were investigated in *Agrobacterium tumefaciens*. Exposure of *A. tumefaciens* to sublethal concentrations of H_2O_2 induced adaptive protection to lethal concentrations of H_2O_2. Similar treatments with organic peroxide and menadione did not produce adaptive protection to subsequent exposure to lethal concentrations of these oxidants. Pretreatment of *A. tumefaciens* with an inducing concentration of menadione conferred cross-protection against H_2O_2, but not to *tert*-butyl hydroperoxide (tBOOH), killing. The menadione induced cross-protection to H_2O_2 was due to the compound's ability to highly induce the peroxide scavenging enzyme, catalase. The levels of catalase directly correlated with the bacterium's ability to survive H_2O_2 treatment. Some aspects of the oxidative stress response of *A. tumefaciens* differ from other bacteria, and these differences may be important in plant/microbe interactions.

Agrobacterium tumefaciens is a soil-borne phytopathogenic bacterium infecting many dicotyledons and causing crown gall tumors worldwide [18]. *A. tumefaciens* is also widely used to generate genetically engineered plants [2].

Aerobic growth generates large quantities of reactive oxygen species (ROS) including superoxide anions ($O_2^{\bullet -}$), peroxide (O_2^{2-}), and hydroxyl radicals ($^{\bullet}OH$) [7]. In addition, the rapid production and accumulation of reactive oxygen species (ROS) known as the oxidative burst has also been shown to be an important plant defense response in plant/microbe interactions [9]. ROS are highly toxic to bacterial cells through their detrimental effects on many biological macromolecules including DNA, proteins, and membrane. Consequently, bacteria have evolved several ways to protect themselves from oxidative damage through the evolution of both enzymatic and nonenzymatic systems that either directly detoxify or repair damage caused by ROS. Also, bacteria have protective pathways that involve stress-induced adaptive and cross-protective responses. Exposure to a sublethal concentration of one oxidant can induce protection against subsequent exposure to killing concentrations of the same oxidant (an adaptive response) or unrelated agents (a cross-protective response) [14]. These responses are highly conserved in many bacteria [3, 4, 14, 20, 21]. Oxidant-induced protective responses often result from the coordinated activation of gene(s) involved in both oxidant detoxification and damage repair [1]. An example of this is found in the plant pathogenic bacterium *Xanthomonas campestris*, which produces elevated levels of a monofunctional catalase (KatA), an alkyl hydroperoxide reductase (AhpCF), and an organic hydroperoxide resistance protein (Ohr) in response to exposure to ROS [13–15]. This increase in detoxification enzymes results in increased resistance to killing by oxidants.

At present little is known about the oxidative stress response of *A. tumefaciens*. Understanding this response could give insights into how *A. tumefaciens* survives in the environment and during interactions with its host. Here, we describe atypical peroxide inducible adaptive and cross-protective responses against peroxide killing in *A. tumefaciens*. The protective levels correlated with the inducer's ability to induce the expression of the peroxide scavenging enzyme, catalase.

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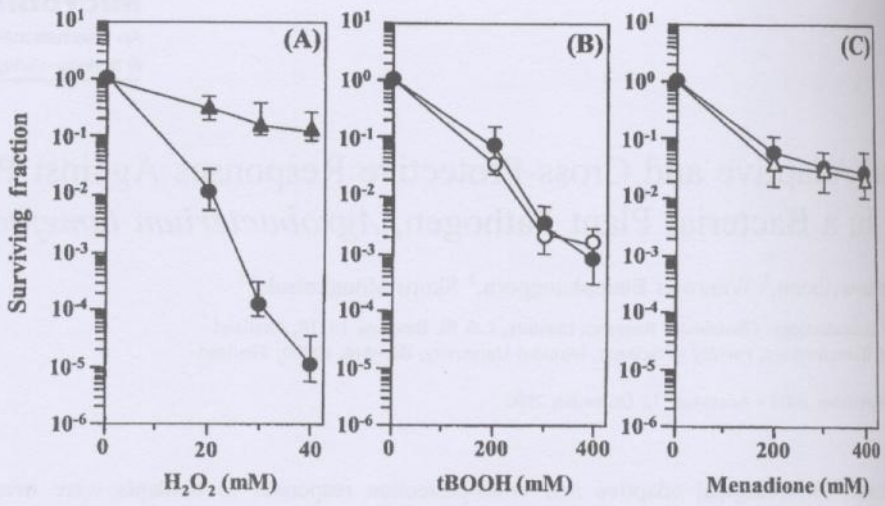

Fig. 1. Induced adaptive responses to peroxides in *A. tumefaciens*. The cultivation conditions, induction, and killing with peroxides were performed as described in Materials and Methods. (A) The H_2O_2 survival curves for uninduced (●), H_2O_2-induced (▲); (B) the tBOOH survival curves of uninduced (●), tBOOH-induced cultures (○); (C) the menadione survival curves of uninduced (●), menadione-induced (△). Values presented are means and standard deviations of three replicates.

Materials and Methods

Bacterial growth conditions. *Agrobacterium tumefaciens* NTL4 [11] was grown aerobically in LB medium at 30°C with continuous shaking at 150 rpm. To ensure synchronous growth, overnight cultures were inoculated into fresh LB medium to give an OD_{600} of approximately 0.1. Exponential phase (OD_{600} about 0.6, after 4 h of growth) cells were used in experiments as indicated.

Determination of resistance levels to oxidants. The peroxide-induced adaptive and cross-protective experiments were performed by adding either 250 μM H_2O_2, 250 μM *tert*-butyl hydroperoxide (tBOOH), or 200 μM menadione to exponential phase cultures. These cultures were grown for an additional 30 min before aliquots of cells were removed and treated with lethal concentrations of either H_2O_2 (20, 30, 40 mM), tBOOH (200, 300, 400 mM), or menadione (200, 300, 400 mM) for 30 min. After treatment, cells were removed and washed once with fresh LB before appropriate dilutions were plated on LB agar. Colonies were counted after 48 h of incubation at 30°C. Surviving fractions are defined as the number of colony-forming units (cfu) recovered after the treatment divided by cfu prior to treatment. All experiments were independently repeated at least three times, and representative data are shown.

Catalase activity assays. Preparation of cell lysates and catalase assays were performed as previously described [19]. Briefly, 20-mL cultures were pelleted and washed once with 50 mM sodium phosphate buffer (PB) pH 7.0. Bacterial suspensions in PB containing 1 mM PMSF, a protease inhibitor, were lysed by brief sonication, followed by centrifugation at 10,000 *g* for 10 min. Cleared lysates were used for catalase assays and total protein determination [19].

Results and Discussion

Adaptive responses against peroxide killing. Inducible protective responses are important for exponential phase cells since they are more susceptible to oxidant killing. We investigated the ability of *A. tumefaciens* to adapt to peroxides and superoxide stresses. *A. tumefaciens* cultures were pretreated with 250 μM H_2O_2 for 30 min before being exposed to killing concentrations of H_2O_2. The results show that the pretreatment increased protection against H_2O_2 killing 10,000-fold (Fig. 1A). By contrast, pretreatment of *A. tumefaciens* with sublethal concentrations of tBOOH or the superoxide-generating agent menadione, did not induce adaptive protection against subsequent exposure to lethal concentrations of these compounds (Fig. 1B, C). The adaptive response towards H_2O_2 treatment is highly conserved and has been observed in many microbes [6, 8, 14, 24]. Although adaptive responses against menadione treatment have been reported in a few microorganisms [5], many bacteria seem to lack this ability [14]. Similarly, adaptation to tBOOH is uncommon in bacteria. It appears that different microbes respond differently to stresses.

Cross-protective responses against peroxide killing. The ability of one type of stress to induce protection against an unrelated stress (cross-protection) is an important survival strategy for bacteria under stressful conditions. Thus, we investigated the ability of different peroxides and superoxide anions to induce cross-protection against unrelated peroxides. *A. tumefaciens* cultures were pretreated with inducing concentrations of menadione or tBOOH and subsequently were treated with lethal concentrations of H_2O_2. The results in Fig. 2A showed that pretreatment with tBOOH did not induce protection against H_2O_2 killing, while menadione pretreatment in-

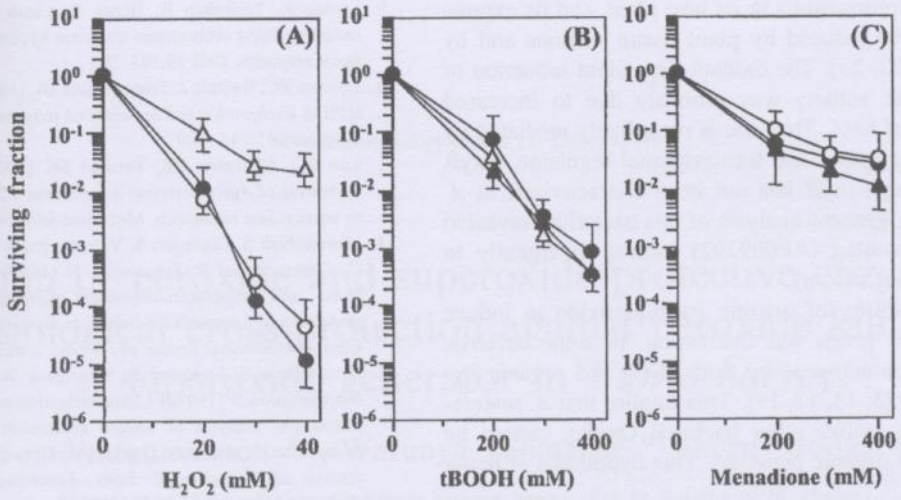

Fig. 2. Induced cross-protective responses in *A. tumefaciens*. The survival curves of exponential phase cultures of *A. tumefaciens* pretreated with H_2O_2 (▲), tBOOH (○), or menadione (△) prior to treatments with lethal concentration of H_2O_2 (A), tBOOH (B), and menadione (C) at indicated concentrations compared with the uninduced culture (●). The values presented are means and standard deviations of three replicates.

duced a 1,000-fold protection against H_2O_2 killing (Fig. 2A). The levels of menadione-induced cross-protection against H_2O_2 killing were lower than the protection levels attained by H_2O_2-adapted cells (Fig. 1A and 2A), suggesting that the adaptive mechanism could be the major protective response against H_2O_2 killing. This observation is in contrast to that of another bacterial phytopathogen, *Xanthomonas*, where menadione is severalfold more effective than H_2O_2 at inducing protection against H_2O_2 killing [12, 14]. The ability of menadione and tBOOH to induce cross-protective responses to oxidant killing was also investigated [14]. In all cases, under the experimental conditions used, no cross-protection was detected (Fig. 2B, C).

Induction of catalase in *A. tumefaciens*. The above data suggested that both adaptive and cross-protective responses against H_2O_2 killing are likely to be a result of H_2O_2 and menadione-induced expression of genes involved in H_2O_2 protection. In bacteria, catalase is the major H_2O_2 detoxification enzyme that catalyzes the conversion of H_2O_2 to water and oxygen. Catalase levels show a direct correlation with the resistance levels to H_2O_2 killing [14]. Exposure of many bacteria to oxidative stress induces the expression of either a bifunctional catalase-peroxidase or a monofunctional catalase [10, 12]. The increase in catalase activity is thought to be responsible for the adaptive and cross-protection responses against H_2O_2 killing. Thus, we measured the catalase activity in response to oxidant pretreatments. Exponential phase cultures of *A. tumefaciens* were pretreated with inducing concentrations of H_2O_2, menadione, or tBOOH, after which total catalase activity was determined. Pretreatment of *A. tumefaciens* with either H_2O_2 or menadione induced an increase in total catalase activity by 3.5 and 2.5-fold, respectively, compared with uninduced levels (Table 1). The magnitude of catalase induction was directly correlated with the ability of these compounds to induce protection against H_2O_2 killing (Fig. 1A and 2A). These findings suggested that catalase activity was the major factor determining the level of resistance to H_2O_2 killing in *A. tumefaciens*. Analysis of the bacterial genome sequence revealed two putative open reading frames identified as monofunctional catalase CatE (ALL41677) and a bifunctional catalase-peroxidase KatA (AAL45436). CatE shows a high degree of sequence identity with atypical growth phase-regulated, stationary phase catalases including *Sinorhizobium meliloti* KatE (76% [16]) and *X. campestris* pv. phaseoli KatE (56% [20]). *A. tumefaciens* KatA has been cloned, characterized, and shown to be a virulence factor in-

Table 1. The effects of peroxide pretreatment on total catalase activity in *A. tumefaciens*

Conditions	Catalase activity[a] ($U \cdot mg\ protein^{-1}$)	Induction level (fold)
Uninduced	7.5 ± 1.6	1.0
H_2O_2-induced (250 μM)	26.0 ± 2.5	3.5
Menadione-induced (200 μM)	19.0 ± 2.0	2.5
tBOOH-induced (250 μM)	8.0 ± 1.5	1.1

[a] Values represent means and standard deviations from three replicates.

volved in tumorigenesis in its host plant, and its expression could be induced by plant tissue sections and by acidic pH [22, 23]. The oxidant-dependent induction of total catalase activity was probably due to increased expression of *katA*. The proc is most likely mediated by the peroxide sensor and transcriptonal regulator, OxyR [17]. Although *oxyR* has not been characterized in *A. tumefaciens*, genome analysis of this bacterium revealed an *oxyR* homolog (AE009392) located divergently to *katA* (data not shown).

The inability of organic hydroperoxide to induce high catalase levels was interesting. In some bacteria, OxyR can be activated by both H_2O_2 and organic hydroperoxide [3, 13, 17, 19]. This implies that *A. tumefaciens* OxyR, unlike other bacterial OxyRs, cannot be activated by organic peroxide. This hypothesis is being investigated.

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Analysis of growth phase regulated KatA and CatE and their physiological roles in determining hydrogen peroxide resistance in *Agrobacterium tumefaciens*

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Abstract

Agrobacterium tumefaciens possesses two catalases, a bifunctional catalase-peroxidase, KatA and a homologue of a growth phase regulated monofunctional catalase, CatE. In stationary phase cultures and in cultures entering stationary phase, total catalase activity increased 2-fold while peroxidase activity declined. *katA* and *catE* were found to be independently regulated in a growth phase dependent manner. KatA levels were highest during exponential phase and declined as cells entered stationary phase, while CatE was detectable at early exponential phase and increased during stationary phase. Only small increases in H_2O_2 resistance levels were detected as cells entering stationary phase. The *katA* mutant was more sensitive to H_2O_2 than the parental strain during both exponential and stationary phase. Inactivation of *catE* alone did not significantly change the level of H_2O_2 resistance. However, the *katA catE* double mutant was more sensitive to H_2O_2 during both exponential and stationary phase than either of the single catalase mutants. The data indicated that KatA plays the primary role and CatE acts synergistically in protecting *A. tumefaciens* from H_2O_2 toxicity during all phases of growth. Catalase-peroxidase activity (KatA) was required for full H_2O_2 resistance. The expression patterns of the two catalases in *A. tumefaciens* reflect their physiological roles in the protection against H_2O_2 toxicity, which are different from other bacteria.

Keywords: *Agrobacterium tumefaciens*; Catalase; Growth phase; H_2O_2; Peroxidase

1. Introduction

Agrobacterium tumefaciens is a soil-borne bacterium that causes crown gall tumors in a wide variety of dicotyledonous plants [1]. In the natural environment, soil bacteria can spend long periods of time in a nutrient-limited state and stress environments. One of the stresses often encountered by *Agrobacterium* is that of exposure to oxidants. During plant–microbe interactions, the

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rapid production and accumulation of reactive oxygen species, primarily H_2O_2, is part of the active plant defense response against microbial invasion [2]. Moreover, oxidative stress can result from exposure to oxidants that are produced by other soil organisms or from chemical contaminants in soil.

Catalase, a heme-containing enzyme, catalyzes the detoxification of H_2O_2 to water and oxygen. Degradation of H_2O_2 not only reduces the direct toxicity of H_2O_2 but also prevents the formation of hydroxyl radicals via the Fenton reaction [3]. Many bacteria have multiple catalases. There are two major types of catalases, monofunctional catalase and bifunctional catalase-peroxidase, which are encoded by non-related genes that are differentially regulated [4]. The physiological roles of these multiple catalases and how they functionally interact with each other to protect the bacteria from peroxide toxicity are not clear. In many bacteria, analysis of mutations in individual catalase genes show that the enzyme plays an important role in protecting bacteria from peroxide stress during exponential phase but the role of the enzyme during stationary phase resistance is less obvious [5–7]. Generally, stationary phase resistance to H_2O_2 and other stresses shows no direct correlation to the levels of various detoxification enzymes [8,9]. Moreover, there are only a few cases where catalase has been shown to contribute to bacterial H_2O_2 resistance during stationary phase [10].

A. tumefaciens possesses two catalase genes, namely *katA* and *catE,* that encode a bifunctional catalase-peroxidase and a homologue of growth phase regulated monofunctional catalase, respectively [11,12]. Inactivation of *katA* leads to hypersensitivity to H_2O_2 killing treatments in actively growing cells and reduces the ability of *A. tumefaciens* to induce tumor formation [13]. Recently, we have shown that pre-exposure of *A. tumefaciens* to sublethal concentrations of H_2O_2 and menadione induced the expression of *katA* and adaptive protection to H_2O_2 toxicity in an *oxyR*-dependent fashion [14,15]. The expression patterns of the two catalases and their roles in protecting the bacteria from H_2O_2 toxicity during the different phases of growth were investigated. The results reveal novel roles for these enzymes, which act synergistically to confer resistance to H_2O_2.

2. Materials and methods

2.1. Bacterial growth conditions

A. tumefaciens NTL4 and the mutant strains were grown aerobically in LB medium at 30 °C with continuous shaking at 150 rpm. To ensure synchronous growth, overnight cultures were inoculated into fresh LB medium to give an optical density at 600 nm (OD_{600}) of about 0.1. Bacterial growth was monitored by measuring the OD_{600} at 2 h intervals. Cells harvested at various growth phases were used in the experiments, as indicated.

2.2. Molecular biology techniques

General molecular genetics techniques including genomic DNA preparation, plasmid preparation, restriction endonuclease digestion, ligation, transformation in *Escherichia coli* and agarose gel electrophoresis were performed using standard protocols [16]. Plasmid purification for DNA sequencing was performed using the QIAprep Kit (Qiagen). DNA was sequenced by the primer walking technique using a BigDye terminator cycle sequencing kit (PE Biosystems) on an ABI 310 automated DNA sequencer (Applied Biosystems). Routinely, *A. tumefaciens* was transformed by electroporation as previously described [14].

2.3. Determination of H_2O_2 resistance level

Resistance of *A. tumefaciens* strains to H_2O_2 killing was quantitatively determined as follows. *A. tumefaciens* strains were grown aerobically in LB broth at 30 °C. Early exponential phase (OD_{600} of 0.2, after 2 h of growth), mid-exponential phase (OD_{600} of 1.0, after 6 h of growth), early stationary phase (OD_{600} of 5.0, after 16 h of growth) and late stationary phase (OD_{600} of 5.0, after 30 h of growth) cells were used in H_2O_2 killing experiments. Aliquots of cells from the cultures were treated with different concentrations of H_2O_2 (10, 20, and 30 mM) for 30 min. After treatment, samples were pelleted and washed once with fresh medium before appropriate dilutions were plated on LB agar plates. Cells that survived various treatments were counted after 24 h incubation at 30 °C. The percent survival is defined as the number of colony forming units (c.f.u.) obtained after the treatment divided by the number of c.f.u. obtained prior to treatment multiplied by 100. All experiments were repeated at least three times and representative data are shown.

2.4. Enzyme activity assays

Preparation of crude bacterial lysates and protein assays were performed as previously described [14]. Briefly, 20 ml cultures were pelleted and washed once with 50 mM sodium phosphate buffer pH 7.0 (PB). Bacterial suspensions in PB containing 1.0 mM PMSF, a protease inhibitor, were lysed by intermittent sonication followed by centrifugation at 10,000*g* for 10 min. The cleared lysates were then used for enzyme assays and total protein determinations. Peroxidase and catalase activity was monitored as described previously [14]. One unit of antioxidant enzyme was defined as the amount of enzyme capable of catalyzing the turnover of 1 μmole of substrate per minute under the assay conditions.

2.5. Catalase activity gel staining

Visualization of catalase isozymes on native PAGE gels was performed as previously described [17]. After gel electrophoresis, gels were soaked in PB containing 50 μg/ml of horseradish peroxidase (Sigma, USA) for 45 min at room temperature. They were then soaked in PB containing 5 mM H_2O_2 for 10 min. After, briefly washing twice with distilled water, they were immediately stained with freshly prepared PB containing 0.5 mg/ml diamino benzidine until the background became dark brown. Areas of catalase activity appeared as clear bands.

3. Results and discussion

3.1. Growth phase dependent variation in KatA and CatE levels

A. tumefaciens has two catalase genes, namely *katA* and *catE* [12]. *katA,* encoding a bifunctional catalase-peroxidase, is regulated by the peroxide sensing/global regulator OxyR [14]. *catE* encodes a monofunctional catalase that shares high score of identity (49%) with a growth phase regulated catalase, KatE, of *E. coli* [18]. To determine if the *A. tumefaciens* catalases were also growth phase regulated, we monitored the total levels of catalase and peroxidase during the different phases of growth. The results in Fig. 1(a) show that total catalase activity increased about 2-fold from early exponential phase (6.5 U mg^{-1} protein at 2 h) to late stationary phase (12.0 U mg^{-1} protein at 30 h). By contrast, the total peroxidase levels increased from 3.5 mU mg^{-1} protein at early exponential phase (2 h) to a peak level of 6.0 mU mg^{-1} protein at mid-exponential phase (6 h) then sharply declined as the culture entered the stationary phase of growth to a final level of 0.9 mU mg^{-1} protein at late stationary phase (30 h). The individual levels of KatA and CatE were then determined during the different growth phases using catalase activity staining gels. Analysis of the catalase activity gel clearly showed that KatA was the major catalase (80–85% of total the catalase level) throughout the exponential phase of growth and displayed a growth phase dependent expression pattern in that the level of KatA (Fig. 1(b)) was highest during mid-exponential phase and declined as the culture entered stationary phase reaching its lowest level during late stationary phase. This pattern paralleled that of the total peroxidase levels (Fig. 1) suggesting that KatA was responsible for the total peroxidase activity. In addition, analysis of the *katA* promoter transcriptionally fused to a promoter-less *lacZ* in a low-copy-number plasmid vector [14] show that the promoter activity was highest during exponential phase then declined during stationary phase, which was similar to the pattern of peroxidase activity (data not shown). By contrast, *A. tumefaciens* CatE expression showed

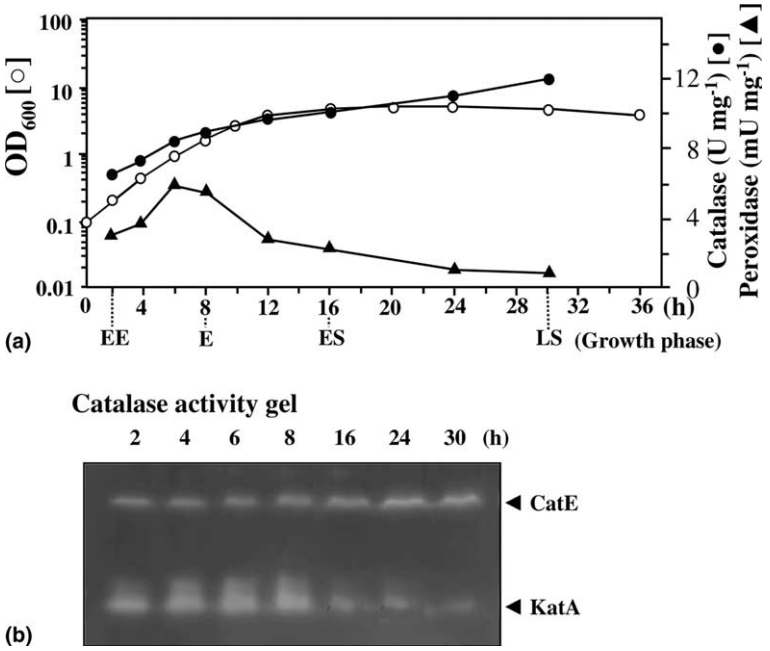

Fig. 1. Growth and catalase and peroxidase levels. In (a), growth curve (○) of *A. tumefaciens* NTL4 was determined by culturing in LB medium at 30 °C with continuous shaking at 150 rpm. At the indicated time, the levels of total catalase (●) and peroxidase (▲) activity were determined in crude lysates as describe in Section 2. The values presented are mean of triplicate assays. EE, early exponential; E, mid-exponential; ES, early stationary; and LS, late stationary phase. In (b), the differential expression of KatA and CatE was monitored by catalase activity gel staining of crude lysates as described in (a). Total protein of 100 μg was loaded in each lane.

an interesting pattern with CatE levels increasing as the cells entered into and during the stationary phase to the point where CatE became the major form of catalase present (80% of the total catalase level) (Fig. 1(b)). Further analyses following the catalase and peroxidase activities in the *A. tumefaciens catE* insertion mutant strain BC04 [19] and the *A. tumefaciens katA* insertion mutant strain PB102 [15] also showed that inactivation of one catalase did not alter the expression pattern of the remaining catalase. The catalase and peroxidase activities in the *catE* strain BC04 followed the same pattern previously observed for KatA expression in the wild-type strain NTL4 (Fig. 1(b)), i.e. the levels peaked (8.5 U mg^{-1} protein) during mid-exponential phase then declined and during stationary (1.5 U mg^{-1} protein) and late stationary phase (2.2 U mg^{-1} protein) (Fig. 2(a)). The PB102 *katA* mutant produced non-detectable levels of peroxidase activity at all growth phases (data not shown). Similarly, CatE expression in the PB102 followed the same pattern that was observed in the wild type strain NTL4 with increasing catalase activity from mid-exponential phase (2.1 U mg^{-1} protein) that peaked during late stationary phase (7.8 U mg^{-1} protein) (Fig. 2(b)). The results obtained with the single catalase mutants indicated that in *A. tumefaciens* inactivation of *katA* does not affect the expression of *catE* and vice versa. The *A. tumefaciens* CatE expression pattern was somewhat similar to that observed for growth phase regulated monofunctional catalases in other bacteria [4,17,20]. The fact that a significant level of CatE was detectable in early exponential phase suggested that the enzyme could also have a physiological role during this phase of growth. However, the exponential phase expression of *A. tumefaciens* CatE contrasts with the expression patterns of growth phase regulated catalases in other bacteria that are under the control of σ^S and tend to be expressed only during stationary phase [21]. As mentioned previously, *katA* is known to be regulated by OxyR [14] while the regulator of *A. tumefaciens catE* is not known. Analysis of the *A. tumefaciens* genome sequence [12] using the BLASTP algorithm [22] did not reveal any open reading frames with significant amino acid sequence similarity to *E. coli rpoS* (data not shown) suggesting that either *A. tumefaciens catE* is regulated by another stationary phase sigma factor(s) that is not related to RpoS or the mechanism of *catE* regulation is completely different from that in *E. coli*.

3.2. The role of KatA and CatE in growth phase dependent resistance to H_2O_2

Since *A. tumefaciens katA* and *catE* were expressed in a growth phase dependent manner, we wanted to more clearly define the role played by each catalase in conferring H_2O_2 resistance during all phases of growth. First, growth curves of *A. tumefaciens* NTL4 and the *katA* and *catE* single and double mutant strains; PB102 (*katA*), BC04 (*catE*) and KC05 (*katA catE*) [19] were determined. All the strains had similar growth rates and growth curves that were indistinguishable from the representative growth curve of the wild-type strain NTL4 shown in Fig. 1(a) (data not shown), indicating that

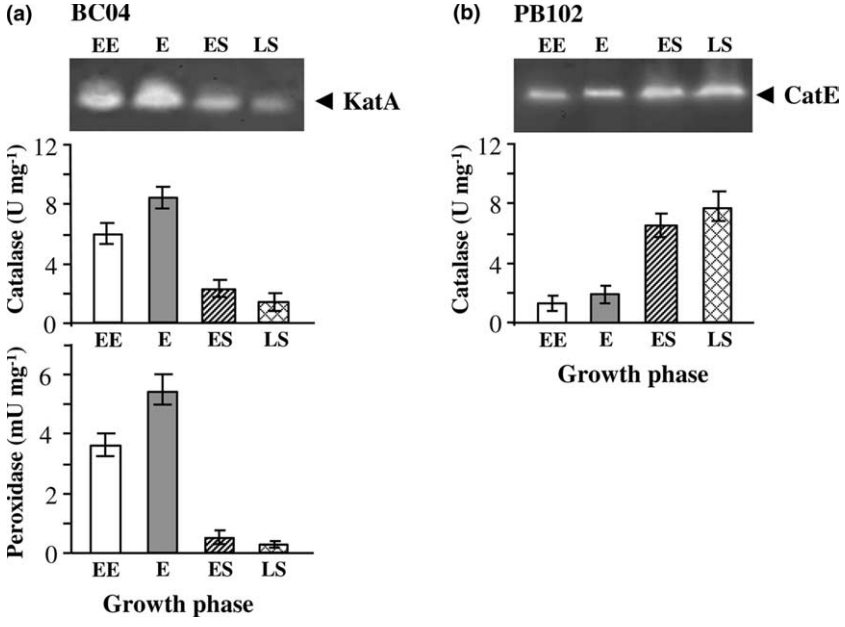

Fig. 2. Catalase and peroxidase levels in *A. tumefaciens catE* (BC04) and *katA* (PB102) mutants. *A. tumefaciens* BC04 (a) and PB102 (b) was grown in LB medium until early exponential (EE), mid-exponential (E), early stationary (ES) and late stationary (LS) phases. Crude extract preparation, catalase activity gel staining and enzymatic assays for catalase and peroxidase activities were performed as described in the Section 2 and Fig. 1(b) legend. Values presented are the means and standard deviations of triplicate assays.

the absence of one or both catalases had no major effect on the aerobic growth of *A. tumefaciens* in rich medium. Next, the survival of *A. tumefaciens* NTL4 and the various catalase mutants, following H_2O_2 killing treatments, was determined during the different growth phases. The results of the H_2O_2 survival studies are shown in Fig. 3. In the case of the wild type strain NTL4, early and mid-exponential phase cells were least resistant to H_2O_2 (Fig. 3(a)), however, as the culture entered stationary phase the resistance to H_2O_2 increased by 10-fold (Fig. 3(a)). This pattern of growth phase dependent increase in the level of H_2O_2 resistance resembles the pattern observed in many other bacteria [9,23–25]. However, it is noteworthy that in *A. tumefaciens* the magnitude of the increase (10-fold) in resistance to H_2O_2 during stationary phase was much lower than the increases observed in other bacteria in which stationary phase cells are much more resistant (10^2-fold higher) to H_2O_2 killing [9,23,24]. Nonetheless, the stationary phase resistance to oxidants could be physiologically important to *A. tumefaciens* in overcoming the plant generated H_2O_2 during the host defense response [2].

The results of the H_2O_2 survival studies using the *katA* and *catE* single and double mutant strains were able to shed some light on the role of these enzymes in the growth phase dependent resistance to H_2O_2. All of the mutant strains showed a pattern of growth phase dependent H_2O_2 resistance that was similar to that of the wild type strain NTL4, i.e. resistance increased as the cultures entered stationary phase, albeit the absolute levels of H_2O_2 resistance of the strains varied markedly (Figs. 3(a)–(d)). Perhaps most surprising was the finding that inactivation of *catE* in strain BC04 had no appreciable effect on H_2O_2 resistance during any growth phase (Figs. 3(a) and (b)), even though total catalase activity in late stationary phase BC04 was only 1.5 U mg^{-1} protein compared to 12.0 U mg^{-1} protein in NTL4 (Figs. 1(a) and 2(a)). This indicated that increased stationary phase H_2O_2 resistance was not simply due to an increase in the level of total catalase activity. Moreover, in both NTL4 and BC04, the fact that the level of KatA was lower during stationary phase (Figs. 1(b) and 2(b)) indicated that KatA alone could effectively protect bacterial cells from H_2O_2 killing, even at the lower enzymatic activity. In spite of this, the fact that CatE was expressed during all growth phases (Fig. 1(b)), combined with the observation that the *katA catE* double mutant strain KC05 was more sensitive to H_2O_2 than the *katA* single mutant strain PB102 (Figs. 3(c) and (d)), indicated that *catE* did play a minor role in the protection of *A. tumefaciens* against H_2O_2 toxicity during all phases of growth. This is in contrast to results in other bacteria that CatE homologues play important roles in the protection against H_2O_2 only during the late exponential and stationary phases of growth [20,21].

In *A. tumefaciens,* KatA catalase-peroxidase has been shown to play an essential role in the protection of actively growing cells from high doses of H_2O_2, as well as in the formation of tumors on host plants [13]. However, the role of *katA* in stationary phase resistance to H_2O_2 has not been extensively studied. It was found that the inactivation of *katA,* in strain PB102, had significant

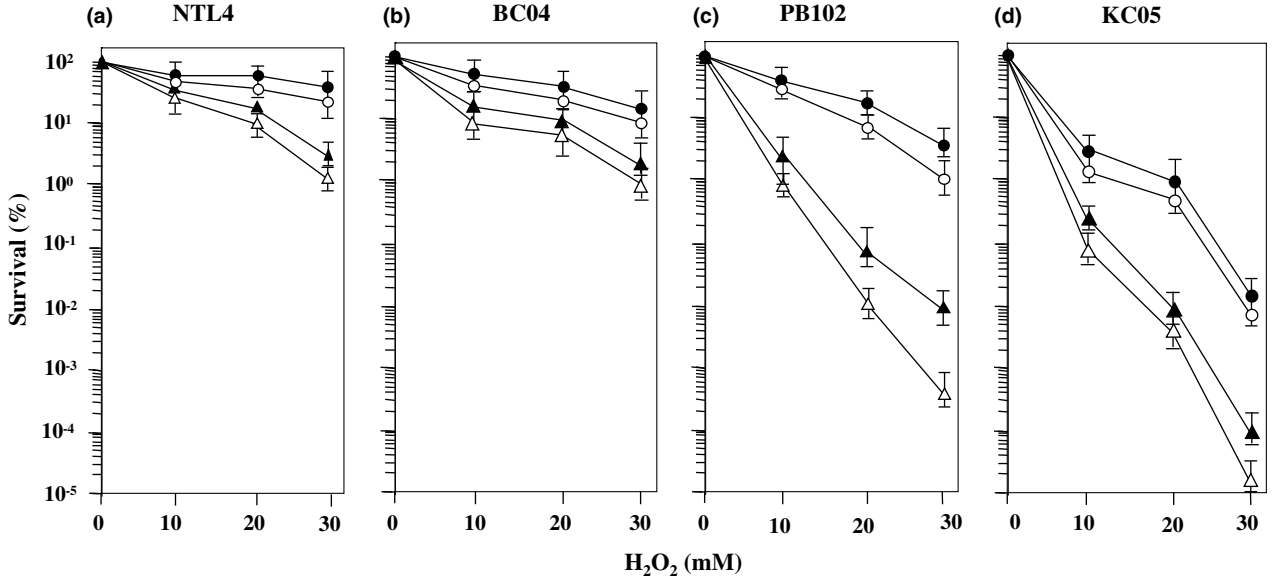

Fig. 3. The H_2O_2 resistance levels of *A. tumefaciens* strains at various growth phases. The survival curves for (a) *A. tumefaciens* NTL4, (b) *catE* (BC04), (c) *katA* (PB102), and (d) *katA catE* (KC05) mutants following H_2O_2 killing treatments are shown. Early exponential (Δ), mid-exponential (▲), early stationary (○) and late stationary (●) phase cells of *A. tumefaciens* strains were treated with 10, 20, and 30 mM H_2O_2 for 30 min. Values presented are the means and standard deviations of triplicate assays.

effects on the H_2O_2 resistance levels during all phases of growth (Fig. 3(c)). As might have been predicted, the most profound effects were seen during early exponential and mid-exponential phase where inactivation of *katA* in strain PB102 resulted in a 10^3- and 10^2-fold reduction in H_2O_2 resistance, respectively, relative to strain NTL4 (Fig. 3(c)). By contrast, during stationary and late stationary phase, PB102 cells were only 10-fold less resistant to H_2O_2 than NTL4 (Figs. 3(a) and (c)). As mentioned previously, the small decrease in stationary phase H_2O_2 resistance of PB102 was probably due, at least in part, to the stationary phase expression of CatE (7.8 U mg^{-1} protein, Fig. 2(b)) since the lack of catalase in the double mutant strain KC05 resulted in a 10^2-fold decrease in stationary phase resistance to H_2O_2 (Figs. 3(a), (c) and (d)). Taken together, the data indicate that KatA and CatE play primary and secondary roles, respectively, in conferring H_2O_2 resistance during both the exponential and stationary phases of growth.

3.3. Complementation analysis of strain KC05 with katA and catE

The role of KatA during stationary phase was interesting in view of the fact that expression of KatA decreased during stationary phase yet still conferred high-level protection against H_2O_2 even in the absence of CatE. Since the two catalases differ with respect to their biochemical activities and were shown, by the mutant studies, to play unequal roles in conferring H_2O_2 resistance, we examined the effect of the expression of plasmid borne KatA or CatE on H_2O_2 resistance in the *katAcatE* strain KC05. *A. tumefaciens* strain KC05 harboring either pKatA (pBBR1MCS-4 [26] containing full-length *katA* [19]) or pCatE (pBBR1MCS-4 containing full-length CatE [19]), as well as KC05 containing only the vector as a negative control, were assayed for both H_2O_2 resistance and total catalase activity during both exponential and stationary phase (Figs. 4(a)

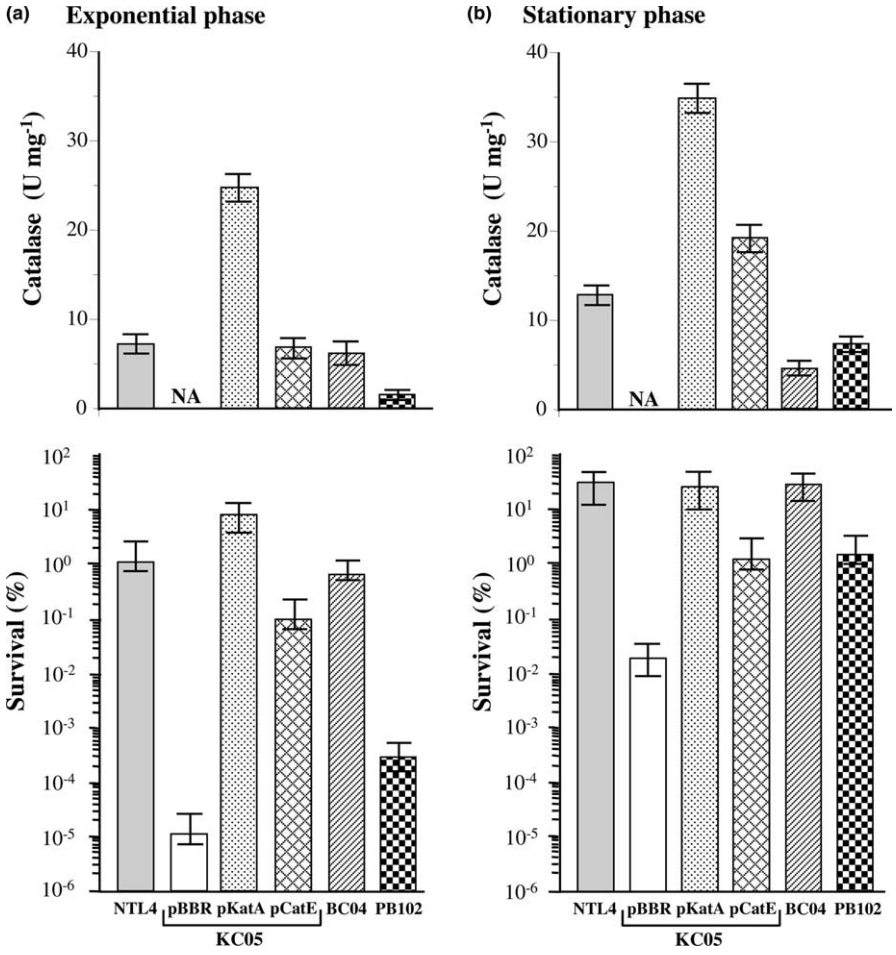

Fig. 4. H_2O_2 resistance and catalase levels of *A. tumefaciens* strains. The levels of resistance against H_2O_2 killing of *A. tumefaciens* (NTL4), *catE* mutant (BC04), *katA* mutant (PB102) and a *katAcatE* double mutant (KC05) harboring plasmids pBBR1MCS-4 (pBBR), pKatA or pCatE are shown. Cells were grown until mid-exponential (a) and late stationary (b) phase. Aliquots of cells were then treated with 30 mM H_2O_2 for 30 min and plated to determine the number of viable cells. The surviving colonies were counted after 24 h incubation. A portion was also harvested and the catalase activity was determined. NA indicates no activity. The values presented are the means and standard deviations of triplicate assays.

and (b)). The results reinforced those of the *katA* and *catE* mutant studies and confirm that bifunctional catalase KatA is responsible for the majority of the H_2O_2 resistance in *A. tumefaciens* during both exponential and stationary phase. Complementation of KC05 with pKatA restored catalase activity and H_2O_2 sensitivity to levels that were equal to or greater than those in the BC04 *catE* mutant and in the wild type strain NTL4 (Figs. 4(a) and (b)). Interestingly, there was no direct correlation between the relative increase in KatA activity and the relative increase in H_2O_2 resistance. During both exponential and stationary phase, KC05/pKatA had 3-fold higher KatA levels, as determined by total catalase activity, than strain NTL4 and yet the increases in the H_2O_2 resistance level were less than 2-fold (Figs. 4(a) and (b)). This suggests that a minimum level of KatA is required for full H_2O_2 protection. Once this level has been attained further increases in the KatA level do not result in further increases in H_2O_2 resistance. Complementation of strain KC05 with pCatE increased the levels of H_2O_2 resistance higher than or equal to PB102 *katA* mutant level at exponential and stationary phases, respectively (Figs. 4(a) and (b)). Interestingly, H_2O_2 resistance levels in KC05/pCatE were consistently lower (≈10-fold) than those of the wild type strain NTL4. This is in spite of the fact that the catalase activities in strain KC05 carrying pCatE were similar to the levels attained in strain NTL4 during exponential phase and 1.5-fold higher than those during stationary phase (Figs. 4(a) and (b)). This clearly indicated that the CatE monofunctional catalase alone could not fully protect *A. tumefaciens* from H_2O_2 toxicity and confirms its secondary role in conferring full H_2O_2 protection during exponential and stationary phase.

3.4. Growth phase dependent, catalase independent, H_2O_2 resistance mechanism

In general, during stationary phase bacterial cells are highly resistant to multiple stresses, including exposure to H_2O_2, through mechanisms that are independent of the action of protective and detoxification enzymes [8,9]. Even though strain KC05 was hypersensitive to H_2O_2 killing at all phases of growth, this strain still showed increased H_2O_2 resistance during stationary phase that was 10^3-fold higher than in exponential phase (Fig. 3(d)). This fact demonstrated the existence of an additional stationary phase dependent H_2O_2 resistance mechanism(s) in *A. tumefaciens* that is independent of catalase activity. At present, the exact nature of the stationary phase mechanism(s) is not known but could involve alterations in membrane components and/or the expression of genes encoding proteins involved in the repair of oxidatively damaged macromolecules such as *msrA* (methionine sulfoxide reductase) or genes encoding general protective proteins such as *dps* (DNA-binding protein at starvation) [27], both of which have been shown to be under growth phase regulation. The involvement of these genes in stationary phase resistance to H_2O_2 is currently being investigated.

4. Conclusions

A. tumefaciens produced two growth phase dependent catalase isozymes, a bifunctional catalase-peroxidase KatA and a monofunctional catalase CatE that are independently regulated. KatA levels were high during exponential phase then declined during the stationary phase of growth, while CatE was detectable in all growth phases and was increased during stationary phase. The catalases contributed unequally to the overall resistance to H_2O_2 with KatA playing a primary role and CatE a secondary role in the protection of *A. tumefaciens* from H_2O_2 at all phases of growth. Interestingly, full protection against H_2O_2 absolutely required a low level of KatA catalase-peroxidase enzyme. *A. tumefaciens* resistance levels to H_2O_2 increased during stationary phase even in the absence of functional catalase indicating an additional unknown stationary phase H_2O_2 resistance mechanism(s) that was catalase independent.

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The role of a bifunctional catalase-peroxidase KatA in protection of *Agrobacterium tumefaciens* from menadione toxicity

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Abstract

Agrobacterium tumefaciens is an aerobic plant pathogenic bacterium that is exposed to reactive oxygen species produced either as by-products of aerobic metabolism or by the defense systems of host plants. The physiological function of the bifunctional catalase-peroxidase (KatA) in the protection of *A. tumefaciens* from reactive oxygen species other than H_2O_2 was evaluated in the *katA* mutant (PB102). Unexpectedly, PB102 was highly sensitive to the superoxide generator menadione. The expression of *katA* from a plasmid vector complemented the menadione-hypersensitive phenotype. *A. tumefaciens* possesses an additional catalase gene, a monofunctional catalase encoded by *catE*. Neither inactivation nor high-level expression of the *catE* gene altered the menadione resistance level. Moreover, heterologous expression of the catalase-peroxidase-encoding gene *katG* from *Burkholderia pseudomallei*, but not the monofunctional catalase gene *katE* from *Xanthomonas campestris* could restore normal levels of menadione resistance to PB102. A recent observation suggests that the menadione resistance phenotype involves increased activities of organic peroxide-metabolizing enzymes. Heterologous expression of *X. campestris* alkyl hydroperoxide reductase from a plasmid vector failed to complement the menadione-sensitive phenotype of PB102. The level of menadione resistance shows a direct correlation with the level of peroxidase activity of KatA. This is a novel role for KatA and suggests that resistance to menadione toxicity is mediated by a new, and as yet unknown, mechanism in *A. tumefaciens*.

Keywords: *Agrobacterium tumefaciens*; Catalase-peroxidase; H_2O_2; Menadione

1. Introduction

Agrobacterium tumefaciens is a Gram-negative soil bacterium that causes crown gall tumors in a wide range of dicotyledonous plants. It is capable of transferring a T-DNA segment of the tumor-inducing plasmid into the plant cell where the plasmid is stably integrated into the host chromosome resulting in the formation of the crown gall [1]. *A. tumefaciens* is widely used in plant transformation for introducing foreign genes into plant cells to generate transgenic plants [2]. During plant/microbe interactions, the initial plant response to microbial interaction involves increased production and accumulation of reactive oxygen species (ROS) including superoxide, hydrogen peroxide and organic peroxide. In order to successfully colonize the host, microbes have to overcome the toxicity of the ROS. Consequently, bacteria have evolved enzymatic and non-enzymatic systems to detoxify ROS. Although the role of ROS production by host plants during *A. tumefaciens* infection is not clear, a recent observation indicates that an *A. tumefaciens* mutant carrying a defective catalase (*katA*) gene showed an attenuated ability to cause tumors [3]. This implies that host-generated ROS and the bacteria's ability to remove them are important in plant/*Agrobacterium* interactions.

Our laboratory has been interested in the ability of *A. tumefaciens* to defend itself from superoxide anions. Menadione is a redox-cycling quinone commonly used in the laboratory. In the living cells, this compound is easily reduced to semiquinone in an NAD(P)H-dependent reac-

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tion. The semiquinone can, in turn, readily reduce O_2 to superoxide anion and generate oxidized quinone which can then re-enter the cycle [4]. Thus, the mechanism of menadione toxicity most likely involves the generation and accumulation of superoxide anions, which can be dismutated to H_2O_2 [4]. Superoxide dismutase is the major enzyme system involved in protection against superoxide anions. This enzyme catalyzes the dismutation of superoxide anions to form H_2O_2 that is subsequently removed by catalases and peroxidases. Catalase is one of the major enzymes involved in the protection of bacteria from H_2O_2 toxicity. The enzyme catalyzes H_2O_2 breakdown to oxygen and water thereby preventing cellular damage caused by H_2O_2 directly or from hydroxyl radicals generated via the Fenton reaction [4]. Several bacteria have two unrelated types of catalases, a bifunctional catalase-peroxidase and a monofunctional catalase encoded by separate, independently regulated genes. The physiological role of these enzymes is thought to be the protection of bacteria from H_2O_2.

We have isolated and functionally characterized the peroxide-sensing transcriptional regulator OxyR and shown that it regulates the expression of *katA*, encoding bifunctional catalase-peroxidase [5]. It has been reported that *A. tumefaciens* KatA plays a role in the adaptive protection of the bacterium against H_2O_2 treatments and also functions as a virulence factor involved in tumorigenesis in infected host plants [3,6,7]. Investigations in several pathogenic bacteria have revealed that a catalase-peroxidase gene plays an essential role in intracellular survival during microbial infection [8,9]. In this paper, we report a novel role for catalase-peroxidase in the protection of *A. tumefaciens* from the toxicity of the redox-cycling agent menadione.

2. Materials and methods

2.1. Bacterial growth conditions

A. tumefaciens NTL4 [10] and the mutant strains were grown aerobically in Luria–Bertani (LB) medium at 30°C with continuous shaking at 150 rpm. To ensure synchronous growth, overnight cultures were inoculated into fresh LB medium to give an OD_{600} of about 0.1. Exponential-phase (OD_{600} about 0.6, after 4 h of growth) and stationary-phase (OD_{600} about 5, after 30 h of growth) cells were used in all experiments, as indicated.

2.2. Molecular biology techniques

General molecular genetics techniques including genomic DNA preparation, plasmid preparation, restriction endonuclease digestion, ligation, transformation in *Escherichia coli*, and agarose gel electrophoresis were performed using standard protocols [11]. Plasmid purification for DNA sequencing was performed using the Qiagen Miniprep kit (Qiagen, France). DNA was sequenced using the primer walking technique, a BigDye terminator cycle sequencing kit (PE Biosystems) and an ABI 310 automated DNA sequencer. Routinely, *A. tumefaciens* was transformed by electroporation under conditions previously described [10].

2.3. Construction of an A. tumefaciens catE mutant

An *A. tumefaciens catE* mutant was constructed by insertional inactivation. A small DNA fragment, internal to the *catE* gene, was amplified with forward oligonucleotide primer BT702 and reverse primer BT703 (see Table 1), which were designed from the nucleotide sequence derived from open reading frame Atu5491 (accession number AAL46177) of the *A. tumefaciens* genome [12], and *A. tumefaciens* genomic DNA as a template. The 200-bp polymerase chain reaction (PCR) product was cloned into pDrive (Qiagen, France) and subcloned as an *Eco*RI fragment into the suicide plasmid vector pKNOCK-Tc [13] to form pKNOCKcatE. The nucleotide sequence of the insert was determined, to assure it was indeed a *catE* fragment, before the plasmid was transferred into *A. tumefaciens* as described elsewhere [13]. Insertion of pKNOCKcatE into the *A. tumefaciens* chromosome by homologous recombination between the *catE* fragment on the plasmid and its counterpart on the chromosome resulted in *catE* insertion mutants that could be selected by their tetracycline-resistant (Tc^R) phenotype. The *catE* mutant designated BC04 was confirmed by PCR with other specific primers that cover the insertion site (data not shown).

2.4. Construction of A. tumefaciens catE katA double mutant

An *A. tumefaciens catE katA* double mutant (KC05) was constructed by transferring pKNOCKcatE into *A. tumefaciens* PB102 (*katA*::pKNOCK-Km [7]) and selecting for the Tc^R and Km^R phenotype. The mutant was confirmed as described for mutant BC04.

2.5. Cloning of A. tumefaciens katA and catE

The full-length *katA* and *catE* genes were amplified from *A. tumefaciens* genomic DNA with two primer pairs, BT774-BT775 and BT790-BT919 (see Table 1), corresponding to open reading frames Atu4642 (accession number AAL45436) and Atu5491 (accession number AAL46177), respectively, in the *A. tumefaciens* genome sequence [12]. The PCR products were cloned into the cloning vector pDrive followed by the determination of the nucleotide sequence and subcloning of the *katA*- and *catE*-specific *Apa*I-*Spe*I fragments into the broad-host-range plasmid pBBR1MCS-4 [14] digested with the same

Table 1
Bacterial strains, plasmids and primers used in this study

Strain/plasmid/primer	Description[a]	Source
A. tumefaciens strains		
NTL4	pTiC58-cured derivative of strain C58Δtet_{C58}, Tc^S	[10]
PB102	NTL4 *katA*::pKNOCK-Km	[7]
BC04	NTL4 *catE*::pKNOCK-Tc	This study
KC05	NTL4 *katA*::pKNOCK-Km *catE*::pKNOCK-Tc	This study
Plasmids		
pDrive	PCR cloning vector, *rep*, phage f1 *ori*, *lacZ*α, Ap^R, Km^R	Qiagen (France)
pKNOCK-Km	Broad-host-range suicide vector; RP4 *oriT*, R6K γ-*ori*, Km^R	[13]
pKNOCK-Tc	Broad-host-range suicide vector; RP4 *oriT*, R6K γ-*ori*, Tc^R	[13]
pKNOCKcatE	pKNOCK-Tc containing *A. tumefaciens catE* DNA fragment	This study
pBBR1MCS-4	Broad-host-range cloning vector; *rep, mob, lacZ*α, Ap^R	[14]
pKatA	pBBR1MCS-4 containing *A. tumefaciens katA*	This study
pCatE	pBBR1MCS-4 containing *A. tumefaciens catE*	This study
pKatG	pBBR1MCS-4 containing *B. pseudomallei katG*	[21]
pKatE	pBBR1MCS-4 containing *X. campestris* pv. *phaseoli katE*	[17]
pAhpCF	pBBR1MCS-4 containing *X. campestris* pv. *phaseoli ahpCF*	[27]
Primers	Sequence (5′-3′)	GenBank accession number
BT702	GTG CTG GAA CGC GTC TGC A	AAL46177
BT703	AGC CGT CTT CGA GTT CAT C	AAL46177
BT774	CCA TCG GAG GGC GAA ATG GAC GC	AAL45436
BT775	ATC GCT TCC GCT CAG ATC AGA TC	AAL45436
BT790	GTT CGT TAT TCC TTG TTG AC	AAL46177
BT919	TGT AGG GAG CTA ACA TGG CCA	AAL46177

[a]Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Tc, tetracycline.

restriction endonucleases to form plasmid pKatA and pCatE, respectively.

2.6. Determination of oxidant resistance by inhibition zone measurement

Analysis of the killing effects of various reagents on *A. tumefaciens* strains was done using an inhibition zone assay [15]. Briefly, 1 ml of an *A. tumefaciens* exponential-phase culture was mixed with 10.0 ml of molten top agar (LB containing 0.7% agar) pre-warmed at 50°C and overlaid onto LB plates (14-cm-diameter Petri dishes containing 40 ml of LB agar). The plates were left at room temperature for 15 min to allow the top agar to solidify. Sterile 6-mm-diameter disks (prepared from Whatman filter paper no. 3) soaked with 5 μl of 1.0 M *tert*-butyl hydroperoxide (tBOOH), 0.5 M H_2O_2 or 1.0 M menadione were placed on the surface of the cell lawn and the zones of growth inhibition were measured after 24 h of incubation at 30°C.

2.7. Determination of oxidant resistance by plate sensitivity assay

The resistance level of the stationary-phase cells to menadione was determined using a modification of the plate sensitivity assay described previously by Delaunay et al. [16]. Serial dilutions of stationary-phase cells were made in 50 mM sodium phosphate buffer pH 7.0 and 10 μl of each dilution was spotted onto a LB agar plate containing 0.2 mM menadione. The plates were incubated at 30°C for 24 h before the results were read.

2.8. Enzyme assays

Crude bacterial lysates were prepared and protein assays were performed as previously described [15]. Briefly, 20-ml cultures were pelleted and washed once with 50 mM sodium phosphate buffer pH 7.0 (PB). Bacterial suspensions in PB containing 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor, were lysed by intermittent sonication followed by centrifugation at $10\,000 \times g$ for 10 min. The total protein concentration was determined for each of the cleared lysates prior to their use in enzyme assays. Superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G6PD), peroxidase and catalase activity was monitored as described previously [17]. One unit of enzyme was defined as the amount of enzyme catalyzing the turnover of 1 μmol of substrate per minute under the assay conditions.

2.9. Statistical analysis

The significance of the differences observed when comparing two strains was determined statistically using Student's *t*-test, one-way analysis of variance. The post-hoc pairwise comparison with a least significant difference test was used when more than two strains were compared. In either case, a value of $P < 0.05$ was considered significant.

3. Results and discussion

3.1. *Hypersensitivity of the* A. tumefaciens katA *mutant to menadione*

In *A. tumefaciens*, inactivation of *katA*, encoding a bifunctional catalase-peroxidase, leads to hypersensitivity to H_2O_2 killing treatments in the growing cells [3]. Recently, we have shown that pre-exposure of *A. tumefaciens* to sublethal concentrations of H_2O_2 and menadione elevates the expression of *katA* in an *oxyR*-dependent fashion and also induces protection against subsequent exposure to lethal concentrations of H_2O_2 [5,7]. The ability of menadione to induce *katA* expression led us to investigate the physiological role of *katA* in the protection of the bacteria against menadione toxicity. The resistance levels against menadione were determined, in the NTL4 wild-type and the *katA* mutant (PB102) [7] strains, using a growth inhibition zone assay. The results show that PB102 was highly sensitive to the menadione treatment as compared to NTL4 (Fig. 1A).

Menadione is a redox-cycling agent that generates superoxide anions. The resulting superoxide anions are then dismutated to form H_2O_2 and other ROS. These compounds can then damage the cell by reacting with proteins, DNA and other cellular components [4]. Therefore, the efficient removal of superoxide anions and H_2O_2 should protect cells from menadione toxicity. Thus, levels of enzymes involved in superoxide anion and H_2O_2 protection such as SOD [4], G6PD [18,19], catalase and peroxidase [4] were determined in NTL4 and PB102 in order to determine if they play a role in resistance to menadione. The results showed that PB102 produced levels of SOD and G6PD activity that were similar to those of wild-type NTL4 (Fig. 2A,B), indicating that these enzymes were not responsible for the increased menadione sensitivity of PB102. By contrast, inactivation of *katA* drastically decreased the level of total catalase activity from 7.0 U mg^{-1} protein in NTL4 to 0.9 U mg^{-1} protein in PB102 (Fig. 2C) while the peroxidase activity was lowered from 3.4 mU mg^{-1} protein in NTL4 to a non-detectable level in PB102 (Fig. 2D). The decrease in catalase and peroxidase activities was probably responsible for the observed menadione sensitivity in strain PB102.

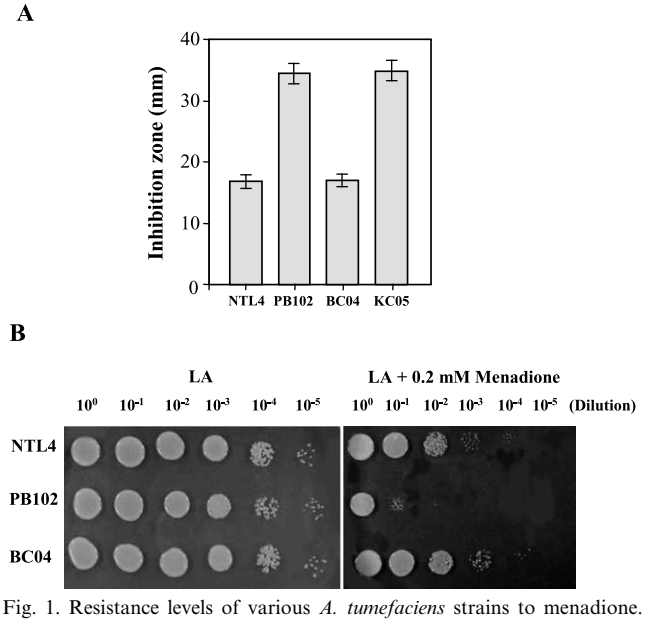

Fig. 1. Resistance levels of various *A. tumefaciens* strains to menadione. The levels of resistance against menadione of *A. tumefaciens* NTL4 wild-type, PB102 (*katA*), BC04 (*catE*) and KC05 (*katA catE*) of exponential-phase (A) and stationary-phase (B) cells were determined by inhibition zone and plate sensitivity assays, respectively, as described in Section 2. The values presented are mean ± S.D. of three independent experiments. LA refers to LB agar.

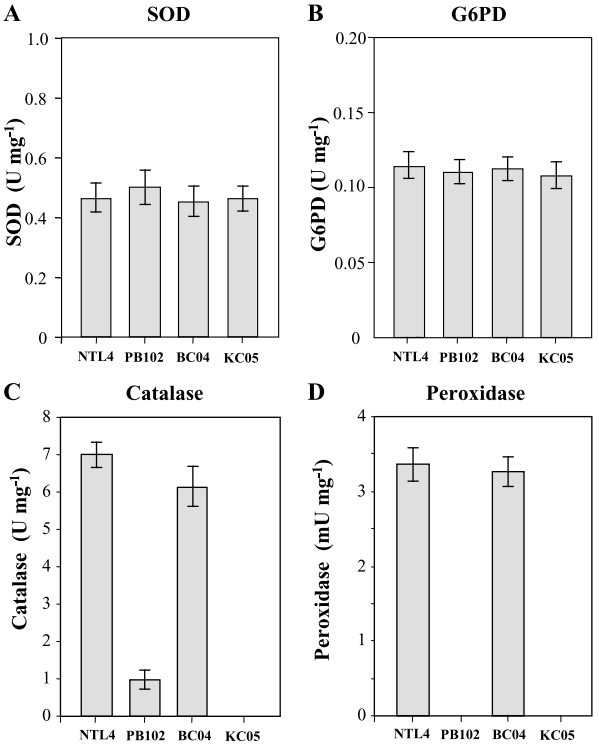

Fig. 2. The levels of antioxidant enzymes in various strains of *A. tumefaciens*. Crude lysate preparation from exponential-phase cultures of *A. tumefaciens* NTL4 wild-type, PB102 (*katA*), BC04 (*catE*) and KC05 (*katA catE*), and enzymatic assays for SOD (A), G6PD (B), catalase (C), and peroxidase (D) were performed as previously described [17]. The values presented are the mean ± S.D. of three independent experiments.

3.2. *The expression of* katA *alone complemented the menadione-sensitive phenotype in PB102*

The results of our analysis of the *katA* mutant, PB102, clearly indicated that KatA plays a significant role in the protection of *A. tumefaciens* from menadione toxicity. However, it was not clear whether the increased sensitivity of PB102 to menadione was a result of a decrease in the levels of catalase and/or peroxidase. In addition to the *katA* gene, encoding a bifunctional catalase-peroxidase en-

zyme, *A. tumefaciens* possesses the *catE* gene, which encodes a monofunctional catalase that is similar to the *E. coli* catalase HPII (KatE) [20]. In order to clearly establish which of these different catalases participates in the protection of *A. tumefaciens* from menadione toxicity, the *A. tumefaciens catE* knockout mutant (BC04) and the *katA catE* double mutant (KC05) were constructed. Growth inhibition zone assays showed that BC04 had a level of menadione resistance that was similar to that of the wild-type strain NTL4 (Fig. 1A). During exponential-phase growth the total catalase activity in BC04 was slightly lower (6.1 U mg^{-1} protein) than that in wild-type NTL4 (7.0 U mg^{-1}) (Fig. 2C), while the peroxidase activities in the two strains were not significantly different (Fig. 2D). In *E. coli*, the level of HPII catalase generally increases upon entry into the stationary phase of growth where it contributes to peroxide stress protection [20]. In order to determine if the functions of *A. tumefaciens* CatE and KatA show growth phase-dependent differences, the menadione resistance level in strains NTL4, BC04 and PB102 was measured during stationary phase. The pattern of resistance between the three strains during the stationary phase was identical to that observed during the exponential phase. No significant difference in menadione resistance levels was observed between BC04 and NTL4 (Fig. 1B), while PB102 was more sensitive than both BC04 and NTL4 (Fig. 1B). The evidence suggested that KatA, and not CatE, was involved in the protection of *A. tumefaciens* from menadione toxicity during both exponential and stationary phases of growth. This is supported by the observation that the phenotype of the *katA catE* mutant, KC05, was identical to that of PB102 (Figs. 1A and 2).

In order to further distinguish the roles of the two catalases in conferring menadione resistance, either KatA or CatE was over-expressed in the *katA* mutant strain PB102 from plasmids pKatA and pCatE, respectively. The H_2O_2 and menadione resistance levels, as well as catalase and peroxidase enzyme activities, were determined in PB102 harboring these plasmids (Fig. 3A–D). As shown in Fig. 3C, total catalase activity in PB102 harboring either pKatA (26.5 U mg^{-1} protein) or pCatE (6.9 U mg^{-1} protein) was significantly higher than the level in PB102 (0.9 U mg^{-1} protein). The expression of either *katA* or *catE* from a plasmid could restore the H_2O_2-resistant phenotype of mutant PB102 (Fig. 3A). However, PB102 harboring pCatE had comparable catalase activity to the NTL4 wild-type (7.0 U mg^{-1} protein), and yet this strain was more sensitive to H_2O_2 than was NTL4 (Fig. 3A,C). The results imply that, at similar levels of catalase activity, CatE catalase is less efficient than KatA in protecting *A. tumefaciens* from H_2O_2. The results in Fig. 3B revealed that only pKatA could complement the menadione-hypersensitive phenotype of mutant PB102. As expected, peroxidase activity (18.0 mU mg^{-1} protein) was detectable in PB102 only if it harbored pKatA (Fig. 3D). Over-expression of CatE, in strain PB102 harboring pCatE, did not significantly increase the menadione resistance level indicating that a general increase in catalase activity alone was not sufficient to confer menadione resistance to mutant PB102 (Fig. 3B). We also investigated the effect of over-expression of *katA* on the menadione resistance level in the *A. tumefaciens* wild-type strain NTL4. NTL4 harboring pKatA was significantly more resistant to menadione than NTL4 and showed elevated total catalase (35.2 U mg^{-1} protein) and peroxidase (22.9 mU mg^{-1} protein) activities (Fig. 3B–D). The results imply that menadione

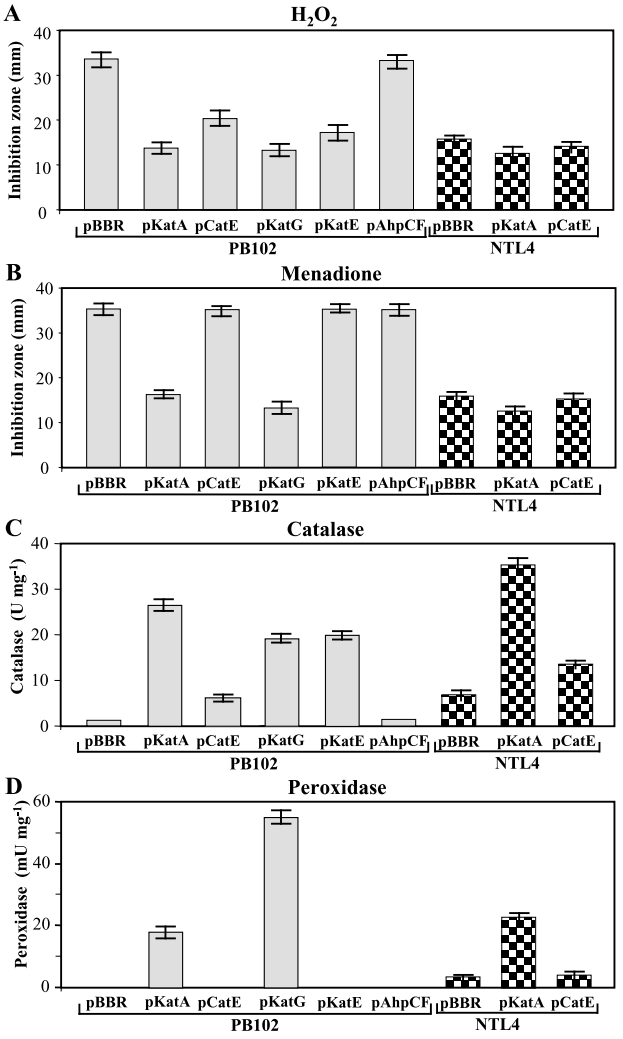

Fig. 3. The correlation between the H_2O_2 and menadione sensitivity, and the levels of catalase and peroxidase in *A. tumefaciens*. The sensitivity levels against H_2O_2 (A) and menadione (B) in *A. tumefaciens* NTL4 and mutant PB102 (*katA*), harboring plasmids expressing several peroxide-scavenging enzymes, were determined by the inhibition zone method. The levels of catalase (C) and peroxidase (D) in crude lysates prepared from exponential-phase cultures were monitored. The values presented are the mean ± S.D. of three independent experiments. The control plasmid pBBR refers to pBBR1MSC-4 containing no insert; pKatA is pBBR1MSC-4 containing *A. tumefaciens katA*; pCatE is pBBR1MSC-4 containing *A. tumefaciens catE*; pKatG is pBBR1MSC-4 containing *katG* from *B. pseudomallei*; pAhpCF is pBBR1MSC4 containing *ahpC* and *ahpF* from *X. campestris*.

toxicity in *A. tumefaciens* is not due to increased H_2O_2 production alone as shown by the inability of high levels of monofunctional catalase to confer increased menadione resistance. Thus, either the combined catalase and peroxidase activities of KatA or its peroxidase activity alone is required in order to protect *A. tumefaciens* from menadione toxicity. This is a novel role for catalase-peroxidase enzymes that has not been previously observed. The mechanism of menadione toxicity in *E. coli* and *Salmonella typhimurium* has been shown to involve the continuous generation of superoxide anions, which are subsequently dismutated to H_2O_2 [4]. While the exact mechanism of menadione toxicity in *A. tumefaciens* is unknown, it probably arises from either a combination of superoxide anions and H_2O_2 or an individual agent reacting with other cellular macromolecules to generate toxic by-products that can be removed by peroxidase enzyme activity.

3.3. Expression of a heterologous catalase-peroxidase gene restored the phenotype of PB102

The finding that only high levels of a bifunctional catalase-peroxidase (KatA) could protect PB102 from menadione toxicity prompted us to investigate whether the protection was due to the enzyme's activities or some other characteristic specific to *A. tumefaciens* KatA. To test this, genes encoding either catalase-peroxidase or monofunctional catalase from unrelated microorganisms were expressed in *A. tumefaciens* PB102 to determine if they could protect the mutant from menadione toxicity. The menadione resistance levels in PB102 harboring either pKatG (containing the *Burkholderia pseudomallei* catalase-peroxidase gene *katG* [21]) or pKatE (containing the *Xanthomonas campestris* monofunctional catalase gene *katE* [17]) were determined. PB102/pKatG produced high levels of catalase (19.1 U mg^{-1} protein) and peroxidase (55.3 mU mg^{-1} protein) (Fig. 3C,D) and was slightly more resistant to both menadione and H_2O_2 than PB102 (Fig. 3A,B). The expression of *katE* in PB102/pKatE enhanced the catalase activity (20.4 U mg^{-1}) and H_2O_2 resistance but failed to complement the menadione-susceptible phenotype of PB102 (Fig. 3A–C). The data confirm the correlation between the bifunctional catalase-peroxidase enzyme activity and menadione resistance phenotype in *A. tumefaciens*.

3.4. Organic hydroperoxide-scavenging enzyme is not involved in the menadione resistance phenotype

Bifunctional catalase-peroxidases belong to class I of the plant peroxidase superfamily and share conserved domains with cytochrome *c* peroxidase, an enzyme that plays a crucial role in the defense against organic hydroperoxide exposure [22–24]. Therefore, the possibility existed that the KatA-dependent mechanism of menadione resistance in *A. tumefaciens* might involve the metabolism of organic hydroperoxides. This possibility was discounted when it was found that the resistance levels to the organic hydroperoxide tBOOH in strains NTL4 and PB102 were found not to be significantly different (data not shown) indicating that KatA plays no major role in the protection of *A. tumefaciens* from organic hydroperoxide toxicity.

A recent observation in *X. campestris* indicates that menadione toxicity may arise from the production and accumulation of organic hydroperoxides [25]. It was found that a high level of alkyl hydroperoxide reductase (AhpCF), an enzyme that metabolizes organic hydroperoxides to their corresponding alcohols, was able to relieve the menadione-hypersensitive phenotype of a *X. campestris oxyR* mutant while a high level of catalase could not [25]. However, searches of the *A. tumefaciens* genome using the BLAST algorithm [26] did not reveal any open reading frames encoding proteins whose primary structures are highly similar to the deduced amino acid sequences of the AhpCF-encoding genes, *ahpC* and *ahpF*. Consequently, we tested whether *X. campestris* AhpCF, expressed from a plasmid (pAhpCF [27]) carrying the genes *ahpC* and *ahpF*, could protect *A. tumefaciens* from menadione toxicity. As shown in Fig. 3B, PB102 harboring pAhpCF showed the same level of menadione resistance as PB102 indicating that, unlike *Xanthomonas*, menadione toxicity in *A. tumefaciens* does not involve the accumulation of organic hydroperoxides.

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Exposure to Cadmium Elevates Expression of Genes in the OxyR and OhrR Regulons and Induces Cross-Resistance to Peroxide Killing Treatment in *Xanthomonas campestris*

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Cadmium is an important heavy metal pollutant. For this study, we investigated the effects of cadmium exposure on the oxidative stress responses of *Xanthomonas campestris*, a soil and plant pathogenic bacterium. The exposure of *X. campestris* to low concentrations of cadmium induces cross-protection against subsequent killing treatments with either H_2O_2 or the organic hydroperoxide *tert*-butyl hydroperoxide (tBOOH), but not against the superoxide generator menadione. The cadmium-induced resistance to peroxides is due to the metal's ability to induce increased levels of peroxide stress protective enzymes such as alkyl hydroperoxide reductase (AhpC), monofunctional catalase (KatA), and organic hydroperoxide resistance protein (Ohr). Cadmium-induced resistance to H_2O_2 is dependent on functional OxyR, a peroxide-sensing transcription regulator. Cadmium-induced resistance to tBOOH shows a more complex regulatory pattern. The inactivation of the two major sensor-regulators of organic hydroperoxide, OxyR and OhrR, only partially inhibited cadmium-induced protection against tBOOH, suggesting that these genes do have some role in the process. However, other, as yet unknown mechanisms are involved in inducible organic hydroperoxide protection. Furthermore, we show that the cadmium-induced peroxide stress response is mediated by the metal's ability to predominately cause an increase in intracellular concentrations of organic hydroperoxide and, in part, H_2O_2. Analyses of various mutants of peroxide-metabolizing enzymes suggested that this increase in organic hydroperoxide levels is, at least in part, responsible for cadmium toxicity in *Xanthomonas*.

Heavy metals are recognized as environmental pollutants and are released from both industrial and agricultural sources. The intensive use of high-phosphate fertilizers in agriculture leads to an increased accumulation of metal ions, especially cadmium, in the soil (34). Cadmium ions are highly toxic to ecosystems, even at very low concentrations. Although cadmium scores negative in the Ames *Salmonella* mutagenicity test, it is a carcinogen in laboratory animals and can induce DNA deletions in *Saccharomyces cerevisiae* (28). Cadmium is a non-redox-reactive heavy metal, and its toxicity is believed to be due to the depletion of glutathione and sulfhydryl groups in proteins (12, 31). Furthermore, cadmium is known to displace Zn and Fe ions from metalloproteins, resulting in their inactivation as well as the release of free Fe that can then catalyze the generation of reactive oxygen species via the Fenton reaction (30).

The effects of cadmium exposure have been examined in some microorganisms. Studies of *S. cerevisiae* indicated that cadmium increases oxidative stress, since strains deficient in antioxidant defense enzymes have a high sensitivity to cadmium and cells grown in the absence of oxygen are more tolerant to cadmium (5, 39). The exposure of bacteria to cadmium induces the expression of genes in many regulons, including genes involved in metal transport (1, 4, 27), DNA repair, the heat shock response, and the oxidative stress response (2, 10, 35).

Xanthomonas campestris pv. phaseoli is a soil bacterium and plant pathogen that often encounters reactive oxygen species (superoxide anions, H_2O_2, and organic hydroperoxides) that are generated either by other soil microorganisms or by host plants as a defense mechanism during microbial invasion (17). Thus, *Xanthomonas* uses both enzymatic and nonenzymatic strategies to survive and proliferate in the presence of these reactive oxygen species. It logically follows that any agents that affect the bacterial oxidative stress response may also alter the organism's ability to survive in the soil environment as well as affecting its pathogenicity. The peroxide-sensing transcription regulators OxyR and OhrR mediate the peroxide stress responses in bacteria (20). For *Xanthomonas*, exposure to hydroperoxide leads to OxyR oxidation, which in turn activates the expression of *ahpC*, *ahpF*, *katA*, and *oxyR* itself (21). OhrR is an organic hydroperoxide sensor and transcription repressor that regulates the expression of *ohr*, encoding a thiol peroxidase (22). These regulators and the products of the genes that they control play crucial roles in the adaptive and cross-protective responses to peroxide stress in *Xanthomonas* (20).

For this study, the effects of cadmium on the *Xanthomonas* peroxide stress protective response and the expression of genes in the *oxyR* and *ohrR* regulons were investigated. A possible mechanism for cadmium induction of peroxide stress-dependent gene expression is discussed.

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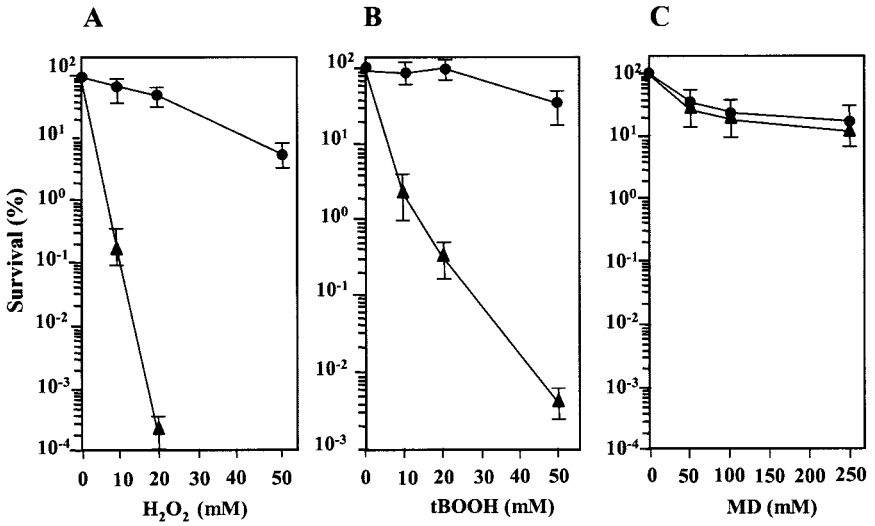

FIG. 1. Cadmium-induced cross-protection in *X. campestris* pv. phaseoli. The growth, induction, and lethal peroxide treatment of cultures were performed as described in Materials and Methods. Survival curves are shown for *X. campestris* pv. phaseoli cultures pretreated with $CdCl_2$ (●) or left untreated (▲) before being exposed to the indicated lethal concentrations of H_2O_2 (A), tBOOH (B), and MD (C). Values presented are the means and standard deviations of three replicates.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *X. campestris* pv. phaseoli was grown aerobically in Silva-Buddenhagen (SB) medium (0.5% sucrose, 0.5% yeast extract, 0.5% peptone, 0.1% glutamic acid; pH 7.0) at 28°C. Overnight cultures were inoculated into fresh SB medium to give an optical density at 600 nm (OD_{600}) of 0.1. Exponential-phase cells (OD_{600} of 0.5 after 4 h) were used in all experiments.

Construction of *X. campestris* pv. phaseoli *oxyR ohrR* double mutant. Genomic DNA extracted from *X. campestris* pv. phaseoli *oxyR* (Gen^r) (26) was transferred into *X. campestris* pv. phaseoli *ohrR* (Amp^r) (33) by electroporation as previously described (26). Extracts of the *oxyR ohrR* double mutant (Gen^r Amp^r) were analyzed by Western blotting to confirm the absence of expression of OhrR and OxyR (data not shown).

Determination of cadmium resistance levels. Analyses of the killing effects of various reagents on *Xanthomonas* strains were performed by the use of inhibition zone assays (23). Exponential-phase cultures (1 ml) were mixed with 10.0 ml of molten top agar (SB medium containing 0.7% agar) held at 50°C and then overlaid onto SB plates (14-cm-diameter petri dishes containing 40 ml of SB agar). The plates were left at room temperature for 15 min to let the top agar solidify. Sterile 6-mm-diameter paper disks, soaked with 5 μl of $CdCl_2$ (0.2 M), were placed on top of the lawn of cells, and the zones of growth inhibition were measured after 24 h of incubation at 28°C.

Determination of the effect of cadmium on oxidant killing treatments. Cadmium-induced cross-protection experiments were performed by adding 75 μM $CdCl_2$ to exponential-phase cultures. These cultures were grown for an additional 30 min before aliquots of cells were removed and treated with killing concentrations (10, 20, and 50 mM) of H_2O_2 or *tert*-butyl hydroperoxide (tBOOH) for 30 min. After treatment, the cells were removed and washed once with fresh SB medium before determinations of cell survival by plating appropriate dilutions on SB agar. Colonies were counted after 48 h of incubation at 28°C. The percent survival was defined as the number of CFU recovered after treatment divided by the number of CFU prior to treatment multiplied by 100.

Northern analysis of *ahpC* and *ohr* expression. Exponential-phase cultures were induced with 75 μM $CdCl_2$ for 15 min. Cells were harvested by centrifugation at 4°C. Total RNAs were extracted from uninduced and cadmium-induced cultures by a modified hot acid phenol method (21). Purified RNAs (10 μg) were separated in a formaldehyde-agarose gel. After electrophoretic separation, the RNAs were transferred to a piece of nylon membrane and hybridized with a radioactively labeled *ahpC* or *ohr* DNA probe, prepared as previously described (23). Prehybridization, hybridization, and high-stringency washes were done according to the methods of Mongkolsuk et al. (21).

Western immunoblot analysis. Western immunoblot analyses of AhpC levels, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotting to polyvinylidene difluoride membranes, and immunodetection with an anti-*Escherichia coli* AhpC polyclonal antibody (32), were performed as previously described (21). Antibody reactions were visualized by use of an alkaline phosphatase antibody detection kit from Promega.

Catalase activity gels. Crude bacterial lysates were prepared by resuspending *X. campestris* cell pellets in 50 mM sodium phosphate buffer (PB), pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride and exposing them to intermittent sonication until the suspensions became clear. The lysates were then centrifuged at 10,000 × g for 10 min, and the cleared supernatants (80 μg of protein) were separated in 7.5% native polyacrylamide gels (37). After electrophoresis, the gels were soaked in PB containing 50 μg of horseradish peroxidase/ml for 45 min at room temperature. The gels were then soaked in PB containing 5 mM H_2O_2 for 10 min. After being washed briefly twice with distilled water, they were immediately stained with freshly prepared PB containing 0.5 mg of diaminobenzidine/ml until the background became dark brown. Catalase activity appeared as a colorless band.

SOD assays. The xanthine-xanthine oxidase-coupled reduction of cytochrome *c* was used to monitor total superoxide dismutase (SOD) activities (19) in crude lysates. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of reduction of cytochrome *c* by 50%.

RESULTS AND DISCUSSION

Cadmium induces cross-protection from killing treatments with H_2O_2 and tBOOH. Exposure to low levels of a stress or chemical can induce increased resistance to a subsequent exposure to the same (adaptive) or an unrelated (cross-protective) stress or chemical. Bacterial adaptive and cross-protective responses are an important physiological adaptation for survival under stressful conditions and have been shown to be crucial protective mechanisms during peroxide stress (25, 36). Thus, we tested the effect of exposure to low cadmium concentrations on the physiological responses of *X. campestris* pv. phaseoli to peroxide stress. First, the effect of cadmium on the H_2O_2 resistance level was determined. *Xanthomonas* cultures were preexposed to 75 μM $CdCl_2$, and the percent survival following a subsequent treatment with a lethal concentration (10, 20, or 50 mM) of H_2O_2 was determined. The results showed that for cadmium-pretreated cells, the percent survival upon exposure to lethal concentrations of H_2O_2 was $>10^5$-fold higher than that for nontreated cells (Fig. 1A). Cadmium-

induced cross-protection against H_2O_2 has been observed in *E. coli*, for which pretreatment of the bacterial culture with $CdCl_2$ increases resistance to multiple stresses (10). Further experiments were done to determine whether cadmium could induce resistance to other oxidative stress-inducing compounds, such as organic hydroperoxide and superoxide anions. Cadmium-pretreated and nontreated cultures were treated with lethal concentrations of the organic hydroperoxide tBOOH and the superoxide-generating agent menadione (MD), and the percentages of survival were determined. Interestingly, cadmium-pretreated cells were 10^4-fold more resistant to subsequent lethal treatments with tBOOH (Fig. 1B) than nontreated cells, while no induced cross-protection from MD was observed (Fig. 1C).

In *Xanthomonas*, there are at least two global peroxide-sensing transcription regulators, OxyR and OhrR, involved in mediating peroxide stress-inducible responses (20). OxyR is essential for most peroxide stress responses and is involved in induced adaptation against H_2O_2 killing, while OhrR regulates the expression of *ohr* in response to organic hydroperoxides (20). In order to test whether the cadmium-induced cross-protection against H_2O_2 and tBOOH killing was dependent on OxyR and/or OhrR, we determined the effect of a cadmium pretreatment on the resistance to H_2O_2 and tBOOH in an *X. campestris* pv. phaseoli *oxyR* mutant (24) and an *X. campestris* pv. phaseoli *ohrR* mutant (23). The results in Fig. 2A clearly show that cadmium-induced cross-protection from H_2O_2 was completely abolished in the *oxyR* mutant. In contrast, the induced cross-protection against tBOOH killing treatments was only partially lost, since cadmium-pretreated *X. campestris* pv. phaseoli *oxyR* was 100-fold more resistant to tBOOH than the nontreated control (Fig. 2B). The inactivation of *ohrR* had no effect on cadmium-induced H_2O_2 protection (data not shown). However, the loss of OhrR led to a reduction in the level of cadmium-induced protection against tBOOH (Fig. 2C). An analysis of the peroxide regulatory mutants clearly showed that the cadmium-induced cross-protection from H_2O_2 was wholly dependent on functional OxyR, while the cadmium-induced cross-protection from tBOOH was more complex and appears to involve genes in both the OxyR and OhrR regulons. These observations were extended by examining the cadmium-induced cross-protection from tBOOH of the *oxyR ohrR* double mutant (Fig. 2D). Cadmium-pretreated *X. campestris* pv. phaseoli *oxyR ohrR* cells were 50-fold more resistant to tBOOH than nontreated cells; however, the relative difference between the resistance levels of the pretreated and nontreated *oxyR ohrR* cells was less than that observed for either *X. campestris* pv. phaseoli *oxyR* or *X. campestris* pv. phaseoli *ohrR* (Fig. 2B, C, and D). These data reinforce the suggestion that genes in both the OxyR and OhrR regulons are required for high-level cadmium-induced resistance to tBOOH. Moreover, the fact that the *oxyR ohrR* double mutant could still mount a partial cadmium-induced cross-protective response to tBOOH indicates that other unknown gene systems participate in the process. Likely candidates for this role are genes encoding glutathione peroxidase-like and bactoferritin comigratory proteins (13, 15), which are found in the *X. campestris* genome and are known to be involved in organic hydroperoxide metabolism (8). These genes, in addition to genes in the OxyR and OhrR regulons, are likely to play some role in the detoxification of and protection against organic hydroperoxides. The regulatory mechanisms controlling these genes have not yet been characterized, so it is not possible to firmly assign them roles in the cadmium-induced cross-protective response to tBOOH.

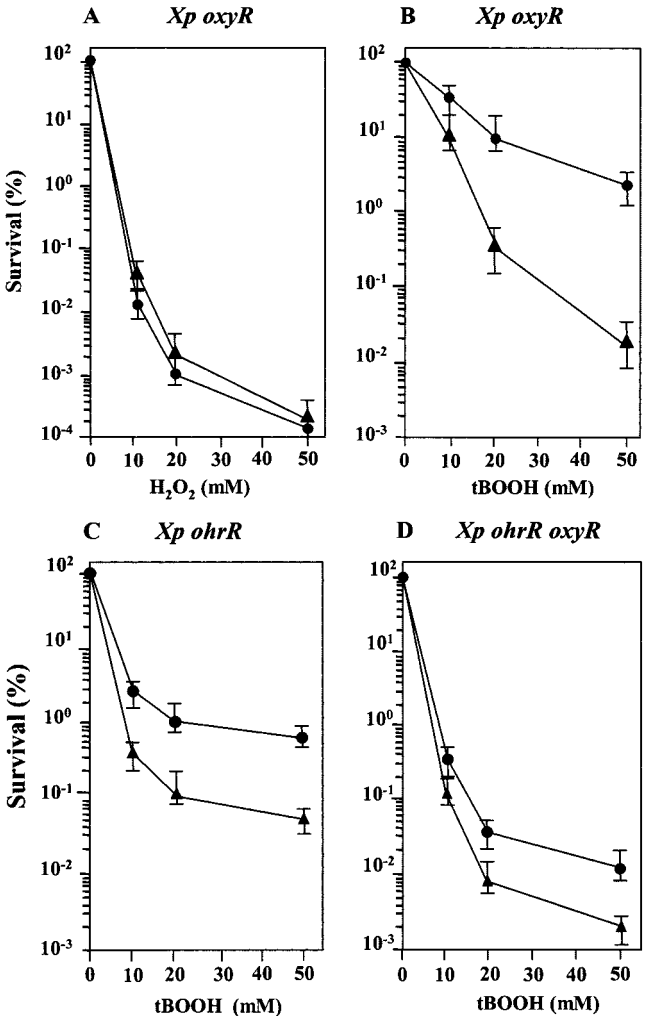

FIG. 2. Cadmium-induced cross-protection in various *X. campestris* pv. phaseoli mutant strains lacking peroxide stress response regulators. Survival curves are shown for exponential-phase cultures of the *oxyR* mutant (*Xp oxyR*) (A and B), the *ohrR* mutant (*Xp ohrR*) (C), and the *oxyR ohrR* double mutant (*Xp oxyR ohrR*) (D) pretreated with $CdCl_2$ (●) or left untreated (▲) prior to exposure to lethal concentrations of H_2O_2 or tBOOH at the indicated concentrations. The values presented are the means and standard deviations of three replicates.

Exposure to cadmium induces high-level expression of peroxide-scavenging enzymes. The ability of bacteria to protect themselves from peroxide stress is often associated with inducible increases in the levels of peroxide detoxification and protection enzymes (25, 36). Since the physiological data indicated that high-level cadmium-induced protection against peroxide depended on functional peroxide-sensing regulators, the effects of cadmium on the levels of peroxide and superoxide detoxification enzymes were examined. Catalases are the major protective enzymes against H_2O_2 toxicity. *X. campestris* possesses two monofunctional catalase isozymes, denoted KatA and KatE (37). The KatA catalase is peroxide inducible and is

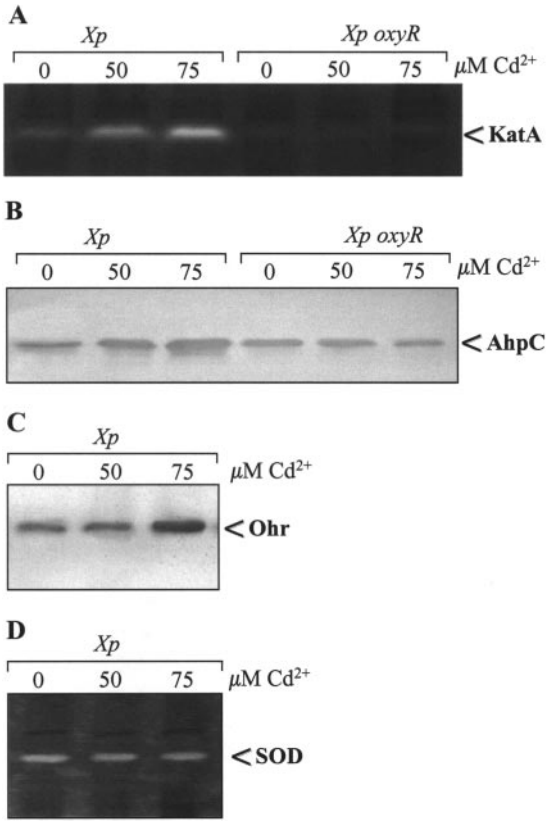

FIG. 3. Induction of peroxide-scavenging enzymes. The levels of KatA catalase (A), AhpC (B), Ohr (C), and SOD (D) in cultures of *X. campestris* pv. phaseoli (*Xp*) and its *oxyR* mutant (*Xp oxyR*), pretreated with the indicated concentrations of $CdCl_2$, were determined by activity gel staining (for KatA and SOD) and Western blot analysis (for AhpC and Ohr) as described in Materials and Methods.

produced during all growth phases (S. Mongkolsuk, unpublished data). KatE is a growth-phase-dependent enzyme whose expression increases as cells enter stationary phase (37). The effect of cadmium exposure on catalase levels in *X. campestris* was determined by catalase activity staining of polyacrylamide gels, which can differentiate between the two isozymes. The results, shown in Fig. 3A, revealed that the exposure of *X. campestris* to 75 μM $CdCl_2$ for 30 min induced a 10-fold increase in the KatA level, as estimated by densitometry. The cadmium-dependent induction of KatA required functional *oxyR* since its expression was abolished in the *Xanthomonas oxyR* mutant (Fig. 3A). We have shown previously that in *Xanthomonas* the level of resistance to H_2O_2 is correlated with the catalase activity (38). The increased level of KatA resulting from exposure to cadmium likely accounts for the observed cadmium-induced cross-protection against H_2O_2 killing. Cadmium-induced protection from organic hydroperoxides showed complex physiological and regulatory patterns. Therefore, the effects of cadmium on the expression of the OhrR-regulated organic hydroperoxide detoxification enzymes alkyl hydroperoxide reductase and Ohr were also evaluated. Alkyl hydroperoxide reductase consists of two subunits, a catalytic subunit, AhpC, and a reductase subunit, AhpF. Western blot analysis with anti-*E. coli* AhpC was performed to determine the level of AhpC in lysates prepared from cadmium-pretreated and nontreated cultures. As shown in Fig. 3B, the level of AhpC increased 10-fold after the culture was exposed to 75 μM cadmium for 30 min, while no cadmium-dependent induction of AhpC was observed for the *oxyR* mutant (Fig. 3B). The effect of cadmium exposure on *ohr* expression was investigated. Northern blots of total RNAs, extracted from *X. campestris* cultures challenged with cadmium for 15 min, were probed with a ^{32}P-labeled *ohr*-specific DNA probe. Densitometer analysis of the Northern results showed an eightfold induction of *ohr* mRNA levels in cadmium-induced cultures compared to those in uninduced cultures (Fig. 3C). The cadmium-dependent induction of *ohr* expression was abolished in the *ohrR* mutant (data not shown). Clearly, cadmium is a potent inducer of peroxide-scavenging enzymes, and this effect is dependent on the functional global peroxide sensor-transcription regulators OxyR and OhrR. The data also indicated that the cadmium-induced cross-protection from peroxide killing was due to cadmium's ability to induce the high-level expression of these peroxide-scavenging enzymes.

The inability of cadmium to induce a protective response against MD killing was surprising since we have previously shown that elevated levels of AhpCF can prevent MD toxicity in an *X. campestris oxyR* mutant (36). However, it has also been shown that residual organic peroxides in combination with MD can dramatically enhance MD's toxicity (29). It is possible that a pretreatment with cadmium at 75 μM continuously generates large quantities of organic hydroperoxides, as evident by its ability to deactivate the OhrR repressor, resulting in increased expression of *ohr*. Therefore, the increased AhpCF levels in cadmium-pretreated *X. campestris* cells may not be sufficient to confer additional protection against MD killing. In *Xanthomonas*, as in other bacteria, SODs play a crucial role in protecting cells against superoxide anion toxicity. A genome analysis of *X. campestris* revealed five putative open reading frames that have high homology scores to genes for known SODs (8). The effect of the cadmium pretreatment of *X. campestris* cultures on the total SOD activity was determined. As expected, the results revealed that a pretreatment with cadmium failed to increase the levels of total SOD activity (Fig. 3D). Thus, the inability of the metal to induce SODs may be responsible for the observed lack of cadmium-induced resistance to MD killing.

High levels of peroxide-scavenging enzymes modulate the cadmium-induced stress response. During the *Xanthomonas* peroxide stress response, the oxidation of OxyR by peroxides is responsible for the transcriptional activation and high-level expression of genes in its regulon, including *ahpCF* and *katA* (18, 21, 38). Furthermore, the oxidation of OhrR by organic hydroperoxides, such as tBOOH and cumene hydroperoxide, derepresses the expression of *ohr* (22). The data shown in Fig. 3 indicate that cadmium is a potent inducer of peroxide-scavenging enzymes. This probably results from the oxidation of OxyR and OhrR and the subsequent activation of genes in their respective regulons. The mechanisms by which cadmium treatment leads to the oxidation of OxyR and OhrR are not known. Cadmium is classified as a non-redox-reactive heavy metal; however, in eukaryotic cells it has been shown to generate intracellular oxidative stress (5, 30). Thus, cadmium may oxidize OxyR and OhrR, either indirectly or directly, through its ability to generate intracellular superoxide anions and/or

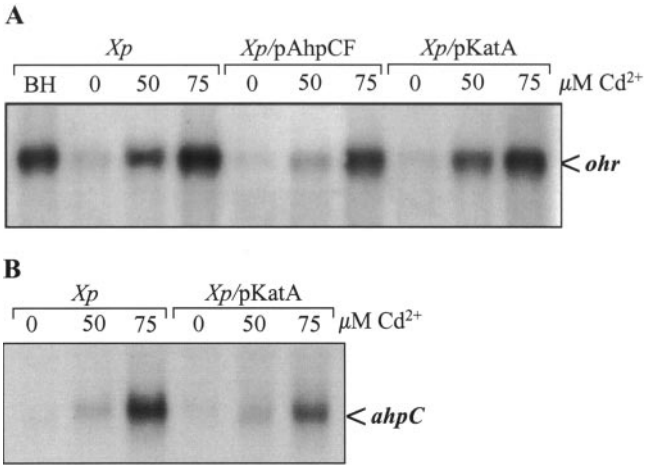

FIG. 4. Effect of high levels of peroxide-scavenging enzymes on cadmium-induced *ohr* and *ahpC* expression. Northern blot results are shown for RNAs prepared from cultures of *X. campestris* pv. phaseoli harboring pBBR1MSC-4 (*Xp*), pAhpCF for the overexpression of AhpCF (*Xp*/pAhpCF), or pKat for the overexpression of KatA (*Xp*/pKatA) that had been pretreated with the indicated concentrations of $CdCl_2$ and hybridized with radioactively labeled *ohr* (A)- and *ahpC* (B)-specific DNA probes. Arrowheads indicate *ohr*- and *ahpC*-specific mRNAs. BH represents induction with 100 μM tBOOH.

peroxides (both H_2O_2 and organic hydroperoxides). For *Xanthomonas*, it was shown previously that the expression of *ohr* is specifically induced by exposure to organic hydroperoxides (23). We reasoned that if exposure to cadmium causes the in vivo generation of organic hydroperoxides that in turn oxidize OhrR, then the high-level expression of an organic hydroperoxide-scavenging enzyme such as AhpCF should reduce the in vivo concentration of organic hydroperoxide, leading to a reduction in the level of oxidized OhrR and a corresponding decrease in the level of *ohr* expression. Exponential-phase cultures of *X. campestris* harboring pAhpCF (an expression vector carrying *ahpC* and *ahpF*) (21) and control cultures harboring the empty vector were treated with 0, 50, and 75 μM $CdCl_2$ for 10 min before the total RNAs were extracted and Northern blot hybridization was performed with a ^{32}P-labeled *ohr* probe. The results shown in Fig. 4A indicate that cadmium induced a high-level expression of *ohr* in cells containing the vector alone. In contrast, in cells overexpressing AhpCF from a plasmid, the level of *ohr* mRNA, as determined by densitometry, was reduced fivefold (for 50 and 75 μM $CdCl_2$ treatments) relative to *X. campestris* containing the vector alone (Fig. 4A). These results strongly favor the hypothesis that the exposure of *X. campestris* to cadmium increases the generation of organic hydroperoxides. Using a similar rationale, we conducted further experiments to determine how cadmium treatment leads to the oxidation of OxyR. We reasoned that if a cadmium treatment results in the production of H_2O_2, then *X. campestris* harboring pKatA (carries *katA*) (6), which expresses high levels of the H_2O_2-metabolizing enzyme catalase, should have lower levels of cadmium-induced *ahpC* expression than a strain harboring the vector alone. However, if organic hydroperoxide alone is responsible for the oxidation of OxyR, then the magnitude of *ahpC* induction should not be affected. Cultures of *X. campestris* and *X. campestris* harboring pKatA were treated with various concentrations of cadmium. Northern blots generated with mRNAs isolated from these cultures were hybridized with an *ahpC*-specific DNA probe. Densitometer analysis of the *ahpC*-specific Northern blot shown in Fig. 4B indicated that the KatA-overexpressing strain contained 50% less *ahpC* mRNA than the wild-type strain after treatment with 75 μM cadmium. The Northern blot results suggest that cadmium-induced production from H_2O_2 contributes significantly to the oxidation of OxyR and the subsequent activation of *katA* and *ahpC* expression in *Xanthomonas*.

Taken together, these results indicate that both organic hydroperoxide and H_2O_2 resulting from cadmium treatment are responsible for the oxidation of OhrR and OxyR and the subsequent enhanced expression of genes in these regulons.

Inactivation of *oxyR* and *ohrR* increases cadmium sensitivity. The ability of cadmium to generate reactive oxygen species (ROS), including organic hydroperoxides, suggests that oxidative stress may be one of the mechanisms responsible for cadmium toxicity in vivo. This notion is consistent with previous reports, mostly for eukaryotic systems, indicating that exposure to cadmium increases the levels of ROS (5). In order to examine this possibility in *Xanthomonas*, we determined the cadmium resistance levels in several peroxide stress response mutants by using an inhibition zone assay. The *ohrR* and *oxyR* mutants showed equal resistances to cadmium by giving inhibition zones of 25 mm in diameter, which were significantly larger (less resistant) than that of wild-type *X. campestris* pv. phaseoli (22 mm). Furthermore, the *oxyR ohrR* double mutant was more sensitive to cadmium than either the *oxyR* or *ohrR* single mutant, as judged by its inhibition zone of 27 mm. This evidence is consistent with investigations of *E. coli* and *Salmonella enterica* serovar Typhimurium (16) and indicates that genes in oxidative stress protective pathways contribute significantly to bacterial resistance to cadmium and that the mechanism of cadmium toxicity involves the production of toxic levels of peroxides. The data also indicate that the OhrR and OxyR regulons each function in a somewhat specialized, nonredundant manner to protect cells from cadmium toxicity. We extended the investigation to determine the cadmium resistance levels in *ahpC*, *katA*, and *ohr* mutant strains. Interestingly, the inactivation of *ahpC* and *ohr*, encoding organic hydroperoxidases, resulted in a significant reduction in the cadmium resistance levels, while the inactivation of *katA*, encoding catalase, had no effect (data not shown). This suggested that H_2O_2 has a minor role in the process and that organic hydroperoxides are the major ROS generated as a consequence of cadmium exposure. This idea is supported by the Northern hybridization results reported earlier showing that the overexpression of AhpCF had a larger negative effect on the cadmium-dependent induction of *ohr* than the overexpression of KatA had on the cadmium-dependent induction of *ahpC* (i.e., 10-fold versus 2-fold, respectively, with 75 μM $CdCl_2$). How then might exposure to cadmium lead to an increase in the levels of lipid hydroperoxides? Cadmium is classified as a non-redox-active metal, and thus the ability of the metal to directly cause peroxidation of membrane lipids resulting in the production of lipid hydroperoxides has only a minor role (14, 31). Cadmium is very reactive toward sulfhydryl groups and causes the depletion of glutathione and the inactivation of enzymes (3, 11). Thus, cadmium ions that enter the

cytoplasm likely cause an increase in the level of lipid hydroperoxides by inhibiting enzymes involved in their metabolism, such as AhpC and Ohr. Both AhpC and Ohr have a cysteine residue at their active site, and mutations in these cysteine residues have been shown to inactivate these enzymes (7, 9). In addition, the depletion of glutathione, a common electron donor for oxidative stress protective enzymes, may lead to oxidative stress conditions, and the generated ROS may react directly with membrane lipids, resulting in the increased production of organic hydroperoxides. Clearly, the cadmium-dependent induction of oxidative stress is a complex process resulting from interactions of the metal with multiple enzyme systems. Ongoing efforts in our laboratory are focusing on identifying the ROS produced in response to cadmium exposure as well as more clearly defining the protective roles of enzymes within the OxyR and OhrR regulons.

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OxyR mediated compensatory expression between *ahpC* and *katA* and the significance of *ahpC* in protection from hydrogen peroxide in *Xanthomonas campestris*

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Abstract

katA and *ahpC*, encoding monofunctional catalase and alkyl hydroperoxide reductase, respectively, play important protective roles against peroxide toxicity in *Xanthomonas campestris* pv. *phaseoli* (Xp). The expression of both *katA* and *ahpC* is controlled by the global peroxide sensor and transcriptional activator, OxyR. In Xp, these two genes have compensatory expression patterns. Inactivation of *katA* leads to an increase in the level of AhpC and a concomitant increase in resistance to *tert*-butyl hydroperoxide (tBOOH). High-level expression of *katA* from an expression vector in Xp also lowered the level of *ahpC* expression. The compensatory regulation of *katA* and *ahpC* was mediated by OxyR, since the compensatory response was not observed in an *oxyR* mutant background. *ahpC* and *katA* play important but unequal roles in protecting Xp from H_2O_2 toxicity. These observations, taken together with a previous observation that an *ahpC* mutant expresses high levels of KatA and is hyper-resistant to H_2O_2, suggest the possibility that inactivation of either gene leads to accumulation of intracellular H_2O_2. This in turn oxidizes reduced OxyR and converts the regulator to the oxidized form that then activates expression of genes in the OxyR regulon.

Keywords: *ahpC*; Catalase; H_2O_2; *katA*; OxyR; *Xanthomonas*

1. Introduction

Xanthomonas campestris is an important bacterial plant pathogen that causes disease in many economically important crops. During infection, a key component of the initial plant defense response against the invading microorganisms is the rapid production and accumulation of reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2) and superoxide anions [1]. Elevated ROS are highly toxic and can cause detrimental effects to living cells through their ability to cause lipid peroxidation, protein modification and DNA damage [2]. In order to survive oxidative stress, bacteria have evolved a variety of enzymes capable of detoxifying ROS and repairing damage generated by them.

X. campestris produces at least one isozyme of manganese superoxide dismutase and two isozymes of

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monofunctional catalase, namely KatA (formerly Kat1) and KatE, to cope with superoxide anions and H_2O_2, respectively [3–5]. KatA is the major catalase produced throughout all phases of growth and its expression is inducible by exposure to oxidants in an OxyR (a peroxide sensor and transcription regulator) dependent manner. KatE is only detected as cells enter the stationary phase of growth or during starvation conditions [4]. The bacterium also produces alkyl hydroperoxide reductase (AhpCF) and an organic hydroperoxide resistance enzyme (Ohr) that function synergistically to catalyze the conversion of organic peroxides to alcohols [6]. These genes are differentially regulated. Expression of *ahpC* is induced by exposure to oxidants in an OxyR dependent manner [7,8] while *ohr* expression is strongly induced only by organic hydroperoxide through the action of OhrR, a transcription repressor and sensor of organic hydroperoxide [9].

In several bacteria, the response to peroxides is complicated and essential for their survival under oxidative stress. An increase in catalase levels upon inactivation of *ahpC* has been reported in both Gram-negative and Gram-positive bacteria including *Pseudomonas aeruginosa*, *X. campestris* and *Bacillus subtilis* [10–12]. These compensatory alterations in gene expression are presumably mediated by transcriptional regulators that sense changes in the level of peroxides, either H_2O_2 or organic hydroperoxides. However, observations in catalase-peroxidase defective strains of *Mycobacterium tuberculosis* and *Burkholderia pseudomallei* revealed an enhanced *ahpC* gene transcription to compensate for the loss of catalase-peroxidase activity [13–15]. Here, we report the characterization of the compensatory expression of *ahpC* and *katA* in *X. campestris* pv. *phaseoli* and show that this process requires OxyR.

2. Materials and methods

2.1. Growth conditions and media

All *Xanthomonas* strains were grown aerobically at 28 °C in Silva-Buddenhagen (SB) medium containing the appropriate antibiotics [16]. All *Escherichia coli* strains were grown aerobically at 37 °C in Luria–Bertani (LB) medium.

2.2. Nucleic acid manipulations

All nucleic acid manipulations were performed according to standard molecular biology techniques [17] or to manufacturers' recommendations. The labeling of the DNA probes with [α-^{32}P]-dCTP was performed using a DNA labeling bead (Amersham Pharmacia Biotech). Southern and Northern Blot analyses were performed as described [16].

2.3. Catalase activity gel staining and assay

Cell lysate preparation and catalase activity gel staining were performed as previously described [4]. Bacterial cells were lysed in 50 mM sodium phosphate buffer pH 7.0 by brief sonication followed by centrifugation at 10,000*g* for 10 min and the supernatants were used for catalase activity gels. Catalase isozymes were separated on native PAGE gels and visualized as previously described [4]. Catalase activity appeared as colourless bands against a dark brown background. The catalase activity assay was carried out spectrophotometrically as previously described [19]. One unit of catalase was defined as the amount of enzyme required to decompose 1.0 μmol of H_2O_2 at 25 °C at pH 7.0.

2.4. Construction of ahpC1 katA mutant

A *X. campestris* pv. *phaseoli ahpC1 katA* double mutant was constructed by transferring the *katA* mutant locus of strain Xp20 [19] into strain Xp *ahpC1* [11] by electroporation using Xp20 chromosomal DNA. The Kan^r and Amp^r, *ahpC katA* mutant, Xp29, was selected and confirmed by Southern blot hybridization (data not shown).

2.5. Determination of oxidant resistance

Analysis of the killing effects of various reagents on *Xanthomonas* strains was performed using inhibition zone assays as described by Mongkolsuk et al. [18]. Briefly, overnight cultures were subcultured as a 5% inoculum into fresh SB broth. One ml of exponential phase cells (4 h culture, $OD_{600\ nm}$ of 0.5) was mixed with 10 ml of molten top agar (SB containing 0.7% agar) held at 50 °C, and overlaid onto SB plates (14 cm-diameter Petri-dishes containing 40 ml of SB agar). The plates were left at room temperature for 15 min to let the top agar solidify. Sterile 6-mm-diameter paper discs soak with 5 μl of either 0.3 M H_2O_2 or 1.0 M *tert*-butyl hydroperoxide (tBOOH), were placed on the surface of the cell lawn. The diameter of inhibition zones was measured after 24 h of incubation at 28 °C.

3. Results and discussion

3.1. Alterations in the phenotype and gene expression patterns of a katA mutant

katA encoding a house-keeping clade II monofunctional catalase from *X. campestris* pv. *phaseoli* (Xp) has been cloned and characterized [3]. The *katA* mutant, Xp20, is highly sensitive to H_2O_2 during exponential and stationary phase growth [19]. In addition, it has been shown that inactivation of *ahpC*, encoding the major

peroxide metabolizing enzyme, alkyl hydroperoxide reductase, results in pleiotropic effects on the peroxide stress response [11]. Thus, the resistance level of Xp20 to the organic hydroperoxide, *tert*-butyl hydroperoxide (tBOOH) was determined by growth inhibition zone assay. Surprisingly, Xp20 was more resistant to tBOOH (1.0 M) than the wild type strain (Xp) and displayed a zone of growth inhibition of 25 mm as compared to a zone of 29 mm for Xp (Fig. 1). In *Xanthomonas*, there are two major organic hydroperoxide detoxification systems, consisting of the organic hydroperoxidase, Ohr (organic hydroperoxide resistance), and the alkyl hydroperoxide reductase, AhpC, that act synergistically to protect cells from organic hydroperoxide toxicity [6,16,18]. In order to further investigate the mechanism responsible for the phenotype, the expression levels of these genes were determined in Xp20 and Xp using Northern blot hybridization. The results in Fig. 2(a) and (b) clearly illustrated that the level of *ahpC* transcripts in Xp20 was approximately 3-fold higher than the level in strain Xp, as determined by densitometry, while the level of *ohr* was not significantly different. The results suggested that the increased level of *ahpC* expression was responsible for the enhanced resistance against tBOOH killing phenotype of Xp20 (Fig. 1). The data also suggested that inactivation of *katA*, a major catalase, was likely to be responsible for the

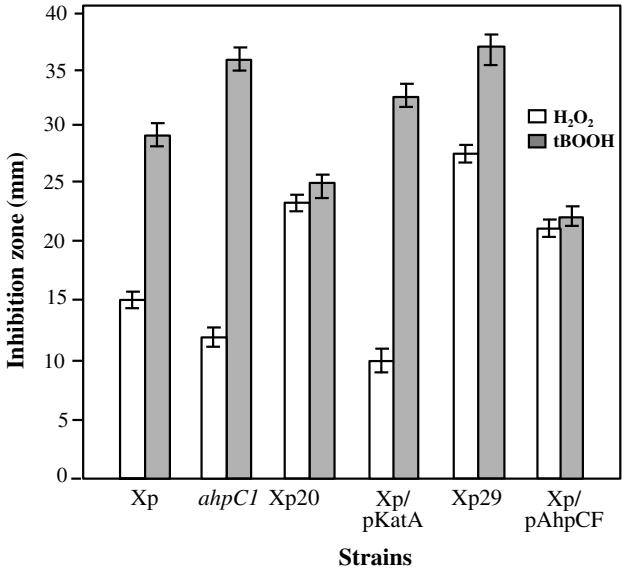

Fig. 1. The levels of peroxide resistance in *X. campestris* pv. *phaseoli* strains. Exponential phase cultures of *X. campestris* pv. *phaseoli* strains were tested for their resistance against H_2O_2 (open bars) and *tert*-butyl hydroperoxide (tBOOH, shaded bars) by inhibition zone assay as described in Section 2. The values presented are the means and standard deviations of three replicates. Xp (wild type harbouring pBBR1MCS-3 vector control), *ahpC1* (Xp *ahpC* mutant harbouring pBBR1MCS-3) Xp20 (*katA* mutant harbouring pBBR1MCS-3), Xp/pKatA (Xp harbouring pKatA), Xp29 (*katA ahpC*; Xp/pAhpCF (Xp harbouring pAhpCF).

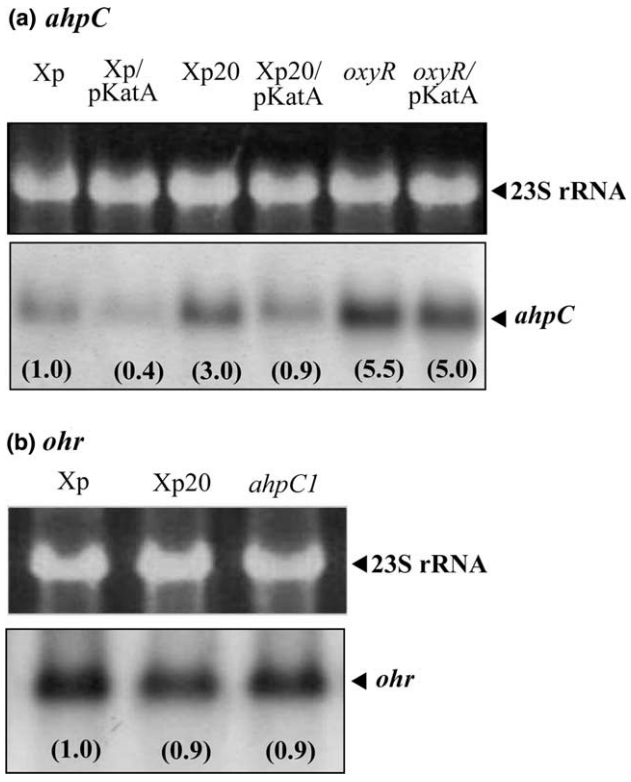

Fig. 2. The levels of peroxide scavenging enzymes and transcripts in *X. campestris* pv. *phaseoli* strains. The levels of *ahpC* transcripts (a) and *ohr* transcripts (b) in *X. campestris* pv. *phaseoli* strains were determined as described in Section 2. Xp, wild type; Xp/pAhpCF (Xp harbouring pAhpCF); Xp20 (*katA*); Xp20/pKatA (Xp20 harbouring pKatA); Xp29 (*katA ahpC*), *ahpC1* (Xp *ahpC*); *oxyR* (Xp *oxyR*); *oxyR*/pAhpCF (*Xp oxyR* harbouring pAhpCF). The level of 23S rRNA as loading control is shown above the autoradiograph of the Northern blot. Number in parenthesis underneath the hybridized band represents the fold induction calculated from densitometric data comparing with the Xp wild type.

elevated *ahpC* expression. This assumption was supported by the observation that expression of KatA, from pKatA (a broad-host-range plasmid containing full-length *katA* in pBBR1MCS-3 [20]), in Xp20 caused a reduction in *ahpC* mRNA to a level that was close to that of the parental wild type strain (Fig. 2(a)). Analysis of the compensatory link between KatA and AhpC expression was extended by the determination of the effect of high-level *katA* expression from pKatA on the level of *ahpC* transcripts in the wild type strain Xp. The results in Fig. 2(a) clearly showed that Xp/pKatA produced a lower level of *ahpC* mRNA relative to Xp harbouring the vector control.

The previous finding that inactivation of *X. campestris ahpC* leads to an increase in KatA levels [11] suggested a two-way compensatory link between *katA* and *ahpC* expression. Thus, the effect of high-level expression of *ahpCF* on the level of KatA was investigated using strain Xp harbouring the AhpCF expression plasmid pAhpCF (pBBR1MSC-3 containing *ahpCF*).

Catalase activity was monitored in exponential phase cells using total catalase activity assays and catalase activity stained gels that could differentiate between different catalase isozymes. High-level expression of *ahpCF* in strain Xp/pAhpCF resulted in a 3.5-fold reduction (i.e. from 5.3 U/mg protein in Xp harbouring the vector control to 1.5 U/mg protein in Xp/pAhpCF) in total catalase activity (Fig. 3) that was due to a decrease in KatA levels (data not shown) (*Note*: In Xp, KatA is the only isozyme that is detectable by catalase activity stained gels during the exponential phase of growth [4]). The changes in the ability to survive peroxide stresses in these strains were also tested. The results in Fig. 1 revealed that Xp containing pAhpCF was more resistant to tBOOH, but significantly more sensitive to H_2O_2 than was Xp containing the plasmid vector. This suggested that the reduction in catalase was responsible for the H_2O_2 sensitive phenotype and that the high level of AhpCF in the strain could not fully compensate for the loss of catalase.

In *X. campestris, katA* and *ahpC* are members of a regulon controlled by the peroxide-sensing transcriptional regulator, OxyR [7,19]. OxyR senses the intracellular concentration of H_2O_2. When the concentration of H_2O_2 reaches a critical level, reduced OxyR is oxidized by H_2O_2 and can then activate transcription of genes in the regulon. It was possible that the compensatory expression of *ahpC* and *katA* was mediated by OxyR most likely in response to the accumulation or dissipation of intracellular H_2O_2 resulting from changes in the relative levels of expression of the peroxide metabolizing enzymes. The finding that an elevated level of KatA in the *ahpC1* mutant did not occur in the *ahpC1 oxyR* double mutant suggests the involvement of OxyR in this compensatory process (Fig. 3 and [11]). The role of OxyR in the compensatory increase in *ahpC* expression in a *katA* mutant was also investigated. The direct investigation of the role of OxyR in the compensatory process was complicated by the fact that inactivation of the *oxyR* alone leads to increased basal expression of AhpC. This is thought to be due to repression of *ahpC* expression by reduced OxyR in the absence of H_2O_2 [21]. Therefore, an alternative approach, that involved testing the effect of high-level expression of *katA* on the level of *ahpC* expression in a wild type (Xp/pKatA) and an *oxyR* (Xp*oxyR*/pKatA, [21]) background, was performed. In Xp/pKatA (Fig. 2(a)), high-level expression of *katA* led to a 3-fold reduction in the level of *ahpC* mRNA. This response was abolished in an Xp *oxyR*/pKatA (Fig. 2(a)) indicating that the compensatory expression of *katA* and *ahpC* is mediated by OxyR. We postulate that the high catalase level in Xp/pKatA makes it more efficient at the removal of endogenously generated H_2O_2 than the wild-type control strain harbouring the vector alone. Thus, the OxyR pool remains reduced thereby repressing the expression of *ahpC* [7].

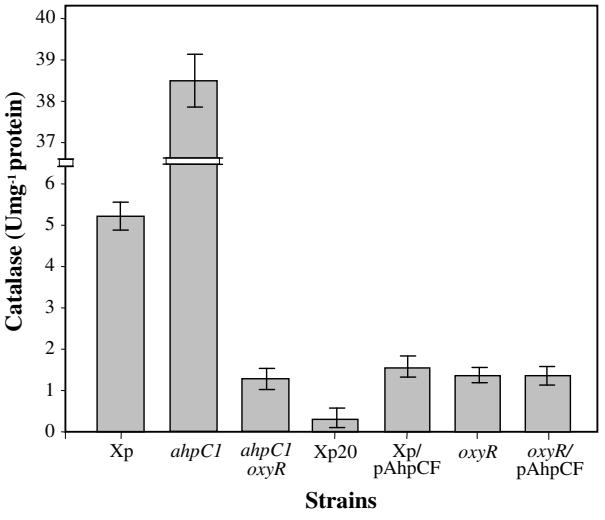

Fig. 3. Total catalase activity in *X. campestris* pv. *phaseoli* strains. Crude lysates prepared from exponential phase cultures were monitored for total catalase activity. The values presented are the means and standard deviations of three replicates. Xp (wild type harbouring pBBR1MCS-3 vector control), *ahpC1* (Xp *ahpC1*), *ahpC1 oxyR* (Xp *ahpC1 oxyR*), Xp20 (*katA* mutant harbouring pBBR1MCS-3), Xp/pAhpCF (Xp harbouring pAhpCF); *oxyR* (Xp *oxyR*); *oxyR*/pAhpCF (Xp *oxyR* harbouring pAhpCF).

3.2. The compensatory increase in KatA in an ahpC mutant is not due to organic hydroperoxide accumulation

While the data clearly indicated that the compensatory expression of *ahpC* and *katA* was mediated by OxyR (Figs. 2 and 3), the nature of the signal responsible for the compensatory regulation remained unknown. Previous studies have shown that *X. campestris* pv. *phaseoli* OxyR could be activated by both H_2O_2 and organic hydroperoxides [16]. Thus, the compensatory expression of *ahpC* and *katA* could be due to increased levels of endogenous organic hydroperoxides and/or H_2O_2 that subsequently oxidized OxyR and activated expression of genes in the regulon. Since *ahpC* can metabolize and confer resistance to both H_2O_2 and organic hydroperoxide, it was possible that inactivation of the gene could lead to the intracellular accumulation of either H_2O_2 and/or organic hydroperoxides [11]. In addition to AhpC, *X. campestris* pv. *phaseoli* produces Ohr, a second organic hydroperoxide-specific hydroperoxidase [6]. The expression of *ohr* is regulated by OhrR, an organic hydroperoxide sensor and transcriptional repressor that specifically senses organic hydroperoxides and not other oxidants [9]. In order to distinguish between H_2O_2 and organic hydroperoxides as the signal molecule responsible for the compensatory response, the levels of *ohr* transcripts were measured in Xp wild type, Xp20 and *ahpC1* strains using Northern blot hybridization analysis (Fig. 2(b)). It was reasoned that the *ohr–ohrR* system should be a valid proxy for the

indirect monitoring of the intracellular accumulation of organic hydroperoxides since increased levels of organic hydroperoxide would be accompanied by increased expression of *ohr*. The results in Fig. 2(b) showed that both the *ahpC1* mutant and the wild type strain *Xp* accumulated similar levels of *ohr* transcripts, indicating that the concentration of intracellular organic hydroperoxide was not increased in strain *ahpC1*. Since OhrR is more sensitive to the presence of organic hydroperoxides, especially to fatty acid hydroperoxides, than OxyR (unpublished data), organic hydroperoxides are unlikely to be responsible for the observed activation of *katA* by OxyR in the *ahpC1* mutant.

3.3. Evaluation of the role of AhpC in H_2O_2 protection

The availability of mutants in both a regulatory gene (*oxyR*) and genes for metabolizing enzymes (*ahpC* and *katA*) allowed their roles in the protection against H_2O_2 to be evaluated. A *katA* mutant (Xp20) was highly sensitive to H_2O_2 and yet it was still less susceptible to exogenous treatments with H_2O_2 than the regulatory, *oxyR* mutant (Fig. 1 and [21]). It was presumed the increased H_2O_2 sensitive phenotype of the *oxyR* mutant, was the consequence of a defect in its ability to induce AhpC expression. In order to elucidate the function of AhpC in the protection of *Xanthomonas* from H_2O_2, the *katA ahpC* double mutant, Xp29, was constructed (see Section 2). The results in Fig. 1 clearly illustrated that Xp29 was more sensitive to H_2O_2 killing treatments than Xp20 suggesting that in *Xanthomonas* AhpC and KatA play important, but unequal roles in the defense against H_2O_2. This observation is consistent with the proposed role of AhpC in protecting *E. coli* from low levels of endogenously generated H_2O_2 [22]. Taken together, we speculated that activation of OxyR in the *ahpC1* mutant most likely resulted from the accumulation of endogenously produced H_2O_2. The same mechanism was also responsible for the compensatory increase in *ahpC* expression in the *katA* mutant.

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Genetic and physiological analysis of the major OxyR-regulated *katA* from *Xanthomonas campestris* pv. *phaseoli*

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katA encodes the major catalase that accounts for 90 % of the total catalase activity present in *Xanthomonas campestris* pv. *phaseoli*. *katA* is located upstream of an ORF designated *ankA* encoding a cytoplasmic membrane protein homologous to eukaryotic ankyrin. Transcriptional analysis of *katA* and *ankA* identified two *katA* transcripts: a major monocistronic *katA* transcript and a minor bicistronic *katA–ankA* transcript. KatA expression was induced in the presence of various oxidants including H_2O_2, organic hydroperoxides and the superoxide-generating agent menadione, in an OxyR-dependent manner. Analysis of the *katA* promoter region showed a putative OxyR binding site located upstream of an *Escherichia coli*-like σ^{70} –35 region that is likely to be responsible for transcription activation in response to oxidant treatment. Gel mobility shift experiments confirmed that purified OxyR specifically binds to the *katA* promoter. A *katA* mutant was highly sensitive to H_2O_2 during both the exponential and stationary phases of growth. This phenotype could be complemented by functional *katA*, confirming the essential role of the gene in protecting *X. campestris* from H_2O_2 toxicity. Unexpectedly, inactivation of *ankA* also significantly reduced resistance to H_2O_2 and the phenotype could be complemented by plasmid-borne expression of *ankA*. Physiological analyses showed that *katA* plays an important role in, but is not solely responsible for, both the adaptive and menadione-induced cross-protective responses to H_2O_2 killing in *X. campestris*.

INTRODUCTION

Catalase is recognized as a principal enzyme in the protection of organisms from H_2O_2 toxicity through its ability to catalyse the conversion of H_2O_2 to oxygen and water. Elimination of H_2O_2 thereby reduces the potential for transition-metal-mediated hydroxyl radical formation via the Fenton reaction (Farr & Kogoma, 1991). Bacteria have several unrelated forms of catalase (Klotz *et al.*, 1997). Inactivation of the genes encoding catalases leads in most cases to increased susceptibility to H_2O_2, and in some cases to a defect in the ability to colonize a host (Visick & Ruby, 1998; Xu *et al.*, 2001). A novel role for catalase in protection against electrophile toxicity has also been reported (Vattanaviboon *et al.*, 2001).

The identity of key regulators mediating the expression of bacterial catalases can be inferred from observations in *Escherichia coli* and *Salmonella typhimurium* showing that the synthesis of catalase-peroxidase (hydroperoxidase I encoded by *katG*) is regulated by OxyR whereas expression of the monofunctional catalase (hydroperoxidase II encoded by *katE*) is under the regulation of a stationary-phase-specific σ^S (Storz & Altuvia, 1994). OxyR is a peroxide sensor and global transcriptional regulator of the peroxide stress response (Toledano *et al.*, 1994; Zheng *et al.*, 1998). The precise role of OxyR in the regulation of catalases seems to vary in different bacteria, particularly in non-enteric species. The expression of monofunctional catalase, encoded by *katB*, in *Pseudomonas aeruginosa* is activated by OxyR (Ochsner *et al.*, 2000), whereas expression of the *Neisseria gonorrhoeae* catalase, encoded by *kat*, is presumably repressed by OxyR (Tseng *et al.*, 2003). *Streptomyces coelicolor* produces multiple catalase isozymes, none of which is regulated by OxyR (Hahn *et al.*, 2002).

Xanthomonas campestris is an important bacterial phytopathogen. Treatment of *X. campestris* pv. *phaseoli* with

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Abbreviations: tBOOH, tert-butyl hydroperoxide; NEM, *N*-ethylmaleimide.

sublethal concentrations of H_2O_2 or a superoxide-generating agent (menadione) induced elevated levels of total catalase activity during both exponential and stationary phase (Vattanaviboon & Mongkolsuk, 2000). The level of total catalase activity correlates with the ability to resist H_2O_2 toxicity (Fuangthong & Mongkolsuk, 1997; Mongkolsuk *et al.*, 1997a). *X. campestris* pv. *phaseoli* produces two detectable isozymes of monofunctional catalase, denoted KatA and KatE, that are encoded by *katA* and *katE*, respectively. KatA is the major catalase produced during all phases of growth, while KatE is detected only as cells enter the stationary phase or under nutrient-starved conditions (Vattanaviboon & Mongkolsuk, 2000). More recently, the *X. campestris* pv. *phaseoli katA* gene, encoding the major catalase, was cloned and characterized (Chauvatcharin *et al.*, 2003). Its putative amino acid sequence is highly homologous (87 % identity) to the clade I catalase from *Pseudomonas syringae*, CatF, whose crystal structure has been solved (Carpena *et al.*, 2003). In this paper, we report expression analysis and demonstrate the physiological importance of *katA* in protection against H_2O_2 toxicity. The involvement of *ankA*, encoding an ankyrin homologue, in the H_2O_2 resistance of *X. campestris* pv. *phaseoli* is also demonstrated.

METHODS

Growth conditions and media. All *Xanthomonas* strains were grown aerobically at 28 °C in Silva–Buddenhagen (SB) medium containing the appropriate antibiotics (Mongkolsuk *et al.*, 1997b). All *E. coli* strains were grown aerobically at 37 °C in Luria–Bertani (LB) medium. Induction experiments were performed with exponential-phase *X. campestris* pv. *phaseoli* cultures (OD_{600} 0·6) treated with 100 μM H_2O_2, menadione, tert-butyl hydroperoxide (tBOOH) or *N*-ethylmaleimide (NEM) (Vattanaviboon *et al.*, 2001) for 15 min for Northern analysis and 30 min for enzyme assays (Mongkolsuk *et al.*, 1997b).

Nucleic acid manipulations. All nucleic acid manipulations were performed using standard molecular biology techniques (Sambrook *et al.*, 1989) or according to the manufacturers' recommendations. The labelling of DNA probes with [α-^{32}P]dCTP was performed using a DNA labelling bead (Amersham Pharmacia Biotech). Southern and Northern blot analyses were performed as previously described (Mongkolsuk *et al.*, 1997b).

Catalase activity gels and assays. Cell lysate preparation and catalase activity gel staining were performed as previously described (Vattanaviboon & Mongkolsuk, 2000). Bacterial cells were lysed in 50 mM sodium phosphate buffer, pH 7·0, containing 1 mM PMSF by brief sonication followed by centrifugation at 10 000 ***g*** for 10 min. Supernatants were used for catalase activity gels and catalase isozymes were visualized on native PAGE gels as previously described (Vattanaviboon & Mongkolsuk, 2000). Catalase activity appeared as colourless bands against a dark brown background. The catalase assay was carried out spectrophotometrically, according to the method of Beers & Sizer (1952). One unit of catalase was defined as the amount of enzyme required to decompose 1·0 μmol H_2O_2 at 25 °C at pH 7·0.

Cloning of full-length *ankA*. The full-length *ankA* gene in pKat29 (Chauvatcharin *et al.*, 2003) was amplified using the oligonucleotide primers BT176 and BT177 (see Table 1). The 620 bp PCR product

Table 1. Bacterial strains, plasmids and primers

Strain/plasmid/primer	Description*	Source
X. campestris* pv. *phaseoli		
Xp	Parental wild-type	Mongkolsuk *et al.* (1998b)
Xp01	Xp *oxyR*::*gen*	Mongkolsuk *et al.* (1998b)
Xp20	Xp *katA*::pGemT	This study
Xp21	Xp *ankA*::pKNOCK-Km	This study
Xp31	Xp *katA*::pGemT, *ankA*::pKNOCK-Km	This study
Plasmids		
pGemT	PCR cloning vector, *rep*, phage f1 *ori*, *lacZα*, Amp^r	Promega
pKat29	pZL1 containing *X. campestris katA* and *ankA*	Chauvatcharin *et al.* (2003)
pKat111	pGemT containing *X. campestris katA* 344 bp DNA fragment	Chauvatcharin *et al.* (2003)
pKNOCK-Km	Broad-host-range suicide vector; RP4 *oriT*, R6K *γ-ori*, Kan^r	Alexeyev (1999)
pKNOCKankA	pKNOCK-Km containing *X. campestris ankA* DNA fragment	This study
pBBR1MCS-5	Broad-host-range cloning vector; *rep*, *mob*, *lacZα*, Gen^r	Kovach *et al.* (1995)
pAnkA	pBBR1MCS-5 containing *X. campestris ankA*	This study
pKatA	pBBR1MCS-5 containing *X. campestris katA*	Chauvatcharin *et al.* (2003)
Primers	**Sequence (5′–3′)**	**GenBank accession no.**
BT148	ACC CAG CTG TAT CGC ATC G	AF461425
BT149	CGC GCT GCC AAG CAT CAG AA	AF461425
BT150	CGG AGC GAT CAA CGT CGG G	AF461425
BT151	CGT GAG CGC GTT GAG CTC C	AF461425
BT176	TTT CGG GAG ATC GCT ATG	AF461425
BT177	GCG GGA CTG GCG CGC TCA	AF461425

*Amp, ampicillin; Gen, gentamicin; Kan, kanamycin; Tet, tetracycline.

was cloned into pGemT-easy and subsequently subcloned into the broad-host-range plasmid pBBR1MCS-5 (Kovach *et al.*, 1995) to generate the *ankA* overexpression plasmid, pAnkA (see Table 1).

Construction of *katA* (Xp20) and *ankA* (Xp21) insertion mutants. The *X. campestris katA* mutant, Xp20, was constructed by insertional mutagenesis using pKat111, consisting of the non-replicative plasmid pGemT containing a 344 bp *katA* internal DNA fragment (Chauvatcharin *et al.*, 2003; see Table 1). pKat111 was transferred into *X. campestris* pv. *phaseoli* wild-type strain (Xp) by electroporation. Homologous recombination between the *katA* fragment on pKat111 and its counterpart on the chromosome resulted in the Amp^r strain Xp20 containing an insertionally inactivated *katA*. Inactivation of *katA* was confirmed by catalase activity gel staining and Southern blots of Xp20 chromosomal DNA digested with *Eco*RI or *Sal*I and hybridized to a *katA*-specific probe (data not shown). The *ankA* mutant Xp21 was constructed by insertional inactivation using the pKNOCK system (Alexeyev, 1999). A blunt-ended 160 bp *Bss*HII fragment from pAnkA was ligated to *Sma*I-digested pKNOCK-Km to form pKNOCKankA. pKNOCKankA was introduced into Xp by conjugation, and the Kan^r *ankA* mutant, Xp21, was selected and confirmed by Southern blot hybridization. The *katA ankA* double mutant, Xp31, was constructed by transferring Xp20 genomic DNA into Xp21 by electroporation and selecting for Kan^r Amp^r exconjugants.

Gel mobility shift assay. ^{32}P-labelled DNA fragments were prepared by PCR using the oligonucleotide primers BT151 and BT150 (see Table 1) and pKat29 (Chauvatcharin *et al.*, 2003) as the template to generate a 254 bp fragment spanning the *katA* promoter region. Gel mobility shift reactions were performed by adding 3 fmol labelled probe in 25 µl reaction buffer [20 mM Tris pH 7·0, 50 mM KCl, 1 mM EDTA, 5 %, v/v, glycerol, 50 µg BSA ml^{-1}, 5 µg calf thymus DNA ml^{-1}, 0·5 mg poly(dI/dC) ml^{-1}]; 400 ng purified OxyR (Loprasert *et al.*, 2000) was added and the reaction was incubated at 25 °C for 15 min. Protein–DNA complexes were separated by electrophoresis on 6 % non-denaturing polyacrylamide gel in 0·5× Tris/borate/EDTA buffer (TBE) at 4 °C.

RT-PCR of *katA–ankA* mRNA. Reverse transcription (RT) of *katA–ankA* mRNA was performed to confirm the bicistronic transcriptional organization of these genes. Total RNA was isolated from *X. campestris* pv. *phaseoli* cultures using the hot acid/phenol method (Mongkolsuk *et al.*, 2002). Purified RNA was treated with 10 U RNase-free DNase I for 30 min to remove contaminating DNA. Primer BT149 (located within *ankA*; see Table 1) was mixed with 10 µg RNA, and 200 U cloned Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega) was added. The mixture was incubated at 42 °C for 60 min. Five microlitres of the mixture was added to a PCR reaction containing primers BT149 and BT148 (located in *katA*; see Table 1). PCR was performed for 35 cycles under the following conditions: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 30 s. The PCR products were analysed by agarose gel electrophoresis.

Primer extension. Total RNA was isolated from uninduced and menadione-induced *X. campestris* pv. *phaseoli* cultures. Primer extension experiments were performed using ^{32}P-labelled oligonucleotide primer BT150 (see Table 1), 5 µg total RNA and 200 U superscript II MMLV reverse transcriptase (Promega). Extension products were sized on sequencing gels next to dideoxy sequencing ladders generated using a PCR sequencing kit with labelled BT150 primer and pKat29 plasmid as the template (Chauvatcharin *et al.*, 2003).

Determination of oxidant resistance. Analysis of the killing effects of various reagents on *X. campestris* pv. *campestris* strains was performed using inhibition zone assays as described by Mongkolsuk *et al.* (1998a). Briefly, overnight cultures were subcultured as 5 % inocula into fresh SB broth and incubated at 28 °C with shaking. One millilitre of exponential-phase cells (4 h, culture OD_{600} ~0·5) was mixed with 10 ml molten top agar (SB containing 0·7 % agar) held at 50 °C, and overlaid onto SB plates (14 cm diameter Petri dishes containing 40 ml SB agar). The plates were left at room temperature for 15 min to let the top agar solidify. Sterile 6 mm diameter paper discs soaked with 5 µl H_2O_2 (0·5 M), tBOOH (0·5 M) or menadione (1·0 M) were placed on top of the cell lawn and the diameters of the inhibition zones were measured after 24 h incubation at 28 °C.

Determination of adaptive and cross-protective resistance to H_2O_2. The induced adaptive or cross-protective resistance to H_2O_2 killing was measured by adding H_2O_2 or menadione (100 µM), respectively, to exponential-phase cultures of *X. campestris* pv. *campestris* strains prior to treatment with lethal concentrations of H_2O_2 (10, 20, 30 mM) for 30 min. After treatment, cells were removed and washed once with fresh SB medium and cell survival was determined by plating appropriate dilutions on SB agar plates. Colonies were counted after 48 h incubation at 28 °C. The surviving fraction was defined as the number of c.f.u. recovered after treatment divided by the number of c.f.u. prior to treatment. Three independent experiments were performed in each case and representative data are shown.

RESULTS AND DISCUSSION

Gene and transcription organization of *katA–ankA*

The cloning and sequencing of *katA* encoding the clade I monofunctional catalase from *X. campestris* pv. *phaseoli* (Xp) has been reported previously (Chauvatcharin *et al.*, 2003). Additional sequencing of DNA surrounding *katA* (GenBank accession no. AF461425) revealed a 600 bp ORF capable of encoding a 199 amino acid protein with a calculated molecular mass of 20·5 kDa. The deduced amino acid sequence was searched against the GenBank database using the BLAST program (Altschul *et al.*, 1997), revealing that the putative protein has a high degree of sequence identity (60 %) with an ankylin-like protein encoded by *ankB* from *P. aeruginosa* (Howell *et al.*, 2000). This ORF was subsequently designated *ankA*. *katA* and *ankA* are located in the same orientation and separated by 59 bp. Analyses of the gene organization of the *kat* loci in various micro-organisms reveal that the *kat–ank* gene organization is found not only in *Pseudomonas* and *Xanthomonas*, but also in several other bacteria such as *Vibrio cholerae* (AE004235), *Campylobacter jejuni* (AL139078) and *Streptomyces coelicolor* (AL939105). The conservation of *kat–ank* organization suggests that these genes could have related functions that are of physiological significance.

The transcriptional organization of *katA* and *ankA* was determined by Northern blots. The results showed that a *katA* probe hybridized to two mRNA bands at 1·6 kb and 2·2 kb that corresponded to the predicted sizes of monocistronic *katA* and bicistronic *katA–ankA* mRNAs respectively (Fig. 1a). Northern blot analysis using an *ankA*-specific probe detected a single positively hybridizing band at 2·2 kb (Fig. 1a), suggesting that *ankA* is

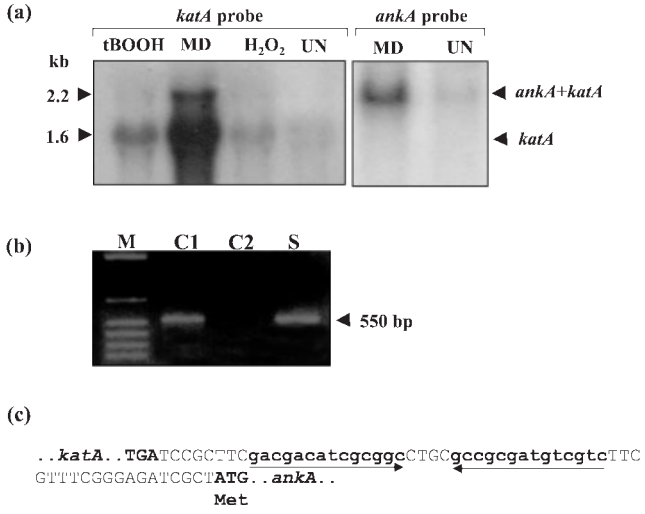

Fig. 1. Expression of *katA–ankA*. (a) Northern blot analysis of total RNA prepared from exponential-phase cultures of *X. campestris* pv. *phaseoli* under uninduced (UN), tBOOH-induced (tBOOH), menadione-induced (MD) and H_2O_2-induced (H_2O_2) conditions hybridized with ^{32}P-labelled *katA* or *ankA* DNA probes. (b) RT-PCR analysis of a total RNA sample (S) extracted from a *X. campestris* pv. *phaseoli* culture as described in Methods. C1, positive control generated using genomic DNA as the template; C2, negative control in which RNase was added prior to the PCR reaction; M, DNA size marker. (c) Nucleotide sequence of the *katA–ankA* intergenic region. The stop codon of *katA* and the start codon of *ankA* are indicated in bold capitals. The inverted repeats of a putative rho-independent transcription terminator sequence are in bold lower case and indicated by arrows.

co-transcribed with *katA*. This was confirmed by RT-PCR experiments that were performed using two specific primers, one located near the 3′ end of the *katA* coding region and another located near the 5′ end of *ankA*. RT-PCR using an RNA sample prepared from an uninduced Xp culture gave rise to a 550 bp product that corresponded to the expected size of a product derived from a *katA–ankA* mRNA template (Fig. 1b). This 550 bp band was absent when the RNA sample was first treated with RNaseA, indicating that the product was not the result of priming to contaminating DNA (Fig. 1b). Thus, *katA* and *ankA* are transcribed as a bicistronic mRNA of 2·2 kb. Densitometer analyses of *katA* Northern blots indicated that the monocistronic *katA* mRNA made up 85 % of the *katA*-encoding mRNA while 15 % consisted of the *katA–ankA* bicistronic message (Fig. 1b). Examination of the DNA sequence in the *katA–ankA* intergenic region shows the presence of an inverted repeat sequence spanning 32 bp followed by a run of three T residues (Fig. 1c). The stem–loop structure resembles a typical rho-independent transcription terminator, suggesting that the major 1·5 kb *katA* monocistronic transcripts are the result of rho-independent transcription termination at this site. Moreover, the minor 2·2 kb *katA–ankA* bicistronic mRNA is likely to result from transcriptional readthrough at this site. The mechanism responsible for the antitermination at the *katA* terminator is not known. It could be mediated by a regulator, as with AmiR in the amidase operon (Wilson *et al.*, 1996). Alternatively, readthrough may be the result of the intrinsic efficiency of the terminator itself (Weisberg & Gottesman, 1999), such that it permits 15 % readthrough transcription into *ankA*.

Oxidant induction of KatA requires a functional OxyR

It has been previously shown that *X. campestris* pv. *phaseoli* produces two monofunctional catalase isozymes, namely KatA and KatE (formerly Kat2) (Chauvatcharin *et al.*, 2003; Vattanaviboon & Mongkolsuk, 2000). The KatA level was found to be high during the exponential phase of growth and declined slightly when cells entered stationary phase. By contrast, KatE levels increased as cells entered stationary phase. In many micro-organisms, catalase activity is induced by exposure to low concentrations of H_2O_2. In order to determine if this was true in *X. campestris* pv. *phaseoli*, total catalase activity and the levels of KatA were determined after exponential-phase cultures were treated with various oxidants. Total catalase activity showed that menadione was the most potent inducer and stimulated an increase in total catalase activity of 9·5-fold. H_2O_2, tBOOH and NEM also induced catalase activity, but to a lesser degree: twofold, fourfold and fourfold, respectively (Fig. 2a). The effect of oxidants on the expression of the two catalases was also assessed using catalase activity-stained gels (Fig. 2a). While oxidant treatments did not significantly change the levels of KatE (data not shown), KatA levels increased in response to the oxidant treatments (Fig. 2a). The magnitude of induction as judged by densitometer analyses of the KatA-specific activity-stained bands showed good correlation with the degree of induction observed using total catalase activity measurements: i.e. menadione was the most potent inducer of KatA, while H_2O_2, tBOOH and NEM stimulated lower levels of induction (Fig. 2a). The results supported the idea that an increase in KatA activity was responsible for the observed increase in total catalase levels following oxidant treatments. Furthermore, the results of Northern blot analyses showing oxidant-induced synthesis of *katA* mRNA suggested that the increased KatA activity was due to increased transcript levels of *katA* (Fig. 1a). Similar oxidant-induced expression of monofunctional catalase has also been observed in *Xanthomomas oryzae* pv. *oryzae*, where menadione was found to be the most potent inducer, followed by H_2O_2 and other oxidants (Mongkolsuk *et al.*, 1996).

Since menadione, H_2O_2, tBOOH, and NEM are known to be potent inducers of other genes in the OxyR regulon in *X. campestris* pv. *phaseoli* (Loprasert *et al.*, 2000; Mongkolsuk *et al.*, 1997b), it was hypothesized that OxyR may also regulate the oxidant-induced expression of *katA*. In order to test this hypothesis, the total catalase activity was

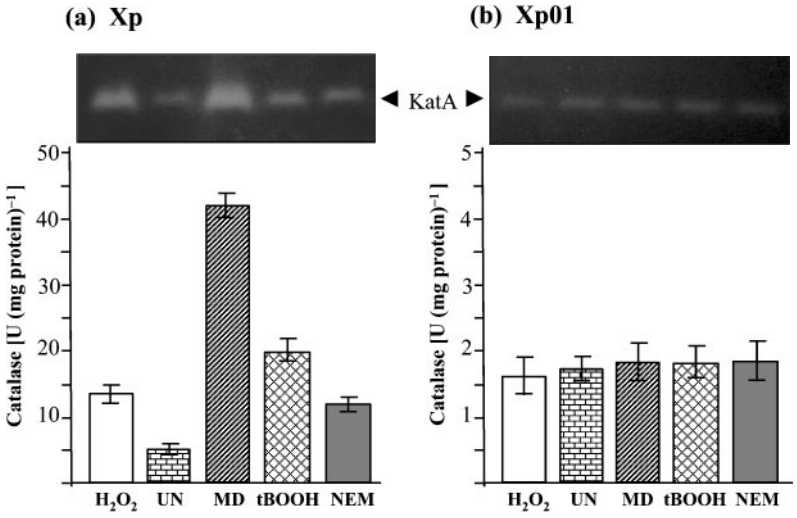

Fig. 2. Effect of oxidant treatment on the expression of *katA*. *X. campestris* pv. *phaseoli* (Xp, a) and an *oxyR* mutant (Xp01, b) were grown to exponential phase and the cells were then induced with H_2O_2, menadione (MD), tBOOH and NEM, and extracts were prepared as descibed in Methods. Crude protein (50 μg for Xp and 100 μg for Xp01) was separated using native PAGE and subjected to catalase activity gel staining (upper panels). The total catalase activity was also determined (lower panels). UN, uninduced control.

measured in the *oxyR* mutant, Xp01, grown in the presence and absence of oxidants. Strain Xp01 showed no oxidant-dependent increases in either total catalase activity or KatA synthesis as determined using activity-stained gels (Fig. 2b). The results indicated that oxidant-dependent induction of KatA synthesis during exponential phase requires a functional *oxyR*.

Analysis of OxyR regulation of the *katA* promoter

A more detailed characterization of OxyR-regulated expression of *katA* was performed. First, the transcriptional start site of the *katA–ankA* operon was mapped. The results of primer extension analyses using total RNA samples prepared from uninduced and menadione-induced cultures showed a single extension product of 78 bases, indicating that *katA* transcription is initiated at the C residue located 21 nucleotides upstream of the *katA* translational start codon (Fig. 3). The proposed σ^{70}-type RNA polymerase consensus binding sequence for a *X. campestris* promoter consists of the −35 element, TTGTNN, separated by 16 to 24 nucleotides from the −10 element, T/AATNAA/T (Katzen *et al.*, 1996). Examination of the sequence upstream of the transcriptional start site revealed the presence of two sequence motifs, TTCTCA (−34 to −29) and GATGAT (−11 to −6), that are separated by 17 bp, and that closely matched the −35 and −10 consensus promoter sequences, respectively (Fig. 3). A significant amount of *katA* primer extension products was detected in the absence of inducer (Fig. 3, uninduced sample) indicating that the gene is constitutively transcribed. This is consistent with other observations (Fig. 2) indicating that even in the absence of oxidant inducers, *katA* is highly expressed during

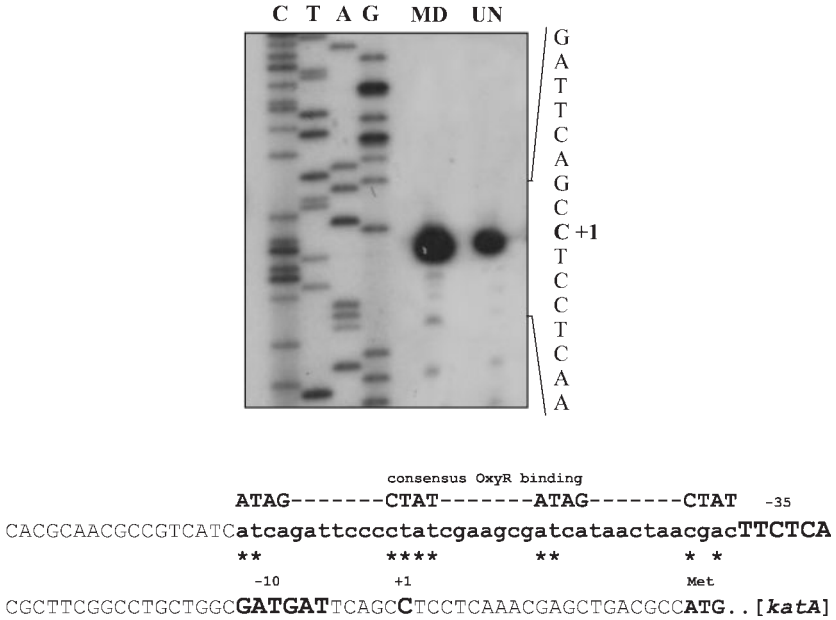

Fig. 3. Localization of the *katA* promoter. Results of primer extention mapping of the transcription start site of the *X. campestris* pv. *phaseoli katA–ankA* operon using total RNA prepared from uninduced (UN) and menadione-induced (MD) *X. campestris* pv. *phaseoli* cultures, sized against a dideoxy sequencing ladder generated using the same primer (upper panel). The *X. campestris* pv. *phaseoli katA* promoter sequence (lower panel) showing the mapped transcription start site (+1) as well as *E. coli*-like σ^{70} −10 and −35 promoter elements (large bold type). A putative OxyR binding site is shown in bold lower-case type and is compared to the *E. coli* consensus OxyR binding sequence.

exponential-phase growth. The amount of primer extension products in the menadione-treated sample was fivefold higher than that in the uninduced sample (Fig. 3). This reinforced the Northern blot hybridization results and confirmed that menadione induction of KatA activity is the result of increased *katA* transcript levels.

The results obtained using the *oxyR* mutant, Xp01, indicated that a functional *oxyR* was required to mediate oxidant-dependent induction of *katA* expression. In *E. coli*, OxyR-mediated activation of gene expression requires the binding of oxidized OxyR to an extended DNA recognition sequence (ATAGntnnnanCTATnnnnnnnATAGntnnnanCT-AT; Toledano *et al.*, 1994) located immediately upstream of the −35 region of the promoter. When bound to this site, OxyR facilitates the binding of RNA polymerase to the promoter, resulting in increased transcription of the gene (Toledano *et al.*, 1994). A DNA sequence that is highly similar (62 % identity) to the *E. coli* consensus OxyR DNA-binding motif, located immediately upstream of the −35 region, was identified in the *X. campestris* pv. *phaseoli katA* promoter (Fig. 3). This sequence is also similar to a previously characterized OxyR binding site within the *X. campestris* pv. *phaseoli ahpC* promoter (Loprasert *et al.*, 2000).

In order to conclusively demonstrate the direct participation of OxyR in the activation of *X. campestris* pv. *phaseoli katA* transcription, purified Xp OxyR and a 254 bp DNA fragment spanning the *katA* promoter, including the putative OxyR binding site, were used in mobility shift assays. The results of these assays demonstrated that OxyR bound to the *katA* promoter (Fig. 4). OxyR binding was inhibited by the addition of excess unlabelled probe fragment (Fig. 4, UP), but not by the addition of excess nonspecific competitor DNA (pBBR1MCS-5) (Fig. 4, UD), indicating that binding was specific for the *katA* promoter. Moreover, the unrelated oxidant-sensing transcription repressor, OhrR (Mongkolsuk *et al.*, 2002), did not bind to the *katA* promoter, indicating that *katA* expression is not under direct OhrR control (Fig. 4, OhrR). These data, coupled with the *in vivo* results, support the hypothesis that *katA* is directly regulated by OxyR.

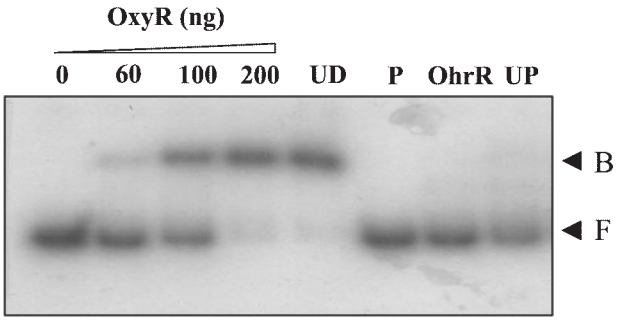

Fig. 4. OxyR binding to the *katA* promoter. A DNA mobility shift assay using a ^{32}P-labelled *katA* promoter fragment and increasing amounts (0 to 200 ng) of purified OxyR (Loprasert *et al.*, 2000) was performed as described in Methods. UD and UP indicate reactions containing 2 μg unrelated DNA (pBBR1MCS-5 plasmid) and 2 μg unlabelled *katA* promoter, respectively, in addition to OxyR (200 ng). P represents ^{32}P-labelled *katA* promoter. OhrR (Mongkolsuk *et al.*, 2002) indicates a reaction containing 2 μg purified Ohr in place of OxyR. F, free probe; B, bound probe.

katA and *ankA* mutants are sensitive to H_2O_2

In order to understand the physiological roles of KatA and AnkA in the *X. campestris* pv. *phaseoli* oxidative stress response, individual *katA* (Xp20) and *ankA* (Xp21) mutants were constructed and their resistance levels against various oxidants were evaluated using the growth inhibition zone method. As expected, Xp20 was highly sensitive to H_2O_2, but not to the superoxide generator menadione (Fig. 5a and data not shown). The increased sensitivity to H_2O_2 could be complemented by expression of *katA* from a vector (Fig. 5a). Interestingly, Xp20 was more resistant to tBOOH

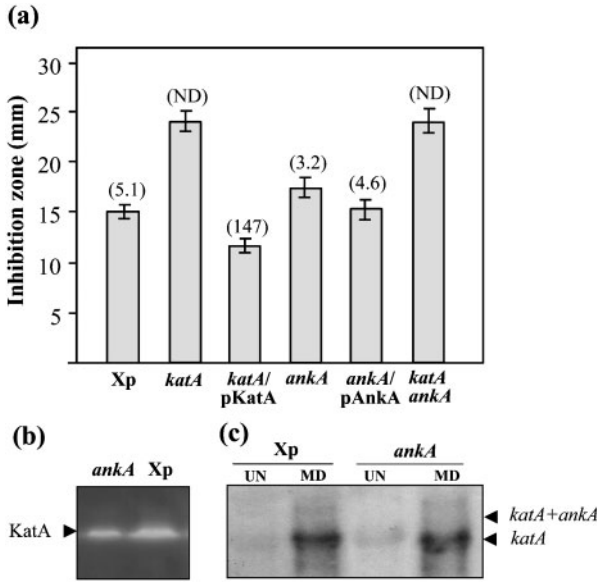

Fig. 5. Resistance levels to H_2O_2 and the expression of *katA* in *X. campestris* pv. *phaseoli* mutant strains. (a) Levels of resistance against H_2O_2 killing in: *X. campestris* pv. *phaseoli* (Xp), Xp20 (*katA*), Xp20 harbouring pKatA (*katA*/pKatA), Xp21 (*ankA*), Xp21 harbouring pAnkA (*ankA*/pAnkA) and Xp31 (*katA ankA*). Cultures were grown to exponential phase. Values in parentheses indicate the total catalase activity (U mg^{-1}) expressed as the means of triplicate assays. Bars represent the standard deviation. 0, activity not detectable. (b) Catalase activity gel of crude proteins (100 μg) prepared from exponential-phase cultures of Xp and Xp21 (*ankA*) and separated by native PAGE. (c) Northern blot analysis of total RNA prepared from exponential-phase cultures of *X. campestris* pv. *phaseoli* (Xp) and the *ankA* mutant (Xp21) under uninduced (UN) and menadione-induced (MD) conditions hybridized with ^{32}P-labelled *katA* DNA probes.

than the parental strain Xp (data not shown). This probably resulted from a compensatory increase in *ahpC* expression levels in response to the loss of *katA*. A similar situation has been previously observed in *X. campestris* pv. *phaseoli*, where inactivation of *ahpC* has been shown to cause an increase in catalase activity (Mongkolsuk *et al.*, 2000). The mechanism of this putative compensatory interaction involving the upregulation of *ahpC* in response to the inactivation of *katA* is currently being investigated.

Since *katA* and *ankA* make up an operon, inactivation of *katA* is likely to have a polar effect on *ankA* expression. Although the overexpression of *katA* in Xp20/pKatA complemented the H_2O_2 sensitivity of Xp20, the phenotype of Xp20 could have been a result of the loss of both KatA and AnkA. In order to test this idea, a *X. campestris* pv. *phaseoli* double *katA ankA* mutant (Xp31) was constructed and its resistance levels to H_2O_2 were evaluated. The results showed that Xp31 and Xp20 had identical phenotypes with respect to H_2O_2 sensitivity and catalase activity (Fig. 5a), suggesting that the observed phenotype of Xp20 was primarily due to the loss of KatA. Unexpectedly, disruption of *ankA* alone in strain Xp21 caused a small but significant decrease in the total catalase level and rendered the cells more sensitive to H_2O_2. The decrease in catalase activity and the increased H_2O_2 sensitivity in Xp21 could be complemented by the constitutive expression of *ankA* from the plasmid pAnkA (Fig. 5a). The small increase in sensitivity to H_2O_2 in Xp21 combined with the observation that the *katA ankA* double mutant, Xp31, did not show increased sensitivity to H_2O_2 relative to the single *katA* mutant, Xp20 (Fig. 5a), suggested that the increased H_2O_2 sensitivity of Xp21 is due to a reduction in the KatA level (Fig. 5b) and not to other independent mechanisms. We also tested whether a mutation in *ankA* altered the steady-state levels of *katA* mRNA. Northern experiments performed using total RNA isolated from Xp20 and Xp21 and hybridized to a *katA*-specific DNA probe indicated that the levels of *katA* mRNA were similar (Fig. 5c), suggesting that inactivation of *ankA* affected KatA levels at the post-transcriptional level. It is possible but highly unlikely that AnkA interacts with KatA and affects the V_{max} of the enzyme, leading to reduction in catalase activity in the *ankA* mutant. A more likely interpretation is that AnkA isinvolved in the anchoring or export of KatA. Alignment of *X. campestris* pv. *phaseoli* AnkA and *P. aeruginosa* AnkB revealed that the two proteins shared 60 % amino acid sequence identity (data not shown). Although AnkA from *X. campestris* pv. *phaseoli* has an extended hydrophobic N-terminal region containing six repeats of proline-alanine residues, topology prediction of the membrane protein using TopPred (Claros & von Heijne, 1994) (available at http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) predicted the presence of a cytoplasmic segment at the first 2 amino acids of the N-terminus followed by a single 21 amino acid transmembrane segment. The remaining portion of the protein (168 amino acid residues) was predicted to reside in the periplasm (data not shown). This structural profile agrees with the data reported previously for *P. aeruginosa* AnkB, suggesting that *X. campestris* pv. *phaseoli* AnkA is localized in the same cellular compartment as AnkB (Howell *et al.*, 2000). In *P. aeruginosa*, AnkB, a membrane-associated protein containing an inner-membrane-spanning motif at the N-terminus, has been proposed to form an antioxidant scaffolding that anchors catalase in the periplasm and provides a protective lattice for effective H_2O_2 detoxification (Howell *et al.*, 2000). It is not known whether this is true in the case of *ankA*. In addition, since both *P. aeruginosa katB* and *X. campestris katA* contain a putative leader sequences identified by the SignalP program (Bendtsen *et al.*, 2004; available at http://www.cbs.dtu.dk) (data not shown), it is possible that one of the functions of ankyrin is in the export of KatA, and inactivation of *ankA* would therefore lead to blockage of the process. This could back up KatA in the cytoplasm and interfere with the translation of *katA* mRNA. The secretion of KatA is being investigated.

katA contributes to H_2O_2-induced adaptive protection and menadione-induced cross-protection against H_2O_2 killing treatments

Physiological adaptation to stresses is an important response for bacterial survival under stressful conditions. The process often involves complex alteration in the expression pattern of genes involved in stress protection and repair of stress-induced damage. The oxidative stress-induced physiological adaptation and cross-protection responses are widely distributed in both Gram-negative and Gram-positive bacteria. We have reported the presence of H_2O_2-induced physiological adaptive and menadione-induced cross-protective responses to lethal concentrations of H_2O_2 in Xp (Mongkolsuk *et al.*, 1998b). The H_2O_2-induced adaptive response is completely abolished in an *oxyR* mutant while the menadione-induced cross-protective response is only partially lost (Mongkolsuk *et al.*, 1998b). The role of OxyR-regulated *katA* expression in these responses was evaluated. Xp20 and its parental strain Xp were grown to exponential phase before being induced with either H_2O_2 or menadione (100 μM) for 30 min. The induced cultures were then treated with lethal concentrations of H_2O_2 for 30 min and the percentage survival relative to an untreated control culture was determined. The results show that Xp20 had significantly impaired H_2O_2-induced adaptive and menadione-induced cross-protection responses against H_2O_2 relative to the parental strain (Fig. 6). In the parental strain Xp, pretreatment with H_2O_2 or menadione induced 100-fold and 1000-fold protection, respectively, against subsequent H_2O_2 killing treatments (Fig. 6a). In the *katA* mutant, Xp20, the levels of induced protection against H_2O_2 killing decreased to 10-fold and 100-fold after induction with H_2O_2 or menadione, respectively (Fig. 6b). This indicated that *katA* has a general role in the protection against killing by H_2O_2 in both uninduced cells and those induced by oxidants. Furthermore, even though uninduced and oxidant-induced

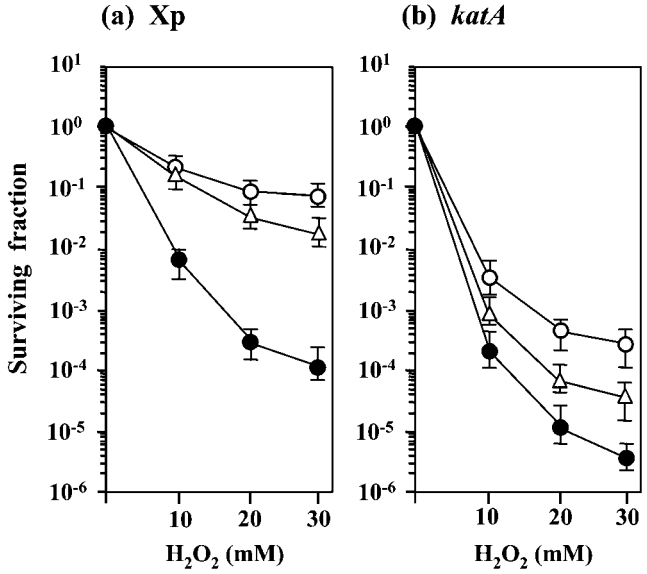

Fig. 6. Adaptive and cross-protective responses against H_2O_2 in Xp20. The survival curves of exponential-phase cultures of Xp (a) and the *katA* mutant Xp20 (b) pretreated with H_2O_2 (△), or menadione (○) prior to subsequent treatments with lethal concentrations of H_2O_2 at the indicated concentrations and compared to an uninduced control culture (●). The values presented are the means and standard deviations of three replicates.

Xp20 cells were 100-fold more sensitive to H_2O_2 killing than the parental strain Xp, strain Xp20 still retained the ability to mount a partial H_2O_2-induced adaptive response. This observation, combined with the fact that inactivation of *oxyR* in *X. campestris* pv. *phaseoli* has been shown to completely abolish the H_2O_2-induced adaptive response (Mongkolsuk *et al.*, 1998b), implies that OxyR-regulated genes other than *katA* must contribute to the H_2O_2-induced adaptive response in Xp20. In *E. coli*, the alkylhydroperoxidase AhpC has an essential role in scavenging H_2O_2 during normal growth (Seaver & Imlay, 2001) and it has been shown that *ahpC* is highly induced by both H_2O_2 and menadione (Loprasert *et al.*, 2000). Thus, it is likely that induction of *ahpC* by oxidants contributed to both the adaptive and menadione-induced cross-protective responses to H_2O_2 killing in *X. campestris* pv. *phaseoli*. The mechanism of menadione-induced cross-protection against H_2O_2 is more complex. The fact that OxyR was only partially responsible for the process (Mongkolsuk *et al.*, 1998b) suggests that, in addition to *katA* and *ahpC*, there must be other genes, independent of OxyR regulation, that are involved. The OxyR-independent menadione induction of these protective systems is currently being investigated.

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ORT COMMUNICATION

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A protects the human pathogen *Burkholderia pseudomallei* inst organic hydroperoxide

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ct The human pathogen, *Burkholderia pseudomalle*, to survive and multiply in hostile environments within macrophages. In an attempt to understand egy to cope with oxidative stress, the physiological d gene regulation of a nonspecific DNA-binding (DpsA) was investigated. Expression of *dpsA* in- *in* response to oxidative stress through increased ption from the upstream katG (catalase–peroxi- promoter, which is OxyR dependent. *dpsA* is also ibed from its own promoter, which is activated by c stress in an OxyR-independent manner. DpsA-de- mutants are hypersensitive to *tert*-butyl hydroper- while overexpression of DpsA leads to increased nce to organic oxidants. *B. pseudomallei* DpsA can otect *Escherichia coli* against organic hydroperox- icity. The mechanism of DpsA-mediated resistance nic hydroperoxides was shown to differ from that l hydroperoxide reductase.

rds Melioidosis · Oxidative stress · Nonspecific inding protein · Organic hydroperoxide

ction

microorganisms have evolved a variety of mech- to protect their DNA from oxidative damage caused tive oxygen species generated during metabolism ent in the environment. In *Escherichia coli*, the died bacterium, conditions of either oxidative or nal stress cause the production of high levels of the nonspecific DNA-binding protein Dps, which effectively protects DNA against oxidants (Martinez and Kolter 1997). *E. coli* Dps and its homologue in *Bacillus subtilis*, MrgA, are highly abundant in stationary-phase cells, where the proteins bind DNA without apparent specificity to form extremely stable complexes (Almiron et al. 1992; Chen and Helmann 1995). Dps forms spherical dodecamers, homologous to ferritins, that sequester and protect DNA from damage due to oxidative stress, nucleases, UV light, and acid stress (Wolf et al. 1999; Choi et al. 2000). *dps* is also expressed during exponential growth, following exposure to low doses of H_2O_2, as part of the OxyR regulon, suggesting that it is critical for survival during oxidative stress (Altuvia et al. 1994).

Burkholderia pseudomallei is a facultative intracellular human pathogen that can cause a fatal disease, melioidosis. Bacterial pathogens are frequently exposed to reactive oxygen species during the course of infection. Exposure to oxygen radicals, in the form of superoxides, hydrogen peroxides, and organic hydroperoxides, can result from release of lysosomal contents within inflammatory cells or their generation by bacterial cellular metabolism (Buettner 1993; Storz and Toledano 1994). The organic hydroperoxide *tert*-butyl hydroperoxide (*t*-BOOH) has been shown to cause DNA base damage in cultured mammalian cells through its ability to react with metals to generate the highly reactive *tert*-butoxyl radical (Altman et al. 1994). Recently, an iron-binding Dps-like protein, Dpr, from *Streptococcus mutans* was found to prevent iron-dependent hydroxyl radical formation (Fenton reactions); the protein incorporates up to 480 iron and 11.2 zinc atoms per molecule (Yamamoto et al. 2002). Therefore, it is possible that the metal-binding ability of Dpr might protect cells against organic hydroperoxide toxicity by preventing hydroxyl radical formation via the Fenton reaction. Elimination of alkyl hydroperoxides is particularly important, because they are highly toxic molecules that can initiate a lipid peroxidation chain reaction that propagates free radicals, leading to DNA and membrane damage (Halliwell and Gutteridge 1984; Akaike et al. 1992). Alkyl hydroperoxide reductase (AhpR) and organic hydroperoxide re-

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sistance protein (Ohr) are the two major microbial enzymes that have been shown to be involved in the detoxification of organic hydroperoxides through the reduction of physiological lipid peroxides, such as linoleic acid hydroperoxide, thymine hydroperoxide and nonphysiological alkyl hydroperoxides, to their nontoxic alcohol forms. Genetic analysis of several bacteria has shown that mutations in the genes encoding these two enzymes lead to an organic-hydroperoxide-hypersensitive phenotype (Storz et al. 1989; Mongkolsuk et al. 1998; Fuangthong et al. 2001).

In its natural environment, whether in a human host or an external environment during transmission, it is likely that *B. pseudomallei* is faced with growth-limiting or potentially lethal conditions, such as nutrient limitation and osmotic and oxidative stress. A facultative intracellular bacterium, *B. pseudomallei* is able to grow under conditions that are usually detrimental to the development of most microorganisms. However, while the ability of *B. pseudomallei* to resist many kinds of stresses is of particular clinical importance, there is currently very little information demonstrating how *B. pseudomallei* overcomes the various stresses it encounters during infection.

In this work, we reveal the protective role of DpsA against exogenous toxic organic hydroperoxides, as well as characterize its gene regulation in response to oxidative and osmotic stresses.

Materials and methods

Bacterial strains and growth conditions

The clinical isolate *B. pseudomallei* P844, *E. coli* DH5α, and their derivatives were grown in Luria-Bertani (LB) medium. *B. pseudomallei oxyR* (R957), *katG* (G221), and *oxyR katG* (RG27) knockout strains were described in previous studies (Loprasert et al. 2002, 2003b). *E. coli* TA4315 is an AhpC-deficient strain (Storz et al. 1989). Plasmid pAhpC consists of a *B. pseudomallei ahpC* fragment in pBBR-Cm (Kovach et al. 1995) and was constructed as previously described (Loprasert et al. 2003a). Pseudomonas agar base supplemented with SR103E (cetrimide, fucidin, and cephaloridine) (Oxoid) was used after conjugation as a selective medium to inhibit growth of *E. coli*. M9 minimal medium was supplied by Gibco BRL. All cultures were grown at 37°C. Tetracycline (60 μg/ml), chloramphenicol (40 μg/ml), specinomycin (100 μg/ml), and erythromycin (100 μg/ml) were used, when required.

Northern analysis

Extraction of total RNA, using a modified hot acid phenol method, and Northern blot analysis of mRNA were carried out as previously described (Mongkolsuk et al. 1996), using a 500-bp *Sph*I-*Pst*I fragment spanning *dpsA* as the probe. Mid-exponential-phase cultures of *B. pseudomallei* were induced by the addition of menadione to a final concentration 200 μM. Both induced and uninduced cultures were then incubated for an additional 15 min prior to the isolation of total RNA.

Construction of a chromosomal *dpsA::lacZ* transcriptional fusion

A minitransposon containing the *dpsA* promoter fused to *lacZ*, pUT-*dpsA*, was constructed using the vector pUT-*Tn5lacZ1* (de Lorenzo et al. 1990). In brief, the kanamycin resistance gene was removed and replaced with the trimethoprim resistance gene (*dfr*) of pGSTp (Shalom et al. 2000). A 600-bp *Eco*RV–*Eco*RI fragment, containing the 5′ end of *dpsA* and 400 bp of upstream sequence, was blunt ended and inserted into the *Sma*I site upstream of *lacZ* in pUT-*Tn5lacZ1*. The resulting plasmid, pUT-*dpsA*, was then conjugally transferred into *B. pseudomallei* and stable trimethoprim resistant transconjugants were selected. Using this method, the *dpsA::lacZ* construct was integrated into the chromosome of both *B. pseudomallei* wild type and *oxyR* mutant strains (Loprasert et al. 2002) creating P844D and R957D.

Induction of *dpsA* promoter by salts

NaCl or KCl (500 mM final concentration) was added to overnight cultures (OD_{600}=0.5) growing in M9 medium containing low (0.05%) glucose. The induced and uninduced cultures were grown for 1 h at 37°C before being harvested for β-galactosidase assays.

β-Galactosidase assays

Cell lysates were prepared using B-PER (bacterial protein extraction reagent, Pierce) and assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactoside (ONPG) as the substrate as previously described (Steers et al. 1965).

Construction of *dpsA* and *dpsA oxyR* mutants

The *dpsA* knockout mutant, D18, was created by insertion of a tetracycline resistant plasmid into *dpsA*. A 180-bp *Eco*RI-*Pst*I fragment from the coding region of *dpsA* was ligated into the suicide vector pKNOCK-Tc (Alexeyev 1999) to create pD180, which was then transferred from *E. coli* S17-1 λ*pir* into *B. pseudomallei* by conjugation. Mutants (D18) containing a single-crossover within *dpsA* were selected on Pseudomonas agar containing 60 μg/ml tetracycline. A *dpsA oxyR* double mutant was created by conjugation between D18 (*dpsA*) and R957 (*oxyR*), resulting the Tc^r Cm^r*dpsA oxyR* mutant strain DR17. Both D18 and DR17 were shown by Southern analysis to contain the desired gene disruptions.

Complementation of the *dpsA* and *dpsA oxyR* mutants

The *dpsA* structural gene and its ribosome-binding site (500 bp) were amplified by PCR and cloned into pGEM-T (Promega) to generate pGEM-D. The primers were D303 (5′AAGGAGTTTCGAGGATGG3′) and D304 (5′TCACGCGAGCAGCGAACG3′). *dpsA* was then removed from pGEM-D and cloned into the *Apa*I-*Spe*I site of pBBR-Sp (spectinomycin resistant), which was created by replacing *cat* (chloramphenicol resistance gene) of pBBR-Cm with the Sp^r gene from pKRP13 (Reece and Phillips 1995), to create pDps, which was then mobilized into the *dpsA* mutant DR18 by conjugation. In order to complement the *dpsA oxyR* mutant DR17, pUT-*oxyR-ery* (erythromycin resistant) was mobilized into the chromosome of DR17 which harbors pDps, creating DR17R/pDps (*dpsA oxyR* Tn*R*/pDps).

Growth on oxidant agar plates

Cultures of the desired strains grown overnight in M9 low glucose medium were adjusted to OD_{600}=1.0 and serially diluted. Ten microliters of each dilution was spotted onto LB agar containing 150 μM *tert*-butyl hydroperoxide (*t*-BOOH) and the extent of growth was observed after 24 h of incubation at 37°C.

Survival in oxidant medium

Overnight cultures in LB medium were subcultured (starting OD_{600}=1.0) into fresh modified M9 (0.2% casamino acids, 0.4% glucose) with and without 150 μM *t*-BOOH and the optical density was measured after 7 h of incubation at 37°C with shaking. The relative growth was calculated by comparing the optical density of treated cultures with comparable untreated cultures.

Growth inhibition zone assays

To test the susceptibility of *E. coli* strains to organic hydroperoxides, disk inhibition assays were done as previously described (Mongkolsuk et al. 1998). Briefly, bacterial cells from an exponential-phase culture (10^8 cells) were added to 3 ml of warm top LB agar. The mixture was then overlayed onto an LB agar plate. When the agar had set, 6-mm paper discs containing 6 μl of 250 μM *t*-BOOH were placed on the cell lawn. Zones of growth inhibition were measured after a 24-h incubation.

Reduction of organic hydroperoxide assay

The reduction of organic hydroperoxide in the growth medium was measured at different times by a reaction using chromogen xylenol orange, ammonium ferrous sulfate, and sulfuric acid as previously described (Shea and Mulks 2002).

Results and discussion

Regulation of *dpsA* expression by OxyR

We have previously shown that *dpsA* is co-transcribed with *katG* upon exposure to oxidative stress (Loprasert et al. 2003b) (Fig. 1A). To test whether the global peroxide sensor OxyR is a regulator of *dpsA* expression, the relative amounts of *dpsA* mRNA in *oxyR*, *katG*, and *oxyR katG* double mutant strains were determined by Northern blot analysis. A lack of OxyR in *oxyR* (R957) and *oxyR katG* (RG27) mutants abolished the induction of *dpsA* following treatment with the superoxide generator menadione (Fig. 1B lane M of *oxyR* and *oxyR katG*). Transcripts of *katG–dpsA* (3.5 kb) and *dpsA* (0.6 kb) were not induced in OxyR-deficient strains when cells were exposed to oxidant (menadione). In the *katG* mutant, a *katG–dpsA* transcript was not detected while *dpsA* mRNA was highly induced, indicating that OxyR can activate *dpsA* expression from the *dpsA* promoter (Fig. 1B lane M of *katG*). It is worthwhile noting that, in the wild-type following oxidant treatment, a transcript of approximately 3.5 kb (*katG–dpsA*) was highly induced while the 0.6-kb mRNA of *dpsA* showed no increase. *dpsA* transcripts were apparent only when *katG* was disrupted, suggesting that OxyR may preferably activate *dpsA* via the *katG* promoter instead of the downstream *dpsA* promoter. Arrangement of *katG* and *dpsA* in an operon would certainly benefit cells by allowing a prompt increase in expression of both genes in response to oxidative stress. While KatG detoxifies the peroxide threat, DpsA would simultaneously protect DNA from peroxide-induced damage.

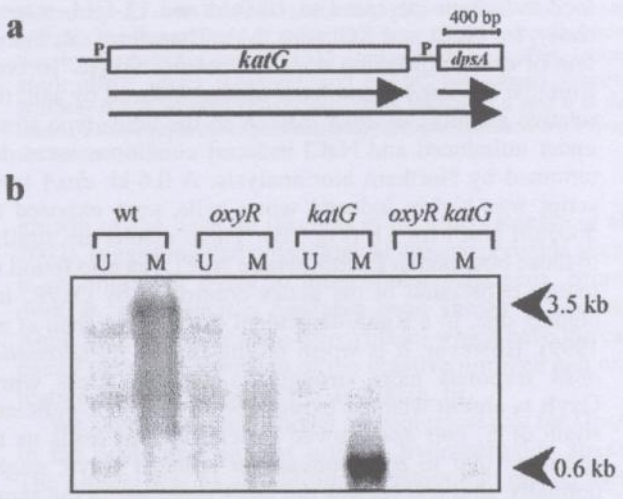

Fig. 1 Gene organization and transcriptional regulation of *katG dpsA* operon expression in response to oxidative stress. **a** The genetic organization of *katG* and *dpsA*. *Arrows* indicate the direction and extent of transcription, *P* promoter regions. **b** Northern analysis of *dpsA* mRNA prepared from *Burkholderia pseudomallei* P844 cells (wt), *oxyR* (R957), *katG* (G221), and *oxyR katG* (RG27) mutants under uninduced (U) and menadione-induced (M) conditions. *Arrowheads* indicate hybridizing mRNAs and their sizes (kb) are shown

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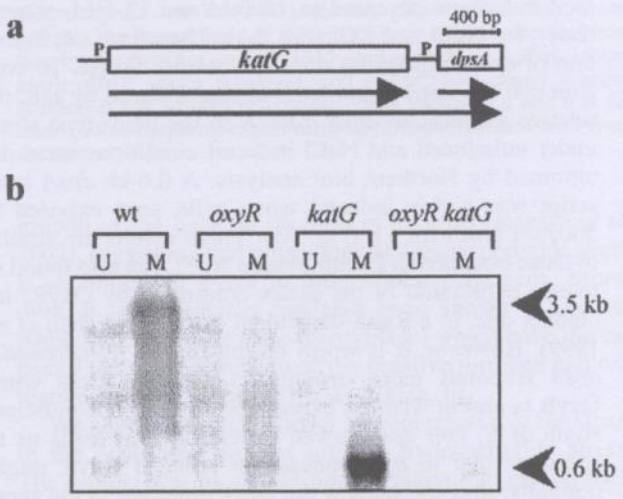

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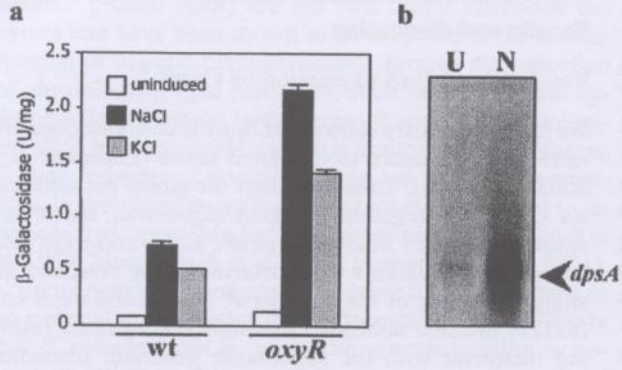

Fig. 2 Regulation of the *dpsA* promoter in response to osmotic stress. **a** β-Galactosidase activities in crude extracts of *dpsA-lacZ* fusion parent P844D (wt), and *oxyR* mutant R957D (*oxyR*) prepared from uninduced cells and cells induced with NaCl and KCl. Each value shown is the mean of three separate experiments: *error bars* standard error from the mean. **b** Northern analysis of *dpsA* mRNA prepared from *B. pseudomallei* P844 cells under uninduced (U) and NaCl-induced (N) conditions

Inducible transcription of *dpsA* by osmotic stress

To determine whether *dpsA* could be induced by oxidative and osmotic stresses, cells were treated with 0.5–10 mM H_2O_2, 500 mM NaCl or 500 mM KCl and *dpsA-lacZ* expression was monitored. Under these conditions, H_2O_2 did not cause any significant induction of the *dpsA* promoter (data not shown). Expression of *dpsA-lacZ* in wild-type (P844D) cells was induced eightfold and sixfold by NaCl and KCl, respectively, compared to an uninduced control. In the *oxyR*-disrupted mutant R957D, the level of *dpsA-lacZ* induction increased to 18-fold and 12-fold, respectively, for NaCl and KCl (Fig. 2A). Therefore, salt induction of *dpsA* expression does not require OxyR. To confirm that the *dpsA* promoter is indeed induced by salt, the relative amounts of *dpsA* mRNA in the wild- type strain under uninduced and NaCl-induced conditions were determined by Northern blot analysis. A 0.6-kb *dpsA* transcript was highly induced when cells were exposed to 500 mM NaCl for 1 h (Fig. 2B). These results are similar to those obtained in *E. coli*, where NaCl was also found to induce expression of the genes controlled by OxyR, including *dps*, in a RpoS-dependent manner (Michan et al. 1999). However, it is worth noting that *B. pseudomallei dpsA* responds more strongly to osmotic stress when OxyR is absent whereas expression in an OxyR-deficient strain of *E. coli dps* showed no effect. This leads us to speculate that in *B. pseudomallei* reduced OxyR might normally bind and repress the *dpsA* promoter in the same manner that it has previously been shown to bind to, and repress expression of, the *katG* promoter in uninduced *B. pseudomallei* (Loprasert et al. 2003b). Therefore, a lack of OxyR would certainly facilitate the RpoS dependence of the *dpsA* promoter by RNA polymerase. Expression of *dps* was also shown to be induced by general stress, e.g. heat shock, exposure to high salt or ethanol, and after glucose starvation in *B. subtilis* (Antelmann et al. 1997).

t-BOOH sensitivity of *dpsA* mutants

The physiological role of DpsA in *B. pseudomallei* was determined by testing the sensitivity of the various mutants to organic hydroperoxide stress. DpsA-deficient mutants exhibited hypersensitivity to *t*-BOOH. The *dpsA* mutant D18 did not grow well on 150 μM *t*-BOOH-containing agar while the growth of *dpsA oxyR* double mutant DR1, was even poorer. In both strains, growth was restored to the wild-type level after complementation with the *dpsA*-containing plasmid pDps (Fig. 3A). The ability of the wild-type and mutant strains to grow in M9 minimal liquid medium containing 150 μM *t*-BOOH was also studied (Fig. 3B) and the results are in good agreement with those determined on agar plates. In the wild-type strain P844, overexpression of DpsA from pDps (strain P844/pDps) increased relative growth following *t*-BOOH exposure (75% compared to 55% for wild-type). In strain D18, which lacks a functional *dpsA*, the relative growth following *t*-BOOH exposure was reduced to 36%. When either DpsA or AhpC was overexpressed in the *dpsA* mu-

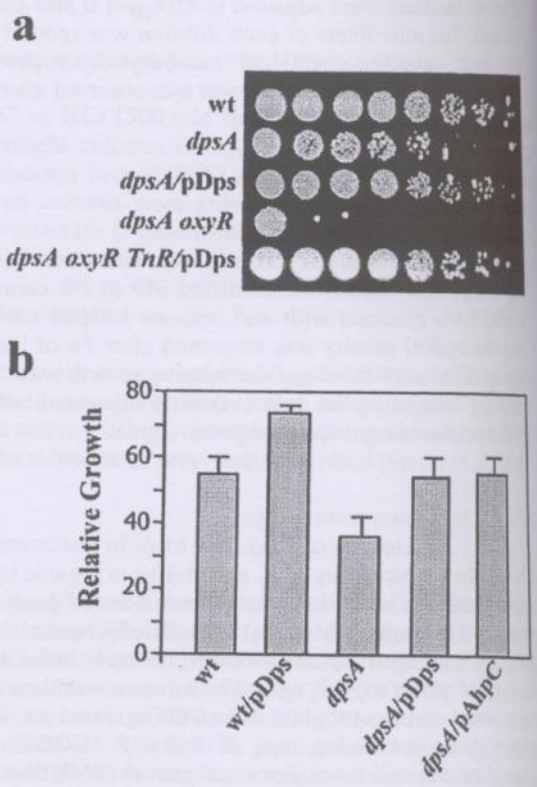

Fig. 3 Sensitivity of *B. pseudomallei* to *t*-BOOH. **a** Growth on *t*-BOOH-containing agar plate of serially diluted *B. pseudomallei* P844 (wt), *dpsA* mutant (*dpsA*), *dpsA* mutant complemented with *dpsA* on plasmid pDps (*dpsA*/pDps), *dpsA oxyR* mutant (*dpsA oxyR*), and the *dpsA oxyR* mutant complemented strain (*dpsA oxyR TnR*/pDps). **b** Relative growth of various strains in *t*-BOOH-containing M9 medium. *B. pseudomallei* P844 (wt), overexpressed DpsA (wt/pDps), *dpsA* mutant (*dpsA*), complemented *dpsA* mutant (*dpsA*/pDps), and *dpsA* mutant with AhpC plasmid pAhpC (*dpsA*/pAhpC). Each value shown is the mean of three separate experiments; *error bars* standard error from the mean

tant strains D18/pDps and D18/pAhpC the relative rate of growth was restored to the wild-type level. This restoration of *t*-BOOH resistance in the *dpsA* strain expressing AhpC was expected since AhpC reduces and detoxifies *t*-BOOH (Storz et al. 1989). Dps-deficient *E. coli* mutants have been shown to be hypersensitive to H_2O_2 (Almiron et al. 1992), *N*-ethylmaleimide (NEM) (Ferguson et al. 1998), and acid stress (Choi et al. 2000). *B. subtilis mrgA* mutants are sensitive to H_2O_2 (Chen and Helmann 1995). To our knowledge, this is the first report demonstrating that Dps protects cells from organic hydroperoxide toxicity. We have previously found that a *B. pseudomallei katG* mutant shows increased sensitivity to H_2O_2, menadione, NEM, and sodium hypochlorite (Loprasert et al. 2003b). In order to rule out the possibility that KatG expression might be reduced in the *dpsA* mutant strain, the sensitivity of this strain to each of the aforementioned oxidants was measured. It was found that the *dpsA* mutant had the same sensitivity, as determined by growth inhibition zone assays, to H_2O_2 (0.5 M), menadione (100 mM), NEM (0.1 M), and sodium hypochlorite (0.6%) as the wild-type (data not shown).

Protection of *E. coli* against *t*-BOOH by *B. pseudomallei* DpsA

To test whether the protective property of DpsA to organic hydroperoxide is specific to *B. pseudomallei*, *dpsA* was overexpressed in the organic-oxidant-sensitive *E. coli* strain TA4315 (Storz et al. 1989) which lacks functional *ahpC*. Growth inhibition studies clearly demonstrated that both *B. pseudomallei* DpsA and AhpC could protect *E. coli* against *t*-BOOH toxicity, suggesting that this is a common property of DpsA (Fig. 4A). Since *B. pseudomallei* AhpC also conferred protection to *t*-BOOH, we were interested in finding out whether both AhpC- and DpsA-mediated protection involve the same or different mechanisms. It has been well documented that AhpC is a reductase that catalyzes the reduction of alkyl hydroperoxide to alcohol (Storz et al. 1989). The levels of *t*-BOOH in the culture medium during growth of *E. coli* strain TA4315 expressing either *B. pseudomallei* AhpC or DpsA from plasmid were therefore determined. As anticipated, the AhpC-expressing strain (TA4315/pAhpC) completely reduced the *t*-BOOH in the culture medium within 20 min, whereas the DpsA-expressing strain (TA4315/pDps) showed no significant reduction of *t*-BOOH levels relative to strain TA4315 carrying plasmid vector pBBR (Fig. 4B). This indicates that the mechanisms of protection employed by DpsA and AhpC during organic hydroperoxide exposure are distinct. It is likely that the binding of DpsA to DNA acts as a physical barrier to organic-oxidant-induced DNA damage in a manner analogous to that observed for hydrogen peroxide protection (Wolf et al. 1999). By contrast, AhpC enzymatically detoxifies the organic hydroperoxides. Analysis of *ahpC* expression in *Legionella pneumophila* and *Salmonella typhimurium* showed that *ahpC* levels increased several-fold during intracellular

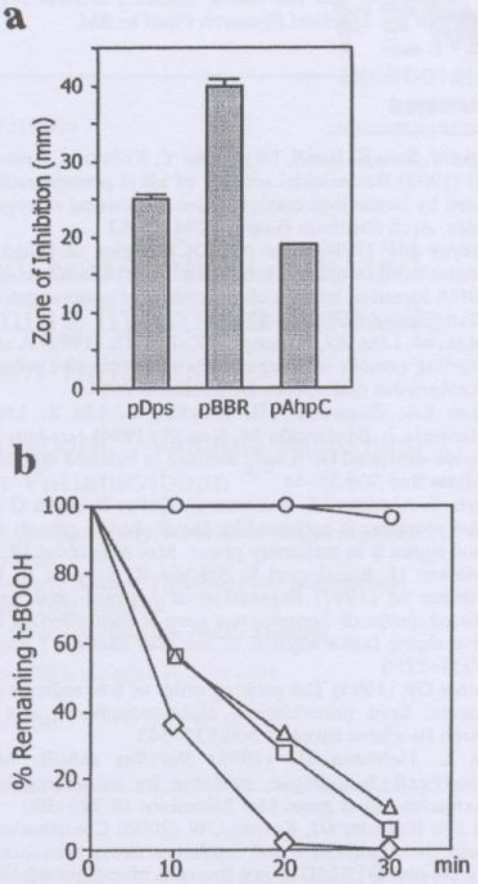

Fig. 4 Growth inhibitory zone and *t*-BOOH degradation assays. **A** Determination of the levels of resistance to *t*-BOOH killing displayed by *E. coli* TA4315 harboring pDps, pBBR vector, and pAhpC. **B** Measurement of the remaining *t*-BOOH after degradation by *Escherichia coli* TA4315 strains harboring pDps (*triangles*), pBBR vector (*squares*), and pAhpC (*diamonds*). Control (circles) is LB without cells. Each value shown in **a** and **b** is the mean of three separate experiments; *error bars* standard error from the mean.

growth of the bacteria (Francis et al. 1997; Rankin et al. 2002), suggesting that physiological organic peroxide stress is an important threat to intracellular pathogens. Moreover, *B. pseudomallei* has also been shown to contain AhpC and KatG (catalase–peroxidase) which have the capacity to relieve a portion of the reactive nitrogen and oxidative stresses (Loprasert et al. 2003a, b).

We have uncovered a novel physiological role for DpsA in the protection against organic hydroperoxide stress. The protein acts as an additional system that can be used by *B. pseudomallei* to guard against attack by host immune systems. This study demonstrates that DpsA is a key component of the stress protection system important for the survival of the infectious pathogen *B. pseudomallei*.

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Novel Roles of *ohrR-ohr* in *Xanthomonas* Sensing, Metabolism, and Physiological Adaptive Response to Lipid Hydroperoxide

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Lipid hydroperoxides are highly toxic to biological systems. Here, the *Xanthomonas campestris* pv. phaseoli sensing and protective systems against linoleic hydroperoxide (LOOH) were investigated by examining the phenotypes, biochemical and regulatory characteristics of various *Xanthomonas* mutants in known peroxide resistance pathways. Analysis of LOOH resistance levels indicates that both alkyl hydroperoxide reductase (AhpC) and organic hydroperoxide resistance enzyme (Ohr) have important and nonredundant roles in the process. Nonetheless, inactivation of *ohr* leads to a marked reduction in LOOH resistance levels. The regulatory characteristics of an *ohr* mutant add further support to its primary role in LOOH protection. Northern analysis shows that LOOH had differential effects on induction of *ahpC* and *ohr* expression with the latter being more sensitive to the inducer. Analysis of the *ahpC* and *ohr* promoters confirmed that the LOOH-dependent induction of these promoters is mediated by the transcription regulators OxyR and OhrR, respectively. Using the in vivo promoter assays and the in vitro gel mobility shift assay, we show that LOOH directly oxidized OhrR at the sensing residue Cys-22 leading to its inactivation. In addition, physiological analysis shows that pretreatment of *X. campestris* pv. phaseoli with a sublethal dose of LOOH induced high levels of resistance to subsequent exposure to lethal concentrations of LOOH. This novel LOOH-induced adaptive response requires a functional *ohrR-ohr* operon. These data illustrate an important novel physiological role for the *ohrR-ohr* system in sensing and inactivating lipid hydroperoxides.

During normal growth *Xanthomonas* spp. are exposed to harmful reactive oxygen species (ROS) including H_2O_2, organic peroxide, and superoxide anions generated from other soil organisms and as a part of active plant defense responses. Lipid hydroperoxides are important components of the ROS produced during the plant defense response (8), and are both highly reactive and toxic to bacterial cells. Plant lipoxygenases catalyze the formation of fatty acid hydroperoxides through the reaction of fatty acid precursors such as linoleic or linolenic acids with molecular oxygen (3, 8). The expression of these enzymes has been shown to be induced in response to microbial invasion and has been linked to the plant microbial defense response (10). Consequently, in order to survive and proliferate during infection, invading bacteria must detoxify lipid hydroperoxides.

To date, very little is known regarding how bacteria protect themselves from fatty acid hydroperoxides. The best-characterized bacterial system for the detoxification of organic hydroperoxides is the alkyl hydroperoxide reductase (AhpC). AhpC catalyzes the reduction of organic peroxides to their corresponding alcohols (24). In many bacteria, inactivation of *ahpC* results in increased sensitivity to organic peroxides and pleiotropic alterations in the oxidative stress response (2, 20, 27, 31, 32). A second system for organic hydroperoxide protection, designated *ohr*, has been discovered in *Xanthomonas* (19). *ohr* confers resistance to organic hydroperoxides, and inactivation of the gene leads to increased sensitivity to organic peroxides (19). *ohr* homologues are widely distributed in both gram-positive and gram-negative bacteria (1, 6, 12, 22, 25, 28). The structure and biochemical mechanism of Ohr have been elucidated (12). Ohr is a thiol peroxidase that catalyses the reduction of an organic hydroperoxide to its corresponding organic alcohol (4). AhpC and Ohr appear to have similar biochemical properties and possibly overlapping physiological functions. The genes are independently regulated. *ahpC* is regulated by OxyR (14, 32), whereas *ohr* is controlled by the transcription repressor OhrR (16). In *Xanthomonas*, AhpC and Ohr were shown to have slightly different organic peroxide substrate preferences (30). Recently, the thiol peroxidases, bactoferritin comigratory protein (BCP), and glutathione peroxidases (Gpx-like) have been reported to contribute to the protection of bacteria from organic peroxide (7, 9). However, the corresponding genes are either found only in a few bacteria and are not well characterized or they have highly specialized physiological roles. Thus, their general role in the protection of bacteria from organic peroxide has yet to be elucidated.

Here, we examined the physiological and biochemical roles of AhpC and Ohr in the protection against lipid hydroperoxide toxicity. The results of the study demonstrate the importance of the *ohrR-ohr* system in the ability to tolerate lipid hydroperoxides and revealed a novel bacterial adaptive response to lipid hydroperoxide exposure. (Parts of this work are from the dis-

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Different sensitivity to LOOH in *ahpC* and *ohr* mutants. Many organic hydroperoxide-metabolizing systems have been studied in bacteria; however, these studies have not addressed the integral roles of gene regulation and bacterial physiology in these defense systems (7, 9, 12, 17, 24, 27, 30). Thus, a growth inhibition zone assay (19) was used to measure the sensitivity to LOOH (prepared as described by Evans et al. [5]) of wild-type *Xanthomonas campestris* pv. phaseoli and various *Xanthomonas* strains. Wild-type *X. campestris* pv. phaseoli was highly resistant to LOOH and exhibited no zone of growth inhibition when exposed to 50 mM LOOH. However, mutants in which the *ahpC* (17) and *ohr* (19) genes were inactivated gave zones of growth inhibition of 6 and 12 mm, respectively. In the double mutant, a zone of inhibition of 16.5 mm was observed. At present, the mechanism responsible for uptake of LOOH is not known. At high concentrations of LOOH diffusion is thought to contribute to the uptake process while at low concentrations of LOOH, the energy-dependent fatty acid uptake system could be involved (21).

We extended these studies to determine the ability of *ahpC1*, *ohr*, and *ahpC ohr* mutants to metabolize LOOH using the Fox assay as described by Ochsner et al. (22) and Shea and Mulks (28). Exponential-phase cultures (optical density at 600 nm [OD_{600}] of 0.5) of the parental strain, *ahpC1*, *ohr*, and *ahpC1 ohr* mutants were incubated with 200 μM LOOH, and the amount of LOOH remaining after a 30-min incubation was determined. The results mirrored the resistance studies in that both *ahpC1* and *ohr* single mutants displayed a decreased ability to metabolize LOOH, with the *ohr* mutant showing the higher degree of impairment, while an *ahpC1 ohr* double mutant was less able to metabolize LOOH than either of the single mutants (data not shown). The ability of the *ahpC1 ohr* double mutant to metabolize LOOH could be restored to levels that were equal to or greater than that of wild type by the overexpression of plasmid-borne *ahpC* and *ohr*, respectively (data not shown), indicating that both enzymes could use LOOH as a substrate. The data suggest that the two systems act through independent pathways with *ohr* being the major protective system and *ahpC* playing a secondary backup role in protecting *Xanthomonas* from LOOH. A possible explanation for this observation could be due to a difference in the cellular locations of the two enzymes. Ohr is structurally related to OsmC, a putative thiol peroxidase that is localized in the periplasmic space (12, 13), and initial studies in our laboratory have shown that Ohr is found in both the periplasm and the cytoplasm (S. Mongkolsuk et al., unpublished observation). By contrast, AhpC is likely to be a cytoplasmic protein (24). Thus, periplasmic Ohr could detoxify LOOH before it entered the cytoplasm, thereby limiting damage to intracellular macromolecules.

LOOH induced the expression of *ahpC* and *ohr*. The LOOH-dependent regulation of *ahpC* and *ohr* is of particular interest, due to the fact that the genes are regulated by different global peroxide-sensing transcriptional regulatory systems and display different patterns of oxidant-induced expression (14, 29). Thus, the effect of treatment with LOOH or the synthetic organic hydroperoxide, *tert*-butyl hydroperoxide (tBOOH), on the expression of these genes in *X. campestris* pv. phaseoli was investigated using Northern blot hybridization analysis. It was found that LOOH was a strong inducer of *ohr* expression. *ohr* was induced by exposure to 10 μM LOOH whereas a similar treatment with 10 μM tBOOH did not induce expression of the gene (Fig. 1A). As inducing concentrations of LOOH increased, there was a parallel increase in the magnitude of induction of *ohr* expression that reached a maximum level of 80-fold (as determined by densitometry), relative to the level in uninduced cells, following treatment with 100 μM LOOH (Fig. 1A). *ohr* expression was also induced by tBOOH, but to a lesser degree (Fig. 1A). Treatment with 100 μM tBOOH induced *ohr* expression by less than 10-fold. When *ahpC* expression was examined, the situation was reversed. As was the case with *ohr*, both peroxides were able to induce *ahpC* expression.

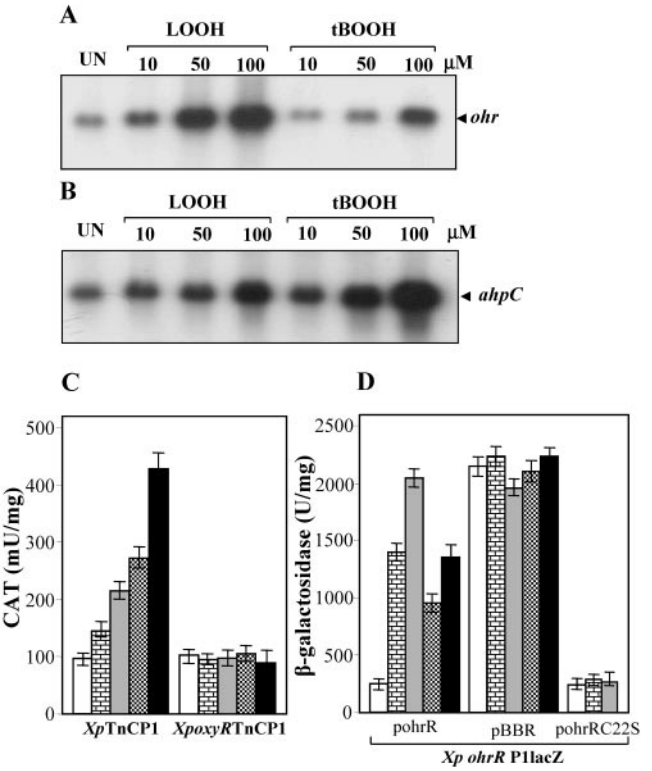

FIG. 1. Induction of *ahpC* and *ohr* expression in *X. campestris* pv. phaseoli by LOOH and tBOOH. Total RNA (10 μg) prepared from *X. campestris* pv. phaseoli cultures induced with 10, 50, 100 μM LOOH; 10, 50, 100 μM tBOOH; and uninduced cells (UN) were loaded into each lane and hybridized with either ^{32}P-labeled *ohr* (A) or *ahpC* (B) probes as previously described (30). The numbers above each lane in both panels A and B indicate the concentrations of peroxide added to the cultures. (C) Induction of the *ahpC* promoter fused to *cat* was monitored by determination of chloramphenicol acetyltransferase (CAT) activity (26) in *X. campestris* pv. phaseoli TnCP1 (*Xp* TnCP1) and an *oxyR* mutant containing TnCP1 (*Xp oxyR* TnCP1). (D) Induction of the *ohrR* P1 promoter fused to *lacZ* was monitored by determining β-galactosidase activity (15) in an *ohrR* mutant containing a P1 *lacZ* fusion (*Xp ohrR* P1lacZ) harboring pohrR, pBBR1MCS-5 (11) (pBBR), and pohrRC22S. For experiments in both panels C and D, exponential-phase cultures were untreated (open bars) or treated with LOOH (100 μM, brick bars, and 200 μM, gray bars), or tBOOH (100 μM, checkered bars, and 200 μM, black bars) for 30 min. Crude lysate preparation and enzymatic assays were performed as previously described (23). The CAT- or β-galactosidase-specific activities from induced cultures and uninduced cultures are shown.

However, tBOOH was the more effective of the two. Treatment with 100 μM tBOOH produced an 80-fold induction in *ahpC* expression levels compared to a 30-fold increase in the *ahpC* levels following treatment with 100 μM LOOH (Fig. 1B). The data clearly showed that the regulation of *ohr* responded more sensitively to the complex organic hydroperoxide, LOOH, than to the simple organic hydroperoxide molecule, tBOOH. By contrast, induction of *ahpC* expression was more sensitive to tBOOH than to LOOH treatments.

We extended these observations by determining the effect of LOOH and tBOOH on the transcription of *ahpC* and *ohrR* by monitoring the promoter activities of these genes using strains containing transcriptional fusions of the *ahpC* promoter with chloramphenicol acetyltransferase (*cat*) (*Xp* TnCP1 [14]) and the *ohrR* P1 promoter with β-galactosidase (*Xp ohrR* P1lacZ [23]) that were constructed by insertion of the reporter gene cassette within the chromosomal copy of *ahpC* or *ohrR*. The results reinforced those of the Northern blot analyses in demonstrating that the *ahpC* promoter was more efficiently induced by tBOOH. Treatment of *Xp* TnCP1 with 200 μM tBOOH resulted in a 4.5-fold increase in *ahpC* promoter activity, relative to an uninduced culture, compared with only a 2.2-fold increase in the presence of 200 μM LOOH (Fig. 1C). Furthermore, induction of the *ahpC* promoter by either organic peroxide depended on the presence of functional OxyR since no induction of the *ahpC* promoter was observed in an *oxyR* mutant background (*Xp oxyR* TnCP1) (Fig. 1C). Analysis of the hydroperoxide dependent induction of *ohrR* P1 promoter activity was complicated by the fact that the *lacZ* reporter gene insertion in this strain inactivates *ohrR*, encoding the *ohr* repressor (23). Thus, it was necessary to first complement this strain with a plasmid-borne copy of *ohrR* (pohrR) (18). As expected, LOOH was more efficient at inducing *ohrR* P1 promoter activity than tBOOH. Treatment of the strain containing the *ohrR* P1*lacZ* fusion (*Xp ohrR* P1lacZ harboring pohrR) with 100 and 200 μM LOOH induced P1 promoter activity by 6.8- and 9.7-fold, respectively, while treatment with the same concentrations of tBOOH resulted in respective increases in P1 promoter activity of 4.5- and 6.4-fold (Fig. 1D). The induction of the P1 promoter was found to be dependent on the presence of functional OhrR since the uncomplemented *ohrR* mutant strain (pBBR) did not show hydroperoxide-specific induction of the P1 promoter (Fig. 1D). The in vivo promoter fusion data supported the Northern blot results and confirmed that the observed increases in the levels of *ahpC* and *ohr* mRNA, in response to organic hydroperoxide treatments, were due to increased rates of *ahpC* and *ohr* transcription. Furthermore, the data show that in the presence of LOOH and tBOOH, the *ahpC* and *ohrR* promoters are induced by separate peroxide sensing regulatory systems. It appears that both OxyR and OhrR can sense changes in lipid hydroperoxide levels with the latter being more sensitive to the presence of the more complex hydroperoxide, LOOH, while OxyR is more sensitive to the simple organic hydroperoxide molecule, tBOOH.

In *Xanthomonas* the mechanism of organic hydroperoxide-dependent derepression of *ohr* transcription is thought to proceed via the oxidation of the highly conserved peroxide sensing cysteine residue, Cys-22, of OhrR (23). In order to test whether Cys-22 is required for LOOH inactivation of OhrR, a plasmid carrying a copy of the mutant *ohrR* (pohrRC22S), in which Cys-22 has been changed to serine (C22S), was transformed into *Xp ohrR* P1lacZ and the ability of LOOH to induce the P1 promoter in this strain was evaluated. The results showed that LOOH-dependent induction of the P1 promoter was abolished in *Xp ohrR* P1lacZ harboring pohrRC22S (Fig. 1D). This indicates that residue Cys-22 of OhrR is essential for LOOH-dependent derepression of the P1 promoter. This favors the idea that in vivo, LOOH or its metabolites mediate the oxidation of residue Cys-22 thus inactivating OhrR.

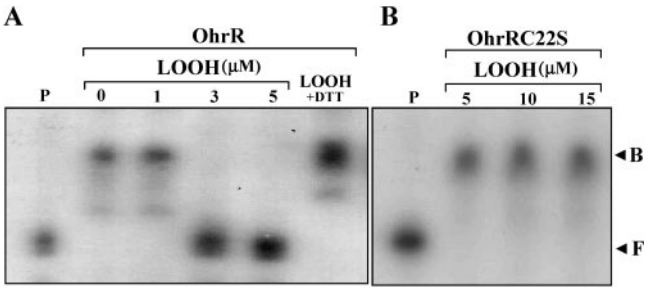

FIG. 2. The effect of LOOH and DTT on OhrR binding to the *ohrR-ohr* promoter. The results of DNA mobility shift experiments, testing oxidation of OhrR by LOOH. The DNA binding reaction contained ^{32}P-labeled P1, the *ohrR-ohr* promoter fragment (18), 3 fmol of purified OhrR or purified OhrRC22S (23). The binding reactions and electrophoresis were performed as previously described (23). In panel A, the binding reactions containing the P1 promoter fragment and OhrR were treated with either various concentrations of LOOH or 5 μM LOOH followed by 10 mM DTT treatment (LOOH+DTT) as previously described (23). In panel B the binding of P1 fragment to 3 fmol of purified OhrR C22S before 5, 10, and 15 μM LOOH were added to the binding reactions. The numbers above each lane indicate the concentration of LOOH added. P indicates free probe. The positions of bound (B) and free (F) probes are indicated.

LOOH oxidizes and inactivates OhrR binding to the promoter. In vivo experiments suggested that LOOH or its metabolites probably oxidized OhrR at Cys-22, but the experiment could not provide a definitive answer regarding the mechanism of LOOH sensing. Thus, gel mobility shift experiments were performed to further characterize the LOOH-sensing mechanism of OhrR. First purified OhrR and OhrRC22S (23) were incubated with a radioactively labeled 170-bp P1 promoter fragment in the presence and absence of LOOH. In the absence of LOOH, OhrR strongly bound to the P1 promoter fragment as shown by the slower-migrating P1 promoter fragment OhrR complex (Fig. 2A). Addition of 3 μM LOOH to the binding reaction completely negated OhrR binding to the P1 promoter fragment. The concentration of LOOH required to completely inhibit the binding of OhrR to the P1 promoter was 100-fold lower than that previously determined for tBOOH (18). Next, we tested whether the inactivation of OhrR by LOOH was due to direct oxidation of the protein by assessing whether the process could be reversed by treatment with a reducing agent (dithiothreitol [DTT]) and determining the effect of LOOH on a nonsensing mutant protein OhrRC22S. The results show that 10 mM DTT reversed the inhibitory effects of LOOH on the binding of OhrR to the P1 promoter (Fig. 2A). In addition, the mutant OhrRC22S had no binding defect as shown by its ability to efficiently bind to the promoter fragment at a similar concentration as wild-type OhrR (Fig. 2B). However, treatment of OhrRC22S with in-

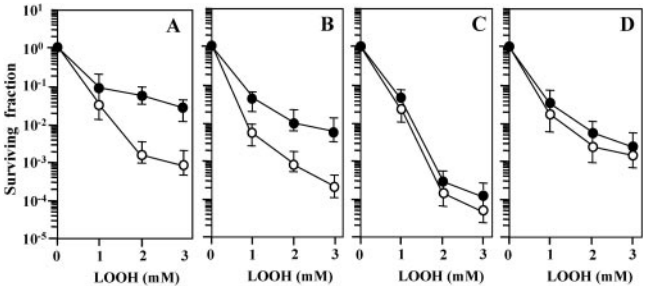

FIG. 3. Induced adaptive protection responses to LOOH in *X. campestris* pv. phaseoli, *ohr* and *ohrR* mutants. The results of LOOH-induced adaptive protection response experiments testing the effect of a 30-min preexposure to 50 μM LOOH on the survival of *X. campestris* pv. phaseoli exponential-phase cultures to subsequent exposure to 0, 1, 2 or 3 mM LOOH for 30 min. Plots of the surviving fraction of cells in cultures that did (●) or did not (○) receive a pretreatment are shown. (A) *Xp*, *X. campestris* pv. phaseoli; (B) *ahpC1* mutant; (C) *ohr* mutant; (D) *ohrR* mutant. Each value presented is the mean and standard deviation of four replicates.

creasing concentrations of LOOH had no effect on the mutant protein's ability to bind to the P1 promoter (Fig. 2B). The results support the idea that LOOH inactivated OhrR through the direct oxidation of the sensing Cys-22 residue. The available in vitro DNA binding data from this and previous studies (18) support the in vivo promoter analyses in showing that OhrR is 80-fold more responsive to the complex hydroperoxide, LOOH, than to the simple hydroperoxide, tBOOH. This favors the idea that OhrR may have evolved to preferentially sense complex organic hydroperoxides such as lipid hydroperoxides via oxidation of the highly conserved peroxide sensing residue Cys-22. The in vivo and in vitro regulatory characteristics of the *ohrR-ohr* operon support its role as the major system for the sensing of and protection from lipid hydroperoxides such as LOOH.

The novel physiological LOOH adaptive response required functional *ohrR* and *ohr*. The adaptive response is an important strategy for microbial survival under stressful conditions; however, an adaptive response to lipid peroxide has not been reported previously. Experiments were done to test if *Xanthomonas* has the capacity to mount an adaptive response to LOOH and whether *ahpC* and *ohr* are involved in the process. *Xanthomonas* cultures that had been pretreated with 50 μM LOOH were exposed to lethal concentrations (1, 2, 3 mM) of LOOH for 30 min and the fraction of surviving cells was determined. The results in Fig. 3A show that LOOH induced cells were 50-fold more resistant to LOOH killing than uninduced cells. This is the first demonstration of a bacterial adaptive response to a lipid hydroperoxide. Similar experiments were then performed using *ohr* and *ahpC* mutants in order to determine the roles of *ohr* and *ahpC* in the LOOH adaptive response. Pretreatment of an *ahpC1* mutant with LOOH induced high-level resistance to subsequent LOOH killing (Fig. 3B). By contrast, a similar preexposure of the *ohr* mutant to LOOH failed to induce increased protection, relative to uninduced cells, against subsequent LOOH killing treatments (Fig. 3C). Clearly, *ohr*, but not *ahpC*, is required for the LOOH adaptive response. We extended the investigation by determining whether proper regulation of *ohr* or simply the presence of functional *ohr* was required for the LOOH adaptive response. In the previous section, we showed that the transcription repressor, OhrR, was involved in LOOH-dependent induction of *ohr*. We therefore tested whether OhrR was also the regulator involved in the LOOH adaptive response. The LOOH adaptive response experiment was repeated using the *ohrR* mutant. As expected, pretreatment of the *ohrR* mutant with LOOH did not induce adaptive protection against subsequent LOOH killing (Fig. 3D) indicating that proper regulation of the operon is required for the LOOH-induced adaptive response. Loss of the induced adaptive protection in *ohrR and ohr*, mutants, but not in *ahpC1* mutant is consistent with the data from the physiological, and gene regulation analyses indicating that the *ohrR-ohr* system plays the major role in protecting *Xanthomonas campestris* pv. phaseoli from LOOH.

An important physiological question is whether *Xanthomonas* is likely to be exposed to LOOH in its natural environment. *Xanthomonas* spp. are important bacterial phytopathogens. During plant microbe interactions, bacteria are likely to be exposed to lipid hydroperoxide produced by plants as part of an active defense response against microbial invasion. It has been shown that increased lipoxygenase, an enzyme involved in lipid hydroperoxide synthesis, is associated with the plant defense response and fatty acid precursors such as linoleic or linolenic acids are abundant in plants (3, 8). Thus, *Xanthomonas* is likely to encounter LOOH during its interaction with host plants. Interestingly, *ohr* homologues have been found in all genomes of bacterial plant pathogens thus far sequenced (Mongkolsuk et al., unpublished observation). This conservation of *ohr* implies its important physiological role in the protection against lipid hydroperoxide exposure during plant-microbe interactions.

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The unique glutathione reductase from *Xanthomonas campestris*: Gene expression and enzyme characterization ☆

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Abstract

The glutathione reductase gene, *gor*, was cloned from the plant pathogen *Xanthomonas campestris* pv. *phaseoli.* Its gene expression and enzyme characteristics were found to be different from those of previously studied homologues. Northern blot hybridization, promoter-*lacZ* fusion, and enzyme assay experiments revealed that its expression, unlike in *Escherichia coli*, is OxyR-independent and constitutive upon oxidative stress conditions. The deduced amino acid sequence shows a unique NADPH binding motif where the most highly conserved arginine residue, which is critical for NADPH binding, is replaced by glutamine. Interestingly, a search of the available Gor amino acid sequences from various sources, including other *Xanthomonas* species, revealed that this replacement is specific to the genus *Xanthomonas*. Recombinant Gor enzyme was purified and characterized, and was found to have a novel ability to use both, NADPH and NADH, as electron donor. A *gor* knockout mutant was constructed and shown to have increased expression of the organic peroxide-inducible regulator gene, *ohrR*.

Keywords: Glutathione reductase; NADPH binding motif; Organic hydroperoxide-responsive promoter; Oxidative stress

Glutathione is a major cellular free thiol-containing compound that is present in animals, plants, fungi, and a large number of prokaryotic species. Glutathione is synthesized by glutathione synthetase and functions as an important cellular antioxidant that can react with a variety of compounds containing electrophilic centers. Apart from its function as an antioxidant, glutathione is also responsible for the maintenance of the intracellular thiol redox status and thus contributes to the function of many biological processes within the cell [1,2]. For most of its functions glutathione must be in the reduced form. Glutathione reductase (Gor) is the enzyme that reduces the oxidized form of glutathione, glutathione disulfide (GSSG), to reduced glutathione (GSH). In *Escherichia coli*, high steady-state levels of glutathione maintain a strong reducing environment in the cell [3]. Glutathione can react with H_2O_2, O_2^-, or $HOO^\cdot$ to form stable glutathione radicals that will then dimerize to form glutathione disulfide. Finally, glutathione reductase can then transfer an electron to glutathione disulfide, to re-form reduced glutathione [4]. Typically, reduction of GSSG to GSH is catalyzed by Gor, which in most cases exhibits a marked preference for NADPH over NADH as the electron donor. One of the most important functions of glutathione is to reduce disulfide bridges in proteins caused by oxidative stress. Although formation of the disulfide bonds is easily reversible, their presence can drastically alter protein function.

Glutathione reductase is a member of an important class of flavoprotein enzymes, the disulfide oxidoreductases, containing two active-site electron acceptors: FAD and a redox-active disulfide. The other members of this class include lipoamide dehydrogenase [5],

☆ *Abbreviations:* *t*-BOOH, *tert*-butyl hydroperoxide; NEM, *N*-ethylmaleimide.

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mercury reductase [6], trypanothione reductase [7], and thioredoxin reductase [8]. These proteins share extensive amino acid sequence similarities, in particular, sequences surrounding the redox-active cysteine residues, implying that they have arisen by divergent evolution from a common ancestor [9]. Glutathione reductase is the most important enzyme in maintaining a high intracellular ratio of reduced:oxidized glutathione (approximately 500:1) [10]. Gor is involved in redox cycles that are important in maintaining the anti-oxidative capacity of cells engaged in a wide variety of functions in which reactive oxygen species may be produced and is considered to be a key enzyme involved in maintaining the redox status of the cell during oxidative stress.

Xanthomonas belongs to an important family of plant bacterial pathogens. The bacterial enzymes and genes involved in the oxidative stress response and in regulating cellular redox status are likely to play important roles in disease development. Therefore, study of the glutathione reductase in this phytopathogen would certainly yield crucial information relating to pathogenesis and how *Xanthomonas* adapts to the host plant environment during infection.

This work reports that *Xanthomonas campestris* glutathione reductase has an atypical NADPH binding motif, in which the most highly conserved arginine residue, that is critical for NADPH binding, is replaced by glutamine. This unique change is specific only to Gor from the *Xanthomonas* genus. Furthermore, recombinant *Xanthomonas* Gor was found to have the ability to utilize both NADPH and NADH as electron donors. A *gor*-disrupted *Xanthomonas* mutant displayed increased expression of the organic peroxide-responsive regulator gene (*ohrR*).

Materials and methods

Bacterial cultures and media. Xanthomonas campestris pv. *phaseoli* was grown aerobically at 28 °C in SB medium as previously described [11,12]. All *E. coli* strains were grown aerobically in Luria–Bertani (LB) broth at 37 °C.

Nucleic acid extraction and analysis, cloning, and nucleotide sequencing. Genomic DNA extraction from *X. campestris* was performed according to the method of Mongkolsuk et al. [13]. Total RNA was isolated by hot-phenol method [13]. Molecular cloning, gel electrophoresis, and nucleic acid hybridizations were performed as previously described [14]. Nucleotide sequences were determined using an automated sequencer, model 310 (Applied Biosystems). *E. coli* and *Xanthomonas* were genetically transformed by a chemical method [14] and by electroporation [13], respectively.

In vitro transcription–translation analysis. Plasmid pGR1800 was used as a template for the expression of cloned gene products using a coupled in vitro transcription–translation *E. coli* S-30 extract system (Promega). A ^{14}C-methylated protein molecular weight standard (Amersham) was used as a standard marker.

Construction of chromosomal gor promoter::cat transcriptional fusion strains. The 540-bp *gor* promoter fragment was generated by PCR amplification using pZL-G1 as the template and primers corresponding to the 5′ region starting 340-bp upstream of the translation start site (5′ CGCGAGCGCCTGCGCATCGG 3′) and 3′ region (5′ CGCT GGCCAACTCGATCTTGC 3′). A *Bam*HI–*Hin*cII *gor* promoter fragment was ligated into *Bam*HI–*Eco*ICRI digested pUC18*Sfi*I *cat*, subsequently the *Sfi*I fragment containing the *gor* promoter and *cat* reporter was excised and ligated into the minitransposon pUT-Tn5 [15] to create pUT-Pgor which was then conjugally transferred into *Xanthomonas* and a stable kanamycin-resistant transconjugant was selected and named *X. campestris* strain TnPgor.

Amplification and sequencing of the conserved region of Gor in Xanthomonas species. Two oligonucleotide primers (5′ CACATCGT GATCGCCACCGG 3′ and 5′ GCCGCAATCGCCACCGGTGT 3′) corresponding to the conserved amino acid regions HIVIATG and TPVAIAA were synthesized and used to PCR amplify *gor* gene-internal fragments from *Xanthomonas vessicatoria*, *Xanthomonas translucens*, and *Xanthomonas hyacinthi* chromosomal DNA. The 560-bp fragments were cloned into pDrive (Qiagen) and their DNA sequence was determined.

High-level production and purification of Gor. High levels of *gor* expression for Gor purification were achieved using a His-tagged gene fusion expression vector system (Qiagen) in *E. coli*. Oligonucleotide primers corresponding to the 5′ (5′ CGGCATGCATGAGT GCGCGTTA 3′) and 3′ (5′ CGAAGCTTCGCAACCAACCAT 3′) non-coding regions of the *Xanthomonas gor* locus were used to amplify *gor* from pZL-G1. The resulting 1400-bp PCR product was then digested with *Sph*I and *Hin*dIII, gel purified, and cloned into pQE30 vector (Qiagen). A clone that expressed high levels of the fusion protein was obtained and named pQEG. A 200-ml culture of *E. coli* harboring pQEG was grown at 37 °C to an optical density at 600 nm of 0.6 and induced with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. The following purification steps were all done at 4 °C. The cells were subsequently pelleted, and the pellet was resuspended in sonication buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl). The suspension was then sonicated for a total of 10 min, with periodic cooling intervals. His-tagged Gor fusion protein was purified using nickle affinity columns according to the manufacturer's recommendations. The purified fusion protein was eluted with 100 mM imidazole in sonication buffer and the homogeneity of the eluted protein fractions was judged by SDS–PAGE. The eluted fractions containing the pure protein were pooled and dialyzed overnight against 20 mM Tris–HCl, pH 7.0, to remove imidazole.

Molecular weight determination of Gor. Protein concentration was measured by the dye binding method [16]. Determination of the molecular weight under denaturing conditions in the presence of SDS was performed as previously described [17]. For molecular weight determination under non-denaturing conditions, the addition of reducing agent (mercaptoethanol) to the protein sample and sample heating were omitted. The native molecular weight of recombinant His-tagged Gor was determined by gel filtration chromatography on a FPLC Akta Purifier (Pharmacia) using a Superdex 75 HR10/30 column (Pharmacia).

Gor enzyme assay. Gor activity was measured by monitoring the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) to thiobis(2-nitrobenzoic acid) by GSH which is produced by Gor according to a previously described method [18].

Disruption of gor gene. A *gor* insertion mutant was created by single recombination of plasmid, pBX170, into the chromosomal copy of *gor*. Specifically, a 1800-bp *Sph*I–*Hin*dIII fragment from pZL-G1 was gel purified and cloned into similarly digested pUC18 resulting in pGR1800. A 800-bp fragment was deleted from pGR1800 by digestion with *Bst*EII, *Hin*dIII, and gap-filled with Klenow polymerase, and ligated to form pBX1000. pBX1000 was further deleted by removing a 830-bp *Xba*I fragment followed by religation to form pBX170. Therefore, pBX170 contains a 170-bp gene-internal *gor* fragment in pUC18. Plasmid pBX170 was then electroporated into *X. campestris* and ampicillin resistant/*gor*-disrupted mutants were selected.

The correct integration of pBX170 into *gor* was verified by Southern blot hybridization (data not shown).

ohrR promoter assay. A previously described mini-Tn5 pP1lacZ construct, in which the *ohrR* promoter has been placed in front of a promoterless *lacZ* gene, was used as an indicator to measure the cellular redox status [19]. β-Galactosidase activity assays were carried out as previously reported [20].

Nucleotide sequence accession number. The nucleotide sequence of the *X. campestris* pv. *phaseoli gor* gene has been deposited in GenBank under Accession No. AY742859.

Results and discussion

Cloning of the X. campestris pv. phaseoli gor gene and its expression

Analysis of multiple amino acid sequence alignments of many Gor proteins revealed the presence of two conserved regions, VGCVPKK and GYIAVE [21], which were suitable for the application of reverse genetics and PCR gene cloning techniques. Degenerate oligonucleotide primers corresponding to the conserved regions were synthesized, taking into account the fact that *Xanthomonas* frequently uses G or C in the last position of codons. One primer corresponding to amino acid region VGCVPKK (5′ GTXGGXTGYGTXCCXAA ZAA 3′) and the second primer corresponding to amino acid region GYIAVE (5′ YTCXACXGCZATZTAXCC IXC 3′) (where X represents G and C, Y represents C and T, Z represents A and G, and I represents inosine) were used to amplify a 400-bp gene-internal portion of the *X. campestris* pv. *phaseoli gor* gene, which was cloned, sequenced, and used as a probe to screen an *X. campestris* pv. *phaseoli* genomic library constructed in a ZipLox vector (BRL Life Technology). A number of positively hybridizing clones were isolated, and plaques were purified. One positive clone, pZL-G1, was completely sequenced. Analysis of the nucleotide sequence revealed the presence of an open reading frame with a predicted amino acid sequence that shared high homology with Gor from a number of different sources. The *gor* gene was then subcloned into pGR1800, and in vitro transcribed and translated using the *E. coli* S-30 system (Promega). A 50-kDa protein band was detected (Fig. 1A) that corresponded to the calculated molecular mass of Gor verifying that the cloned *gor* could be in vitro translated to yield a full-length protein. Next, we examined the transcription pattern of *gor* in *X. campestris* using Northern blot hybridization experiments. The results, shown in Fig. 1B, revealed that *X. campestris* pv. *phaseoli gor* is transcribed as a 1.5-kb monocistronic mRNA. The level of *gor* mRNA was unaltered when cells were exposed to the oxidative stress inducing agents; diamide, paraquat, *N*-ethylmaleimide (NEM), cadmium, and nickel (Fig. 1B). *gor* promoter activity was also monitored in exponential phase cells of the *X. campestris* strain TnPgor, a strain that contains a

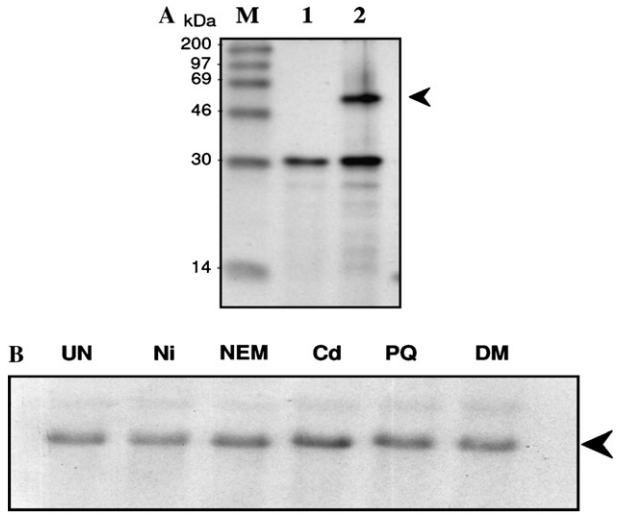

Fig. 1. In vitro translation products (A) and constitutive expression (B) of *gor*. (A) In vitro transcription-translation of pGR1800-encoded proteins with *E. coli* S-30 extracts. Lane M, protein molecular mass markers; lane 1, pUC18; and lane 2, pGR1800. The arrow indicates in vitro translation products of *gor*. The second band at around 30 kDa is the product of the ampicillin resistance gene. (B) Northern blot of total RNA isolated from *X. campestris* uninduced (UN) or induced with 0.2 mM Ni ($NiCl_2$), NEM (*N*-ethylmaleimide), Cd ($CdCl_2$), or PQ (paraquat), and 2 mM DM (diamide). The membrane was probed with a radioactively labeled *gor* DNA fragment. Ten micrograms of total RNA was loaded in each lane. The arrow indicates the 1.5-kb mRNA of *gor*.

chromosomal *gor::cat* transcriptional fusion, that had been exposed to 2 mM of either menadione, H_2O_2, *tert*-butyl hydroperoxide (*t*-BOOH), cumene, or paraquat for 30 min. Consistent with the mRNA analysis, no significant change in *gor* promoter activity was observed in the presence of any of the oxidants tested (data not shown). Moreover, exposure of cells to 10 μM paraquat for up to 24 h resulted in no increase in *gor* promoter activity (data not shown). This is in contrast to the situation in the yeast, *Schizosaccharomyces pombe*, where *gor* expression has been shown to increase upon exposure to oxidants such as: organic hydroperoxide, diamide, and the superoxide generator, menadione [22].

The constitutive expression of *X. campestris gor* raised the question of whether *gor* is in the OxyR regulon as is the case in *E. coli* [23]. To answer this question Gor enzyme and promoter activities were measured in *X. campestris* wild type, an *X. campestris oxyR* knockout mutant, and an *X. campestris oxyR5* strain that has spontaneous mutations at G197 and L301 of OxyR that render it constitutively active [24,25]. Both Gor enzyme activity and *gor* promoter activity were not significantly different in the three strains (data not shown) indicating that *X. campestris gor* expression differs from that of *E. coli gor* in that it is not regulated by OxyR. Similar OxyR-independent expression of *gor* has thus far only been observed in the photosynthetic bacterium

Rhodobacter capsulatus, where *gor* expression was found not to be induced by H_2O_2 [26].

Enzyme kinetic study and the coenzyme binding motif analysis of Gor

A His-tagged Gor protein fusion was constructed as described in Materials and methods. His-tagged *Xanthomonas* Gor was expressed at high level in *E. coli* harboring pQEG and purified using nickle affinity column chromatography. The purity of each eluted protein fraction was determined by SDS–PAGE (Fig. 2). Both SDS- and non-denaturing PAGE indicated that the recombinant *Xanthomonas* Gor enzyme ran as a single band of approximately 50 kDa. This was confirmed using gel filtration column chromatography by FPLC which indicated that the enzyme was active as a monomer of 50 kDa in size (data not shown). This is atypical of the known Gor from various sources which are generally dimeric enzymes [27]. The only monomeric Gor reported to date is from the photosynthetic alga *Chlamydomonas reinhardtii* [28].

The kinetic parameters of the recombinant *Xanthomonas* Gor catalyzed reduction of oxidized glutathione were determined (Table 1). Interestingly, the K_m for NADH of *Xanthomonas* Gor was 55.5 μM which is approximately 3.5- and 36-fold lower than those of human erythrocyte [29] and *E. coli* [30] Gor, respectively. Surprisingly, *Xanthomonas* Gor utilized both NADH and NADPH with nearly equal affinity (K_m of 52.6 μM for NADPH versus 55.5 μM for NADH) (Table 1). This was unusual given that the Gor enzymes that have been studied in detail either use NADPH exclusively or show only a very low affinity for NADH [27]. Only Gor from *Chromatium vinosum* has thus far been reported to preferentially utilize NADH (K_m of 60 μM for NADH versus a K_m of 3000 μM for NADPH) [31]. The deduced amino acid sequence of *Xanthomonas* Gor was compared with other Gor sequences from various sources including bacteria, plant, and human using the Clustal W program [32], in order to identify sequence differences that might explain the enzymes' unique NADH/NADPH specificity (data not shown). *Xanthomonas* Gor showed a high degree of sequence identity with Gor sequences from *E. coli* (45%), *Haemophilus influenzae* (45%), human (44%), and *Pseudomonas aeruginosa* (40%). All active-site amino acid residues, as well as those involved in FAD binding and GSSG binding, were conserved among the different Gor homologs [27]. Most Gor homologs contained the highly conserved NADPH binding site sequence ($GxGYIAx_{18}Rx_5R$) where the first arginine residue (R200) in the Rx_5R motif is virtually 100% conserved. However, *X. campestris* Gor was found to have a unique NADPH binding site sequence ($GxGYIAx_{18}Qx_5E$) in which the highly conserved arginine residues are replaced by glutamine (Q200) and glutamic acid (E206) (Fig. 3). While this unique NADH/NADPH binding sequence is likely the reason for *Xanthomonas* Gor's ability to utilize both electron donors, the mechanism by which this is made possible remains unknown. A previous study of human glutathione reductase found that NADH also binds to Gor but with less affinity than NADPH, (i.e., a 60-fold higher K_m than that for NADPH) due to its lack of a 2′-phosphate group [30] that can interact with the positively charged residues R218 and R224 [30]. In *E. coli* Gor, replacement of R218 and R224 with M and L, respectively, substantially decreased the enzyme's affinity for NADPH and resulted in a catalytically less favorable configuration for bound NADPH [30]. In the NADH-dependent enzymes, like dihydrolipoamide dehydrogenase, conserved E residues replace the R residues in equivalent positions of the NADH binding motif where they were suggested to be involved in binding the 2′-OH group of the ribose moiety of NADH [30]. Therefore, E206 in *Xanthomonas* Gor may facilitate NADH ribose group binding thus allowing the enzyme to use NADH as a cofactor. Rationalizing how the Qx_5E motif facilitates NADPH binding is more difficult since Q200 is an uncharged residue and E206 is negatively charged, so both do not favor binding of the negatively charged phosphate group of NADPH.

A comparison of a total of 86 deduced Gor amino acid sequences, that included those identified from 209 completed microbial genomes as well as all the Gor protein sequences deposited in the SwissProt database, revealed that the $Q200x_5E206$ NADH/NADPH binding motif was present only in Gor from two *Xanthomonas* species, *X. campestris* pv. *campestris* and *X. axonopodis*

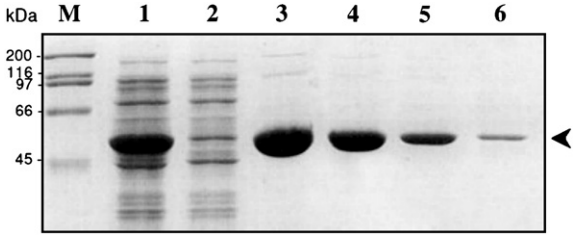

Fig. 2. SDS–PAGE of Gor at different stages of purification. Lane M, molecular mass standard; lane 1, crude extract; lane 2, after nickle affinity column; and lanes 3–6, eluted fractions. Each lane was loaded with 5 μl protein. Arrow indicates Gor protein bands.

Table 1
Kinetic parameters of glutathione reductase from *Xanthomonas*

Parameter	NADPH	NADH
K_m (μM)	52.6	55.5
k_{cat} (min^{-1})	2250	1950
k_{cat}/K_m (min^{-1} μM^{-1})	42.8	35.1
V_{max} (U/ml)	39.2	39.2
Specific activity (U/mg)	45	39

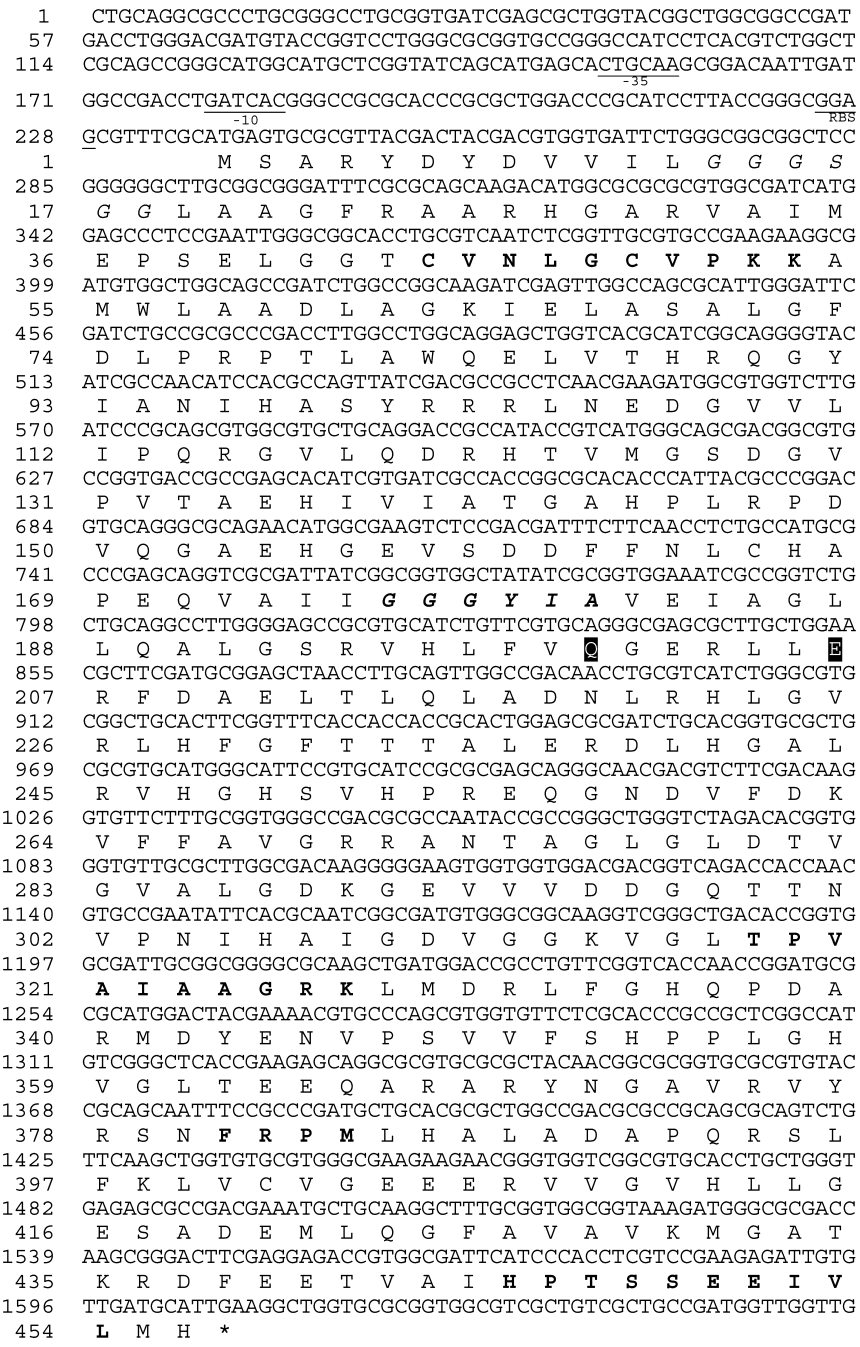

Fig. 3. Nucleotide sequence and predicted amino acid sequence of *Xanthomonas gor*. The putative −35, −10 promoter regions, and ribosome binding site (RBS) are underlined. Regions of residues important for GSSG binding are shown in bold letters. Residues involved in NADPH binding are in italic and bold. Q and E residues that replace the most conserved R at the NADPH binding sites are marked by white letters on a black background.

pv. *citri*, while all other Gor sequences contained the highly conserved NADPH binding motif ($GxGYIAx_{18}Rx_5R$) in which R200 was absolutely conserved among Gor from all sources except *Xanthomonas*. In order to determine if the Qx_5E sequence motif was shared between other members of the genus, *Xanthomonas* DNA fragments spanning the Qx_5E region within *gor* in *X. vessicatoria*, *X. translucens*, and *X. hyacinthi* were amplified by PCR, cloned, and sequenced. The sequences from all three *Xanthomonas* species contained the Qx_5E binding motif indicating that the Gor NADH/NADPH binding specificity is common to members of the genus. *X. campestris* Gor also differed from Gor of other organisms in respect to its specific activity, that was comparatively low relative to the specific activities of Gor isolated from other sources [30,33,34]. Presumably, the relatively low specific activity of *Xanthomonas* Gor may be compensated for by

the enzyme's unique ability to utilize both NADH and NADPH.

Increased expression of an organic peroxide-inducible regulator gene (ohrR) in gor mutants

In order to define the physiological role of *Xanthomonas* atypical Gor, the expression of the well-characterized organic peroxide-inducible *ohrR* promoter system [19,35–37] was used as an indicator of the cellular redox state in *Xanthomonas* wild type and *gor* mutant strains. The organic hydroperoxide resistance protein (Ohr) was first identified in *X. campestris* [12] and its expression is regulated by a novel transcription repressor, OhrR (Fig. 4A) [37]. Expression of the *ohrR-ohr* operon is highly induced by organic peroxide through the oxidation of a highly conserved cysteine residue that prevents the protein from binding to its target promoter region [19,37]. Thus, expression of the *ohrR-ohr* operon is a sensitive indicator of oxidative stress that is induced either by exposure to organic oxidants in the external environment or those generated as a result of internal cellular processes. The question of whether *Xanthomonas* atypical Gor affects the cells' ability to respond to oxidative stress was investigated through the use of a highly sensitive *ohrR* promoter-*lacZ* fusion system. A mini-Tn5 pP1lacZ construct was transferred to both wild type and *gor*-disrupted mutant strains of *X. campestris* pv. *phaseoli* and their response towards organic peroxide exposure was determined and compared (Fig. 4B). In the absence of peroxide, *ohrR* promoter in *Xanthomonas* lacking Gor exhibited marginally higher β-galactosidase activity (Fig. 4B, uninduced) when compared to the wild type level indicating that the absence of Gor enzyme causes the intracellular environment to become more

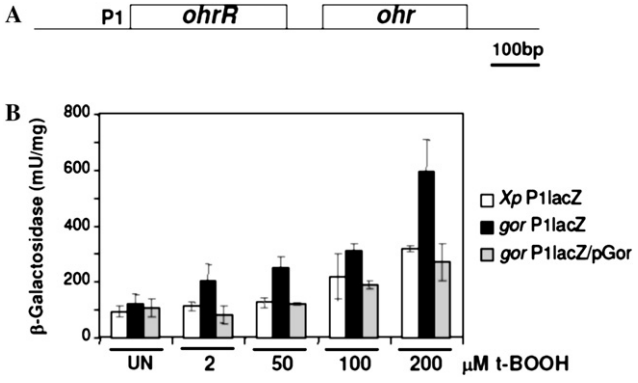

Fig. 4. Diagram of the genetic organization of *ohrR* and *ohr* (A). Expression of the *ohrR* promoter in *Xanthomonas* wild type and *gor* mutants when exposed to organic hydroperoxide (B). β-Galactosidase activities in crude extracts of an *ohrR-lacZ* fusion in parental (*Xp*P1lacZ) and *gor* mutant (*gor* P1lacZ), as well as the complemented strain (*gor* P1lacZ/pGor) when uninduced (UN) or induced with 2, 50, 100, or 200 μM *t*-BOOH. Each value shown is the mean of three separate experiments and error bars indicate standard error of the mean.

oxidized and the OhrR mediated derepression of the *ohrR* promoter. The situation became more pronounced when both strains were exposed to higher concentrations of organic peroxide (*t*-BOOH). The *ohrR* promoter in *gor* mutants responded more strongly to all concentrations of *t*-BOOH (Fig. 4B, 25–100 μM *t*-BOOH). Complementation of the *gor* mutant *gor* P1lacZ with a plasmid-borne *gor* in strain *gor* P1lacZ/pGor reduced *ohrR* promoter activity to the level in the wild type background. The result demonstrated that *Xanthomonas* Gor indeed plays a key anti-oxidative stress role in maintaining the reduced cellular redox state.

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Brief note

Protection of *Xanthomonas* against arsenic toxicity involves the peroxide-sensing transcription regulator OxyR

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Abstract

Arsenic has been shown to mediate its toxicity through induced generation of reactive oxygen species. Here, we examined the role of oxidative stress-inducible genes (*katA*, *ahpC* and *ohr*) and their regulators (*oxyR* and *ohrR*) in the response to arsenic treatment in a plant pathogenic bacterium, *Xanthomonas campestris* pv. phaseoli (*Xp*). Overproduction of peroxide-scavenging enzymes (KatA, AhpCF and Ohr) did not enhance arsenic tolerance in wild-type *Xp*. Furthermore, inactivation of *katA*, *ahpC*, *ohr*, and *ohrR* genes had no effect on the level of arsenic resistance. By contrast, an *oxyR* mutant (*Xp oxyR*) showed increased sensitivity to both pentavalent arsenate and, to a greater extent, trivalent arsenite. The resistance of cells to arsenite treatment was significantly affected by the level of iron. Cells were 10-fold more sensitive to arsenite killing in the presence of excess iron, while removal of iron by an iron chelator (2,2′-dipyridyl) protected *Xanthomonas* from arsenite toxicity. The arsenite-sensitive phenotype of *Xp oxyR* could be complemented by the expression of functional OxyR from a plasmid vector, but not by the expression of other known OxyR-regulated peroxide-scavenging enzymes such as KatA and AhpCF, Ohr and OhrR. The data suggested that as yet unidentified, OxyR-regulated gene(s) are involved in conferring arsenic resistance in *Xp*. To our knowledge, this is the first report showing that the peroxide-sensing regulator OxyR is involved in arsenic resistance.

Keywords: Arsenic resistance; OxyR; KatA; AhpCF; Ohr; OhrR

1. Introduction

Arsenic is a toxic metal that is found in both natural environments and in sites contaminated by fungicides, pesticides and herbicides. Arsenic mainly exists in two oxidation states, arsenite As(III) and arsenate As(V). Arsenite is more toxic than arsenate and readily reacts with the thiol and nitrogen groups of proteins, thus disrupting their function. In addition, the production of reactive oxygen species (ROS) associated with arsenic toxicity has been reported [5,13]. Exposure to arsenic may exert an effect on soil and plant-pathogenic bacteria. Plants induce oxidative stress in infecting bacteria by generating ROS such as H_2O_2, superoxides, and organic peroxides, to inhibit microbial invasion [2]. In response to certain peroxides, bacteria have evolved enzymatic oxidant-scavenging systems, including catalase (KatA) and alkyl hydroperoxide reductase (AhpCF), to defend against host-derived oxidative killing. The genes encoding these enzymes are regulated by OxyR, a global regulator of the peroxide stress regulon [14]. Ohr is an additional protective enzyme against organic peroxide toxicity [9]. The regulation of Ohr expression is independent of OxyR and is controlled by the negative regulator OhrR [16].

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Table 1
Strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Reference or source
Xanthomonas campestris pv. phaseoli strains		
Xp	Wild type	Laboratory collection
Xp HR	Spontaneous multiple peroxide resistant mutant	[3]
Xp oxyR	Gen^r, *oxyR* mutant	[10]
Xp katA	Amp^r, *katA* mutant	Laboratory collection, unpublished
Xp ahpC	Kan^r, *ahpC* mutant	[12]
Xp ohr	Tet^r, *ohr* mutant	[9]
Xp ohrR	Tet^r, *ohrR* mutant	[16]
Plasmids		
pOxyR	Amp^r, *oxyR* coding region cloned into pBBR1MCS-4	[7]
pOxyRC199S	Amp^r, pOxyR mutated to convert Cys199 to Ser	[7]
pOxyR5	Amp^r, OxyR locked in the oxidized form	[11]
pKatA	Kan^r, *katA* coding region cloned into pUFR047	[8]
pAhpCF	Amp^r, *ahpCF* coding region cloned into pUFR047	[6]
pOhr	Amp^r, *ohr* coding region cloned into pUFR047	[9]
pOhrR	Amp^r, *ohrR* coding region cloned into pBBR1MCS-4	[16]

On the molecular level, the regulation of oxidant-responsive regulons during arsenic stress is poorly understood. Here we examined the role of oxidative stress-inducible genes and their products in response to arsenic exposure in the plant pathogenic bacterium *Xanthomonas campestris* pv. phaseoli. We found that arsenite resistance is dependent on functional OxyR, suggesting that OxyR plays an important role in the defense against arsenite exposure in *Xanthomonas*.

2. Materials and methods

2.1. Bacterial strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. Cells were grown aerobically at 28 °C in Silva–Buddenhagen (SB) medium containing the appropriate antibiotics. SB medium contains 0.5% yeast extract, 0.5% peptone, 0.1% glutamic acid, and 0.5% sucrose (pH 7.0). Ampicillin (200 $\mu g\,ml^{-1}$), gentamicin (15 $\mu g\,ml^{-1}$), kanamycin (15 $\mu g\,ml^{-1}$), and tetracycline (15 $\mu g\,ml^{-1}$) were added as required.

2.2. Arsenic resistance of Xanthomonas strains using inhibition zone assay

Cells from an overnight culture (10^8) were subcultured into 10 ml of SB medium and grown at 28 °C with shaking for 4 h. Exponential phase cells (10^8) were added to 10 ml of prewarmed (50 °C) top agar (0.7% SB agar) and layered onto SB agar plates containing 40 ml of medium (14-cm-diameter petri dishes). After the top agar solidified, sterile 6-mm-filter paper disks containing 5 µl of a solution containing varying concentrations of either trivalent sodium arsenite ($NaAsO_2$) or pentavalent sodium arsenate (Na_2AsO_4) were placed on the surface. Plates were incubated at 28 °C for 24 h and the diameters of the inhibition zones were measured. All assays were performed in triplicate.

2.3. Survival assay

Exponential phase cells (10^8) were treated with 150 mM $NaAsO_2$ at room temperature. After 30-min treatment, samples were washed once with fresh SB medium before appropriate dilutions were plated on SB agar plates containing 0.1% pyruvate. Cells that survived were counted after incubation at 28 °C for 48 h. The percent survival is defined as the number of colony forming units (CFUs) obtained after the treatment divided by the number of CFUs obtained prior to treatment multiplied by 100. In some experiments, cells were treated with arsenite in the presence of 200 µM $FeCl_3$ or in the presence of an iron chelator, 200 µM 2,2′-dipyridyl. All assays were performed at least three times and representative data are shown.

3. Results and discussion

3.1. The dose-response of wild-type Xp to arsenical compounds

To determine the basal level of resistance of wild-type *Xp* to arsenic, inhibition zone assays of cells exposed to filter disks soaked in varying concentrations of sodium arsenite (25, 50, 100, 200, 400, and 800 mM $NaAsO_2$) and sodium arsenate (250, 500, 1000, 1500, and 2000 mM Na_2AsO_4) were performed. No clear inhibition zone was observed at 25 mM arsenite and 250 mM arsenate. Clear inhibition zones were observed at concentrations above 25 mM arsenite and 250 mM arsenate, the diameters of which increased with increasing concentrations of arsenical compounds (Fig. 1). *Xp* showed a higher tolerance to arsenate than to arsenite. An

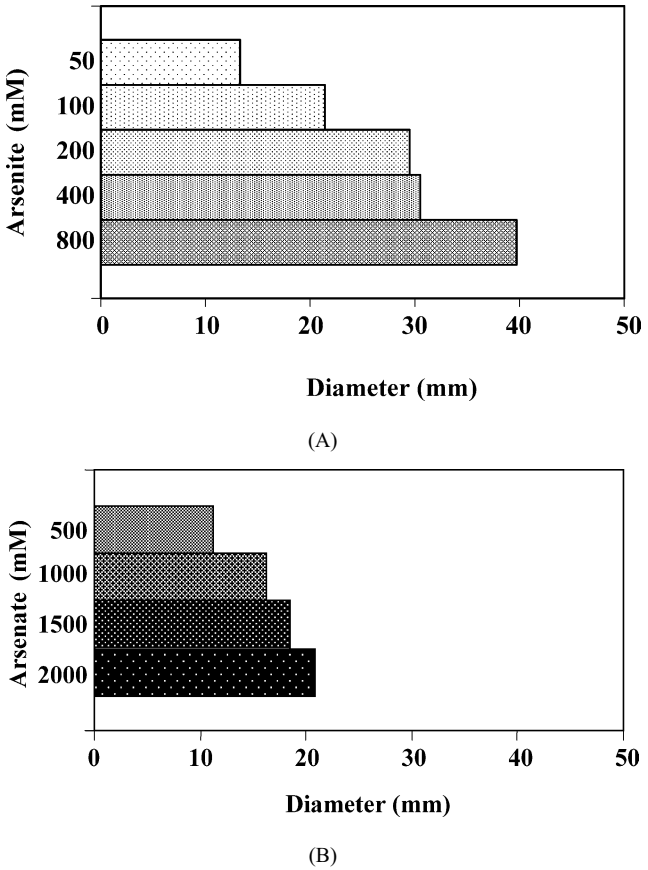

Fig. 1. Determination of the basal levels of resistance to arsenic in wild-type *Xanthomonas*. Sensitivity to arsenic was determined by inhibition zone assay as described in Section 2. Exponential phase cells were spread on SB agar plates containing filter disks impregnated with 5 µl of the indicated concentrations of trivalent sodium arsenite (A) or pentavalent sodium arsenate (B). The inhibition zones were measured after incubation at 28 °C for 24 h. The diameters of the inhibition zones are indicated in mm. Results represent the means of triplicate experiments.

inhibition zone could be detected at 500 mM of sodium arsenate (Fig. 1B) whereas a clear inhibition zone could be observed at a much lower concentration of sodium arsenite (50 mM) (Fig. 1A). These results are consistent with the well-documented fact that arsenite is more toxic than arsenate [15]. Sodium arsenite and sodium arsenate at concentrations of 100 mM and 1 M, respectively, which yielded manageable inhibition zone sizes, were chosen for use in subsequent experiments.

3.2. Role of oxidative-inducible genes and regulatory genes in response to killing concentration of arsenic

Arsenic has been shown to induce ROS, which mediate its toxicity [5,13]. In order to investigate the effects of peroxide-scavenging enzymes (KatA, AhpC, and Ohr) and peroxide response regulatory proteins (OxyR and OhrR) on arsenic resistance, the resistance levels to 100 mM sodium arsenite and 1 M sodium arsenate were determined in wild-type *Xp* and various mutant strains (Table 2). Inactivation of

Table 2
Inhibition zone assays[a] of mutant strains compared to those of wild type at lethal concentrations of arsenic

Strain	100 mM As(III)	1 M As(V)
Xp	21.0 ± 0.8	16.1 ± 0.8
Xp oxyR	26.4 ± 1.0	19.5 ± 1.5
Xp katA	21.1 ± 0.9	15.4 ± 0.7
Xp ahpC	21.1 ± 0.8	16.5 ± 1.2
Xp ohrR	22.3 ± 1.2	16.3 ± 0.5
Xp ohr	21.3 ± 1.0	17.0 ± 0.6
Xp HR	21.8 ± 0.8	ND
Xp/pKatA	22.2 ± 0.7	15.9 ± 1.4
Xp/pAhpCF	21.2 ± 0.9	16.6 ± 0.7
Xp/pOhr	20.8 ± 0.9	16.1 ± 1.0
Xp oxyR/pOxyR	22.6 ± 0.06	ND
Xp oxyR/pOxyRC199S	28.3 ± 0.15	ND
Xp oxyR/pOxyR5	22.8 ± 0.07	ND
Xp oxyR/pOhrR	26.0 ± 0.14	ND
Xp oxyR/pKatA	26.9 ± 0.9	ND
Xp oxyR/pAhpCF	28.3 ± 1.2	ND
Xp oxyR/pOhr	25.5 ± 1.6	ND

[a] Inhibition zone assays were performed as described in Section 2. Exponential phase cells were spread on SB agar plates containing filter disks impregnated with 5 µl of 100 mM of sodium arsenite As(III) or 1 M sodium arsenate As(V). The inhibition zones were measured after incubation at 28 °C for 24 h. The diameters of the inhibition zones are indicated in mm. Results represent the means and standard errors of triplicate experiments. ND: not determined.

katA, *ahpC*, *ohr*, and *ohrR* (*Xp katA*, *Xp ahpC*, *Xp ohr*, and *Xp ohrR* strains, respectively) resulted in no significant alterations in arsenic resistance levels compared to wild-type *Xp* as judged by the diameter of the inhibition zones against either arsenite or arsenate. By contrast, an *oxyR* mutant (*Xp oxyR*) showed increased sensitivity to both arsenite and arsenate relative to wild-type *Xp*. In addition, the arsenite-sensitive phenotype of *Xp oxyR* could be complemented by the plasmid-borne expression of functional OxyR, as observed in *Xp oxyR*/pOxyR. These data indicated that OxyR plays a crucial role in protection of *Xanthomonas* from arsenic toxicity.

3.3. Overexpression of katA, ahpCF, ohr did not confer increased protection against arsenic toxicity

Overproduction of the peroxide-scavenging enzymes, catalase, alkyl hydroperoxide reductase, and organic hydroperoxide resistance protein in strains *Xp*/pKatA, *Xp*/pAhpCF, and *Xp*/pOhr, respectively, did not enhance arsenic tolerance compared to the parental strain *Xp* (Table 2). Additionally, inhibition zones obtained from the multiple peroxide-resistant mutant *Xp* HR and wild-type *Xp* were similar (Table 2), despite the fact that *Xp* HR has 100-fold-increased levels of catalase [3]. These results suggested that wild-type levels of these protective enzymes are sufficient to counter the ROS that are accumulated as a consequence of arsenic exposure.

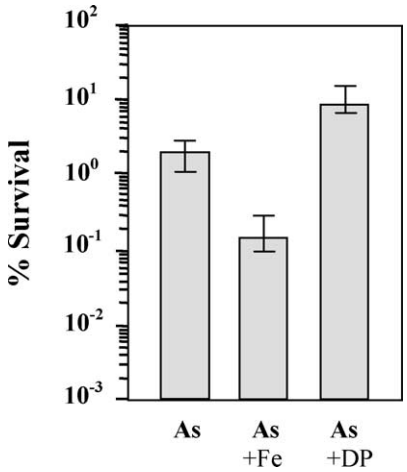

Fig. 2. Effect of iron level on arsenite toxicity in the *Xp oxyR* mutant. Survival assays were performed as described in Section 2. Exponential phase cells were treated with either 150 mM sodium arsenite (As), 150 mM sodium arsenite in the presence of 200 μM $FeCl_3$ (As + Fe) or 200 μM 2,2′-dipyridyl (As + DP). Values presented are means and standard deviations of three replicates.

3.4. Arsenite resistance requires the active oxidized form of OxyR

It has been reported that the redox status of OxyR determines its function such that expression of *oxyR*-regulated genes is repressed by reduced OxyR while the oxidized form acts as a transcriptional activator [11]. It is also known that a conserved cysteine residue at position 199 of OxyR is involved in the redox sensing [11]. We tested the ability of two mutant OxyRs, OxyRC199S, containing a cysteine 199 to serine substitution, and OxyR5, that is permanently locked in the oxidized form, to complement the arsenite sensitive phenotype of *Xp oxyR*. As shown in Table 2, *Xp oxyR* expression of OxyRC199S from pOxyRC199S had no effect on the arsenite-sensitive phenotype. However, the arsenite-sensitive phenotype of *Xp oxyR* was complemented when OxyR5 was expressed from pOxyR5 [11]. These data confirm that the oxidized form of OxyR is required for the maintenance of wild-type levels of arsenite resistance in *Xp*. Furthermore, the arsenite-sensitive phenotype in *Xp oxyR* could not be rescued by the introduction of plasmids encoding *katA*, *ahpCF*, or *ohr*. This suggests the existence of unidentified OxyR-regulated genes that are involved in conferring arsenic resistance to *Xp*.

3.5. Role of iron in arsenite toxicity in Xanthomonas

Studies have provided experimental evidence that superoxide anions and H_2O_2 are generated in various cellular systems in the presence of arsenite [13]. Iron is known to be involved in the generation of highly toxic hydroxyl radicals from superoxide anions and H_2O_2 via the Fenton reaction [4]. In addition, arsenic-induced release of iron from ferritin has been reported and likely contributes to arsenic toxicity [1]. We determined the effect of iron on arsenite toxicity using percent survival assays in a *Xp oxyR* mutant. Survival of arsenite-treated cells was negatively affected by iron (Fig. 2). The *Xp oxyR* mutant, treated with 150 mM arsenite in the presence of 200 μM $FeCl_3$ showed a 10-fold lower rate of survival than cells treated with arsenite alone. By contrast, co-treatment of cells with arsenite and an iron chelator (200 μM 2,2′-dipyridyl) had a protective effect against arsenite toxicity (Fig. 2). Similar findings were observed in the wild-type *Xp* (data not shown). These data provided evidence that iron plays an important role in arsenite toxicity in *Xanthomonas*.

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NOTES

Important Role for Methionine Sulfoxide Reductase in the Oxidative Stress Response of *Xanthomonas campestris* pv. phaseoli

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A methionine sulfoxide reductase gene (*msrA*) from *Xanthomonas campestris* pv. phaseoli has unique expression patterns and physiological function. *msrA* expression is growth dependent and is highly induced by exposure to oxidants and *N*-ethylmaleimide in an OxyR- and OhrR-independent manner. An *msrA* mutant showed increased sensitivity to oxidants but only during stationary phase.

Xanthomonas spp. are soil bacteria that are the causative agents of bacterial blight diseases in many economically important crops. Bacteria are constantly exposed to harmful reactive oxygen species (ROS) that originate from many sources, such as aerobic respiration, chemical pollutants in the environment, and the initial defense responses of plants to microbial invasion. ROS are highly reactive and can damage biological macromolecules, including proteins, nucleic acids, and lipids. Methionine residues in proteins are particularly susceptible to oxidation by ROS resulting in formation of racemic mixtures of methionine-*S*-sulfoxide and methionine-*R*-sulfoxide. Most eukaryotic and prokaryotic cells possess repair enzymes, such as peptide methionine sulfoxide reductases (Msr proteins), which catalyze the thioredoxin-dependent reduction of either free methionine sulfoxide [Met(O)] or protein-bound Met(O) to methionine. *Escherichia coli* and several other bacteria have two methionine sulfoxide reductases, namely, MsrA and MsrB, encoded by two structurally unrelated genes (20). MsrA and MsrB have distinct substrate specificities. MsrA uses only the S epimer, while MsrB uses the R epimer of Met(O) as a substrate (6, 17).

In bacteria, the physiological function of the Msr proteins has not been fully elucidated. In general, *msrA* is recognized as a gene required for bacterial virulence and survival under some stressful conditions (4, 18, 20). Examination of *msrA* expression patterns could give important clues as to its physiological function(s). While different bacteria appear to display different *msrA* expression patterns in response to various conditions, in no case has a regulator of *msrA* expression been identified. Moreover, there is little correlation between the gene expression pattern and any possible physiological role for the gene. For example, MsrA has been shown to play a significant role in the protection of several microorganisms from oxidative stress, and yet in none of these bacteria has the gene been shown to be oxidative stress inducible (5, 18–20, 22, 26). In many microorganisms, the mechanism of regulation of *msrA* expression and the physiological function(s) of the gene product remain to be elucidated.

In this paper, the expression patterns of *msrA* in *Xanthomonas campestris* pv. phaseoli were examined. The gene has novel patterns of growth-phase-dependent and oxidative-stress-inducible expression. The oxidative-stress-inducible expression of *msrA* is not regulated by known stress sensors and transcriptional regulators. Physiological analysis of an *msrA* mutant indicated that the gene plays an important role in the protection against oxidative stress.

Nucleotide sequence accession number. The nucleotide sequence determined in this study was assigned GenBank accession number AF404824.

Cloning, genome organization, and transcription of the *msrA* locus from *X. campestris* pv. phaseoli. The isolation of a genomic clone (pA301) containing *talA*, encoding a transaldolase, from *X. campestris* pv. phaseoli was reported previously (24). Analysis of the nucleotide sequence downstream of *talA* revealed the presence of an unidentified open reading frame (ORF) and a truncated ORF with high homology to the C-terminal region of MsrA. A fragment containing this truncated gene (0.45-kb SphI fragment from pA301) was used as a probe to isolate a DNA fragment containing full-length *msrA* from an existing genomic library constructed in λZip-lox (11). A positively hybridizing plaque was purified and excised into plasmid pA8. Analysis of the nucleotide sequence revealed that the fragment contained the putative *msrA* that was predicted to encode a 216-amino-acid polypeptide with a molecular mass of 23.5 kDa and a pI of 5.39. The deduced amino acid sequence of *Xanthomonas* MsrA showed a high degree of identity to both eukaryotic and prokaryotic peptide methionine sulfoxide reductases (MsrA). Analysis of the *Xanthomonas* MsrA amino acid sequence showed the presence of a conserved consensus sequence, GCFWG, that is thought to comprise the active site

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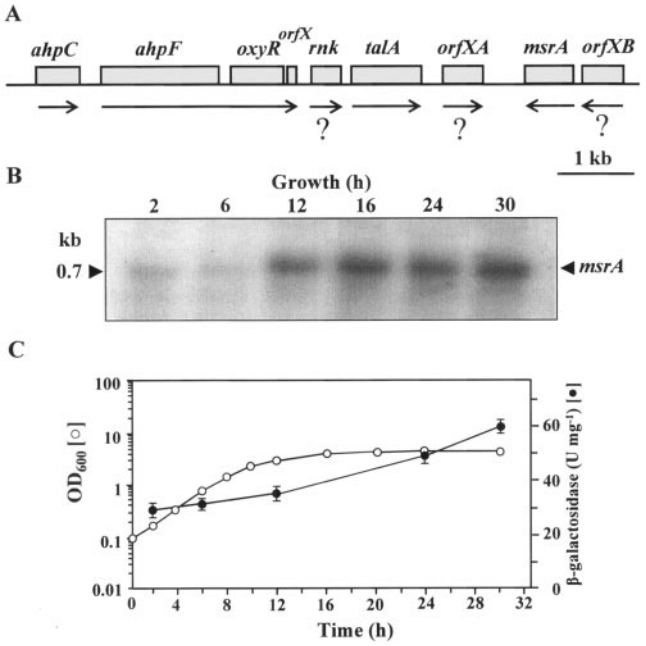

FIG. 1. Gene organization and growth-phase-dependent expression of *msrA*. (A) Physical and transcription maps of *msrA* in *X. campestris* pv. phaseoli. The arrows indicate the orientations and lengths of the transcripts. Question marks indicate uncharacterized genes. (B) Results of a Northern blot hybridization showing *msrA* expression at different growth phases. At the indicated times, RNA samples were prepared from cultures of *X. campestris* pv. phaseoli. RNA isolation, gel electrophoresis, and Northern blotting were done as previously described (15). Ten micrograms of total RNA was loaded into each lane. The blot was probed with a radioactively labeled *msrA* fragment (15). (C) Growth curve and expression analysis of *X. campestris* pv. phaseoli strain Xp08 containing an *msrA* promoter-*lacZ* fusion. The growth curve (○) of Xp08 (*msrA*::*lacZ*) in SB medium was determined at 28°C with continuous shaking at 150 rpm. At the indicated times, samples were removed and crude lysates were prepared and assayed for β-galactosidase activity (14). OD_{600}, optical density at 600 nm.

of the enzyme (17), and two cysteine residues at the C terminus which correspond to Cys-198 and Cys-206 of *E. coli* MsrA that have been shown to be involved in catalysis (17, 23).

msrA was located between two ORFs of unknown function (*orfXA* and *orfXB*) on the *X. campestris* pv. phaseoli genome (Fig. 1A). Nonetheless, the genes in this region showed an interesting organization. Comparison of the sequence of the *msrA* region of *X. campestris* pv. phaseoli with those of *X. campestris* pv. campestris and *Xanthomonas axonopodis* pv. citri showed that the gene organization *hemK-ahpC-ahpF-oxyR-orfX1-rnk-talA-orfXA-msrA-orfXB* (Fig. 1A) was conserved among the three bacteria (2). *msrA* is located in a region rich in genes involved in the oxidative stress response. We have shown that, in addition to *msrA*, *ahpC* and *ahpF*, encoding the catalytic and the reductase subunits of alkyl hydroperoxide reductase, respectively, and the peroxide sensor and transcription regulator OxyR are essential for the peroxide stress protection response (10, 13). Moreover, *talA* also plays an important role in protecting the bacteria from a superoxide generator, menadione (MD) (24).

A partial transcription map of the region is also shown (Fig. 1A). The transcripts encoding *ahpC*, *ahpF-oxyR-orfX1*, and *talA* have been previously determined (13, 24). Northern analysis, using a 340-bp SmaI-SphI *msrA*-specific fragment as a probe, indicated that *msrA* was transcribed on a 0.7-kb monocistronic mRNA (Fig. 1B).

Growth-phase-dependent expression of *msrA*. The expression of *msrA* during the different stages of bacterial growth has not been well studied. In some bacteria, *msrA* appears to play important roles in oxidative stress protection (4, 18, 20, 26). Hence, the timing of its expression is likely to be important, since the levels of resistance to oxidative stress vary significantly at different stages of growth (11). The growth-phase-dependent expression patterns of *msrA* were investigated by use of both Northern blot analysis and *msrA* promoter-*lacZ* fusion analysis. The results of Northern hybridizations showed that *msrA* was expressed at low levels during exponential-phase growth (Fig. 1B). The expression increased eightfold (as judged by densitometer analysis of Northern blots) as the culture entered the stationary phase and during the stationary phase. These results were independently confirmed by use of an *msrA* promoter-*lacZ* fusion construct. A promoterless *lacZ* was transcriptionally fused to *msrA* on the *X. campestris* pv. phaseoli chromosome (*msrA*::*lacZ*) to yield strain Xp08 by using the R6K-derived suicide plasmid pVIK112 (7) inserted with the 273-bp DNA fragment of the *msrA* coding region (corresponding to nucleotides 121 to 393) at EcoRI and SmaI sites. The plasmid was introduced into *X. campestris* pv. phaseoli by electroporation. Xp08 was selected by its kanamycin resistance and was confirmed by Southern blot analysis (data not shown). *msrA* promoter activity (β-galactosidase activity) was monitored in Xp08 throughout the different growth phases. As shown in Fig. 1C, the β-galactosidase activity increased twofold (from 30 to 60 U mg^{-1} protein) as growth proceeded from exponential to stationary phase, with peak β-galactosidase levels being attained as cells entered the stationary phase and during the stationary phase of growth. These results are consistent with those of the Northern hybridization analysis and indicated that the expression of *msrA* is stationary phase dependent. A similar pattern of *msrA* expression in *E. coli* has been observed (18). Generally, soil bacteria spend long periods in a nutrient-limited state and have evolved mechanisms to survive under starvation conditions that involve increasing the expression of genes that protect them from the various starvation-associated stresses (21). The growth-phase-dependent expression pattern of *msrA* suggests that it belongs to the starvation stress response genes. *msrA* is likely to play an important physiological role(s) during stationary phase. The mechanism(s) controlling stationary-phase-dependent gene expression in *Xanthomonas* is not known; analysis of the bacterial genome did not show any ORFs with high homology to RpoS, suggesting that other sigma factors or additional regulatory mechanisms may control stationary-phase-dependent *msrA* expression. Interestingly, the regulator of stationary-phase expression of *E. coli msrA* is also not known, but it has been shown that the regulator is not σ^S (18).

Oxidative stress induction of *msrA* expression. In several bacteria, *msrA* has been shown to be important in protecting bacteria from oxidative stress, probably by repairing oxidized Met residues (27). However, in the bacteria thus far investigated, *msrA* expression has not been shown to be induced by

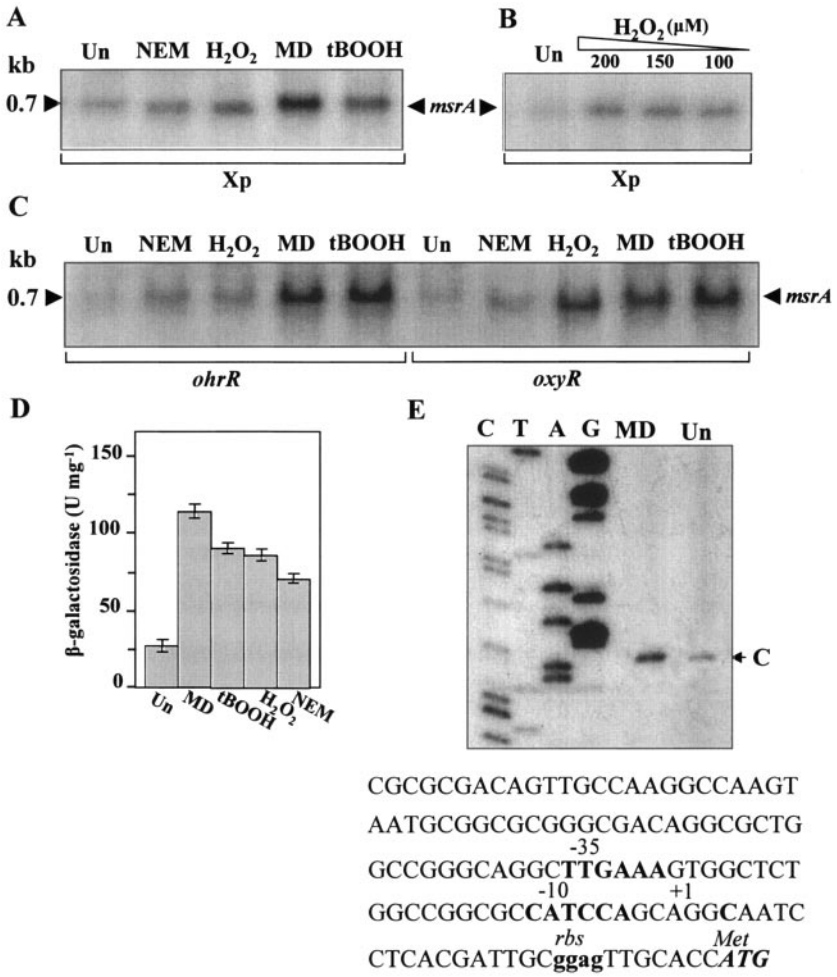

FIG. 2. Oxidant-inducible *msrA* expression and localization of the *msrA* promoter. *X. campestris* pv. phaseoli exponential-phase cultures were treated with 100 μM NEM, 200 μM H_2O_2, 200 μM MD, or 200 μM tBOOH for 10 min before total RNA was isolated, and Northern blots were prepared and probed with a radioactively labeled *msrA*-specific probe. Ten micrograms of total RNA was loaded into each lane in all Northern blot hybridization experiments. (A) Northern blot showing *msrA* expression in response to oxidant treatments in *X. campestris* pv. phaseoli (Xp). The arrow indicates the size of the *msrA* mRNA. Un, uninduced. (B) Northern blots of *msrA* expression in response to various concentrations of H_2O_2. (C) Northern blot showing *msrA* expression in *X. campestris* pv. phaseoli *oxyR* and *ohrR* mutants treated with various oxidants. The *ohrR* and *oxyR* mutants were grown and treated with oxidants as described for panel A with the exception that the *oxyR* mutant was treated with 100 μM H_2O_2, 100 μM MD, or 100 μM tBOOH. (D) Xp08 (*msrA-lacZ*) was grown and treated with various oxidants. Crude lysate preparation and β-galactosidase levels were determined as previously described (14). (E) Primer extension of RNA extracted from uninduced (Un) and MD-induced cultures. The experiment was performed using ^{32}P-labeled oligonucleotide primer BT110 (5′CTAACGTTGTTTGAAGGCG3′) as previously described. C, T, A, and G are sequence ladders generated by using the same primer. The arrowhead indicates the *msrA* transcription start site. Putative −35 and −10 regions are shown in bold, capital letters. A putative ribosome binding site (*rbs*) is marked in bold, lowercase letters, and the translation initiation codon ATG is in bold italics.

oxidative stress (5). This suggests that the constitutive basal expression of *msrA* is sufficient to confer protection against oxidative stress generated from internal and external sources. In *X. campestris* pv. phaseoli, as in other bacteria, exposure to sublethal levels of oxidants leads to a severalfold increase in the expression of oxidative-stress-protective enzymes, such as catalase (KatA), alkyl hydroperoxide reductase (AhpC), and organic hydroperoxide resistance thiol peroxidase (Ohr) (1, 13, 15). This inducible response plays an important role in protecting the bacterium against stresses. Thus, *msrA* expression in response to exposure to various oxidants was investigated by Northern blot analysis. Exponential-phase cultures of *X. campestris* pv. phaseoli grown in SB medium (15) were treated with *N*-ethylmaleimide (NEM), H_2O_2, MD, or *tert*-butyl hydroperoxide (tBOOH) for 10 min. RNA samples were extracted (15) and analyzed by Northern blot hybridization using a radioactively labeled *msrA* probe. The results in Fig. 2A show that pretreatment of the cultures with MD induced *msrA* expression 10-fold, while tBOOH, H_2O_2, and NEM pretreatments produced intermediate levels of induction of sixfold, threefold, and twofold, respectively. The oxidant induction of *msrA* promoter was done using an *msrA* promoter-*lacZ* fusion. A similar pattern of oxidant induction of *msrA* promoter was obtained in Xp08 (*msrA*::*lacZ*), with menadione being the most potent inducer, followed by H_2O_2, tBOOH, and NEM (Fig. 2D). In *Xanthomonas*, MD, H_2O_2, and tBOOH have all

been shown to be potent inducers of genes in the OxyR regulon, while tBOOH also induces genes in the OhrR regulon (13, 15). NEM induction of *msrA* probably results from the depletion of thiol antioxidant molecules and the inactivation of oxidant scavenging enzymes that lead to oxidative stress. The observed pattern of oxidant-inducible *msrA* expression in *Xanthomonas* differs from previous reports of other bacteria, in which induction of *msrA* expression has been observed in response to a shift in pH (26), exposure to phenolic compounds (22), and treatment with cell wall-active antibiotics but not to oxidative stress (19).

Since *X. campestris* pv. phaseoli *msrA* displayed a unique oxidant-inducible expression pattern, we attempted to identify the regulator involved in controlling the expression of the gene. The oxidant-inducible expression pattern of *msrA* was similar to the patterns observed for many OxyR-regulated genes (10, 12). In *Xanthomonas*, OxyR is a peroxide sensor and a global transcriptional regulator of peroxide stress and OxyR-regulated genes are involved in the detoxification of H_2O_2 (*katA*) and organic hydroperoxides (*ahpC*) (1, 10, 13). Thus, analyses of the effects of oxidants on the expression of *msrA* in the wild type and an *oxyR* mutant were performed. The results shown in Fig. 2C clearly showed that the pattern of oxidant-induced *msrA* expression was not affected by inactivation of *oxyR*, since *msrA* transcription was highly induced by tBOOH. It should be noted that the inducing concentrations of oxidants were lowered to 100 μM for H_2O_2, MD, and tBOOH and 50 μM for NEM due to the *oxyR* mutant's inherent hypersensitivity to oxidants relative to the wild type (16). The effect of inactivation of the organic-hydroperoxide-sensing transcription repressor, *ohrR*, on *msrA* expression was also tested (14). The results of Northern blot analyses using the parental strain and an *ohrR* mutant showed that the profiles of oxidant induction of *msrA* in the two strains were similar (Fig. 2A and C). From these results, we concluded that both *oxyR* and *ohrR* are not responsible for the regulation of *msrA* expression. The evidence suggests the existence of an unidentified regulator(s) that could sense and respond to oxidative stress by increasing transcription of *msrA*.

The relative levels of peroxide induction in the wild type and *oxyR* and *ohrR* mutants reveal interesting patterns. The magnitude of H_2O_2 and tBOOH induction of *msrA* was lower in the parental strain than in either the *oxyR* or *ohr* mutant strain (Fig. 2B and C). This is due to the inability of the *oxyR* mutant to induce expression of the catalase and alkyl hydroperoxide reductase genes, which are responsible for H_2O_2 and organic hydroperoxide detoxification, respectively. Similarly, the *ohrR* mutant that has decreased *ohr* expression due to a polar effect of the mutation in *ohrR* (14) thus has a reduced capacity to metabolize organic hydroperoxide. Thus, in the regulatory mutants, intracellular peroxide levels were higher due to lower levels of peroxide detoxification enzymes. This would result in increased protein oxidation in the mutants relative to the parental strain, which in turn may stimulate higher levels of *msrA* induction. At present, the regulator(s) of oxidant-induced *msrA* expression has not been identified, and it remains to be seen whether such a regulator directly or indirectly senses oxidants and/or oxidized proteins.

In order to localize the *msrA* promoter region, the transcription initiation sites of *msrA* mRNA, isolated from uninduced and MD-induced cultures, were mapped by primer extension. The results shown in Fig. 2E showed a single predominant primer extension product corresponding to a transcription initiation site located 27 nucleotides upstream of the *msrA* translation start. Analysis of the sequence upstream of the transcription start site revealed the presence of a −10 promoter sequence, TTGAAA, separated by 17 nucleotides from a −35 promoter sequence, CATCCA. The *msrA* promoter −35 and −10 regions matched the consensus sequences for *X. campestris* promoters at 6/6 and 4/6 nucleotides, respectively (8). No sequences similar to the consensus binding sites for either OxyR or OhrR were found in the vicinity of the *msrA* transcription start. This was consistent with the results of the Northern blot analyses that indicated that OxyR and OhrR are not involved in the regulation of *msrA*. In addition, the primer extension results clearly showed that MD pretreatment increased *msrA* transcription initiation (Fig. 2E). Thus, the increase in the steady-state level of *msrA* mRNA after MD treatment is at least in part due to increased transcription of *msrA*.

Analysis of the physiological role of *msrA*. As mentioned previously, the physiological roles of *msrA* appear to differ in different bacteria. In order to determine the physiological role of *msrA* in *X. campestris* pv. phaseoli, an *msrA*-disrupted mutant was constructed by insertional inactivation using the nonreplicative plasmid pKStet (a tetracycline resistance derivative of pBluescript KSII [Stratagene]) containing a 220-bp internal fragment of *msrA*. The *msrA* mutant strain Xp07 was isolated, and the insertional inactivation of the gene was confirmed by both PCR and Southern blot analysis (data not shown). First, the aerobic growth rates of the mutant and the parental wild-type strain were determined in complex medium (Silva Buddenhagen [SB]) and minimal medium (M9). No significant difference between the growth rates of the strains was observed (data not shown). Thus, the loss of *msrA* function caused no adverse effects on bacterial growth. Recent reports suggested that in some bacteria, inactivation of *msrA* led to increased sensitivity to oxidative stress, indicating the importance of the gene in protecting bacteria from the stress. The level of resistance of the *msrA* mutant against various oxidants was determined as previously described (3) and compared with that of the parental strain. Exponential- and stationary-phase cultures were serially diluted and overlaid on SB agar plates containing the appropriate concentrations of oxidants, including H_2O_2, tBOOH, MD, and NEM. The surviving colonies were counted after 48 h of incubation at 28°C. During exponential-phase growth, Xp07 and the parental strain had similar levels of resistance to all oxidants tested (Fig. 3A). However, high-level expression of *msrA* from the plasmid pMsrA (broad-host-range plasmid pBBR1MCS-2 [9] containing the *msrA* gene) in Xp07 resulted in a small (7- to 10-fold) increase in the levels of resistance to H_2O_2, tBOOH, and NEM (Fig. 3A) relative to those of the parental strain. This observation suggested that the enzyme does not play a major role in protecting *X. campestris* pv. phaseoli from oxidant killing during the exponential phase of growth. Nonetheless, high-level expression of *msrA* does provide additional protection against oxidant killing.

Analysis of growth-phase-dependent oxidant resistance levels indicated that *msrA* played an important protective role against oxidant killing during the stationary phase of growth.

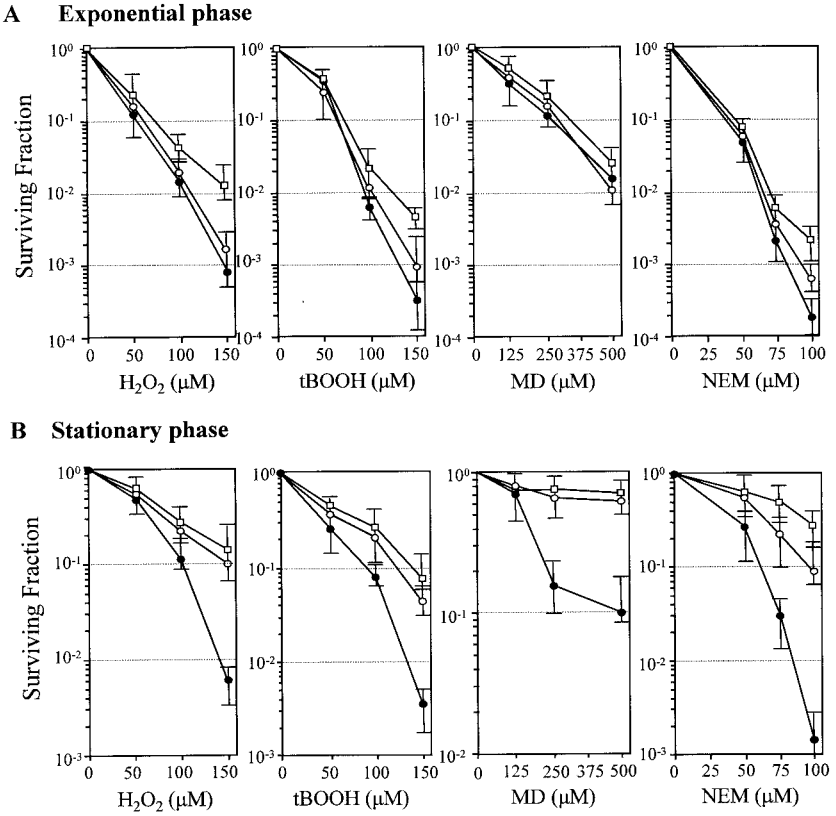

FIG. 3. Determination of oxidant resistance levels in *X. campestris* pv. phaseoli strains. *Xanthomonas* strains were grown in SB medium for 4 h for exponential-phase experiments and 30 h for stationary-phase experiments. Determination of oxidant resistance levels was done using a plate sensitivity assay as previously described (3) with some modifications. Essentially, cell aliquots were serially diluted in 50 mM sodium phosphate buffer, pH 7.0, prior to being plated on SB agar alone and SB agar containing the indicated concentration of oxidants. The colonies were scored after incubation at 28°C for 48 h. The surviving fraction was defined as the number of CFU from the oxidant-containing plate divided by the number of CFU from the SB agar plate. Experiments were repeated three times, and means and standard deviations are shown. The survival curves of the *msrA* mutant (Xp07 [●]), Xp07 harboring pMsrA (□), and the parental wild type (○) are shown.

The stationary-phase cells of *msrA* mutant strain Xp07 were 10- to 100-fold more sensitive to H_2O_2, tBOOH, MD, and NEM treatments than the parental strain (Fig. 3B), and the oxidant sensitivity phenotype of Xp07 could be complemented by pMsrA. Analysis of related *X. campestris* pv. campestris genome has shown the existence of *msrB*. The phenotypes of *msrA* mutant could not be complemented by expression of *msrB* in an expression vector (data not shown).

In *Xanthomonas*, as well as in other bacteria, stationary-phase cells are highly resistant to oxidant killing (11). The mechanisms responsible for stationary-phase resistance to oxidants are not fully understood but are thought to be independent of the levels of scavenging enzymes. It has been previously shown that the activities of oxidant-scavenging enzymes, such as catalase and superoxide dismutase, decreased as *Xanthomonas* cultures entered into the stationary phase and during the stationary phase of growth (11, 25). This is likely to lead to intracellular accumulation of oxidants and a subsequent increase in the oxidation of macromolecules. Thus, during stationary phase, enzymes that are involved in the various repair processes, such as MsrA, become important in protecting cells from intracellular oxidants. *Xanthomonas msrA* is the only protein oxidation repair system thus far studied that shows a good correlation between the gene expression pattern and its physiological role. During normal growth, exponential-phase cells are less likely to be damaged by oxidants, due to the presence of high levels of oxidant-scavenging enzymes. However, during exponential phase, the bacteria are still highly susceptible to extracellular oxidants. The oxidant-inducible expression of *msrA* during exponential phase provides the cells with additional MsrA to repair damage caused by exposure to extracellular oxidants. This is reflected in the low level of *msrA* expression during exponential phase. As growth continues into stationary phase, a decline in scavenging enzyme activities (11, 25) leads to an increase in the intracellular accumulation of oxidants and hence the need to increase *msrA* expression to repair oxidized proteins.

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NOTES

Multinucleated Giant Cell Formation and Apoptosis in Infected Host Cells Is Mediated by *Burkholderia pseudomallei* Type III Secretion Protein BipB

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Here we have assessed the role of a type III translocator protein, BipB, in the cell biology and virulence of *Burkholderia pseudomallei*. Genetic inactivation of *bipB* reduced multinucleated giant cell formation, cell-to-cell spreading of bacteria, and induction of apoptosis of J774A.1 macrophages. The *bipB* mutant was also significantly attenuated following intranasal challenge of BALB/c mice, whereas virulence was fully restored by complementation with a functional *bipB* gene.

Burkholderia pseudomallei, the etiological agent of melioidosis in humans and animals, is a gram-negative bacterium. Melioidosis is endemic in southeast Asia and tropical Australia and has been reported sporadically elsewhere (6). Currently, there is no vaccine against melioidosis. Uniquely among intracellular bacterial pathogens, *B. pseudomallei* induces host cell fusion leading to multinucleated giant cell (MNGC) formation in tissue culture models of infection (14). This novel phenotype may be relevant to pathogenesis, since granuloma formation and generation of MNGC are also found in tissues of humans with melioidosis (23). In addition to inducing MNGC formation, *B. pseudomallei* is able to spread from cell to cell and induce apoptotic death in infected host cells (14). The molecular mechanisms of these pathogenic characteristics have not been elucidated.

Analysis of the *B. pseudomallei* genome and several other studies have demonstrated the presence of a type III secretion system (TTSS) (for reviews, see references 3, 12, 17, 20, and 22). A knockout mutant of *B. pseudomallei* lacking a functional *bipD* gene, a homologue of *Salmonella enterica* serovar Typhimurium *sipD*, on the TTSS3/*bsa* cluster of TTSS exhibited reduced replication in murine macrophage-like cells (20), was significantly attenuated in BALB/c mice and gave partial protection against subsequent challenge with wild-type *B. pseudomallei* (19). These data correlated with the recent report that the TTSS3/*bsa* cluster is required for the pathogenicity of *B. pseudomallei* (21). In addition to BipD, *B. pseudomallei* BipB and BipC (46 and 30% amino acid identity to *Salmonella* SipB and SipC, respectively) have been identified in the TTSS3/*bsa* cluster (3). Here, we report on the role of BipB in the pathogenesis of infection with *B. pseudomallei*. With *Salmonella* organisms, purified SipB integrates into artificial membranes and induces liposome fusion (10), and it is required for inducing apoptosis in murine macrophages (11). By analogy with SipB, therefore, we investigated the role of BipB for MNGC formation, cell-to-cell spreading, and induction of apoptosis in infected host cells. We also examined the virulence of a *B. pseudomallei bipB* mutant in a murine model of melioidosis.

Construction of a *B. pseudomallei bipB* mutant. Analysis of the *B. pseudomallei* genome (http://www.sanger.ac.uk/Projects/B_pseudomallei), by use of the *sipB* sequence from *S. enterica* serovar Typhimurium as the query in a TBLASTX search, identified a coding sequence of 1,860 bp encoding the predicted BipB protein of 620 amino acids. In order to determine the function of BipB in *B. pseudomallei*, a chromosomal *bipB* mutant of *B. pseudomallei* was constructed. In brief, a 250-bp internal fragment of the *bipB* gene was amplified from *B. pseudomallei* K96243 genomic DNA by use of primers BipB-45 (5′-AACCAGGCCACGCAGCAG-3′) and BipB-46 (5′-CGT CTTCTGCATCTCCTC-3′). The amplified fragment was cloned into a suicide vector, pKNOCK-Tc (1), kindly provided by M. F. Alexeyev. This constructed plasmid was introduced from *Escherichia coli* S17-1λpir (7) into *B. pseudomallei* K96243 by conjugation. Transconjugants were selected by plating on pseudomonas agar supplemented with SR103 (Oxoid, United Kingdom) containing tetracycline. The isolated mutant, designated *B. pseudomallei* BS46 (*bipB*::pSSB-1), was verified by PCR and Southern blot hybridization to ensure insertion of the *bipB* suicide plasmid at the correct location (data not shown). For complementation analysis, the amplified *bipB*

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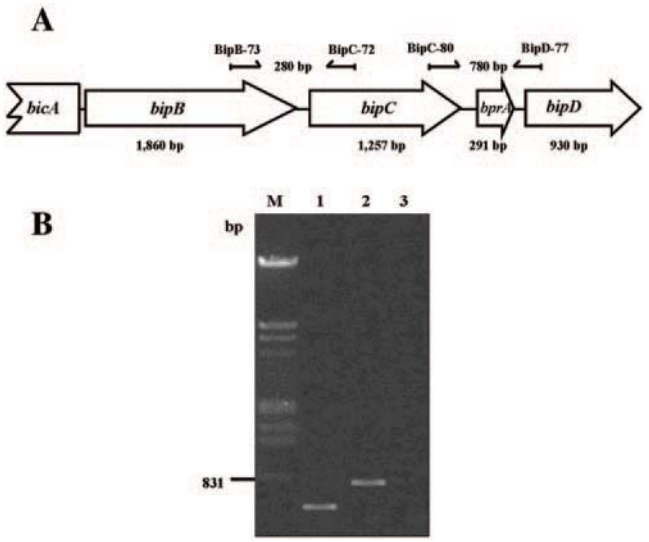

FIG. 1. The *B. pseudomallei bipB* operon. (A) Physical map of *bipB-bipC-bprA-bipD* gene organization together with locations of primer pairs BipB-73–BipC-72 and BipC-80–BipD-77 for RT-PCR analysis of *B. pseudomallei bipB* operon. (B) Ethidium bromide-stained gel showing the amplified DNA of RT-PCR products from primer pairs BipB-73–BipC-72 (lane 1) and BipC-80–BipD-77 (lane 2). Lane 3 is an RNA sample subjected to PCR to ensure no DNA contamination in the RNA preparation. Lane M shows lambda DNA markers.

gene was cloned into pBBR1MCS (15) and introduced into *B. pseudomallei* BS46. To confirm that *B. pseudomallei* BS46pBipB contained the *bipB* gene, the DNA plasmid was extracted and sequenced (data not shown).

To determine whether *bipB* was cotranscribed with the downstream genes *bipC-bprA-bipD*, reverse transcription-PCR (RT-PCR) was undertaken. Extraction of total RNA, by use of the modified hot acid phenol method, was carried out as described previously (2). In brief, mid-exponential-phase cultures were harvested and extracted with hot acid phenol. Total RNA was precipitated and resuspended with RNase-free distilled water. For RT-PCR analysis, *bipB-bipC-bprA-bipD* was reversed transcribed into cDNA (Invitrogen) and then amplified with different primers, namely, BipB-73 (5′-CTGCTCGGCG ATCTGCTCAA-3′), BipC-72 (5′-ACCGCCTTGTCGCCCT G-3′), BipC-80 (5′-GAGCAGAAAGAGGACGAGA-3′), and BipD-77 (5′-CGCAGATCGTCGTCGGTCA-3′) (Fig. 1A).

As depicted in Fig. 1B, *B. pseudomallei bipB-bipC-bprA-bipD* was transcribed in a single transcriptional unit. It is likely that *B. pseudomallei* BS46 is a polar *bipB* mutant. To investigate whether this mutation does not have effect on expression of other secreted proteins, Western blot analysis using anti-BopE (kindly provided by M. P. Stevens, United Kingdom) to detect BopE in whole-cell and secreted protein fractions of *B. pseudomallei* BS46 and wild-type strains was undertaken. BopE, homologous to the *Salmonella* SopE, was an effector protein secreted by the *B. pseudomallei* TTSS (18). BopE was detected in both whole-cell and secreted protein fractions of *B. pseudomallei* BS46 (data not shown). This suggests that the TTSS of *B. pseudomallei* BS46 is still functional to express and secrete other proteins such as BopE.

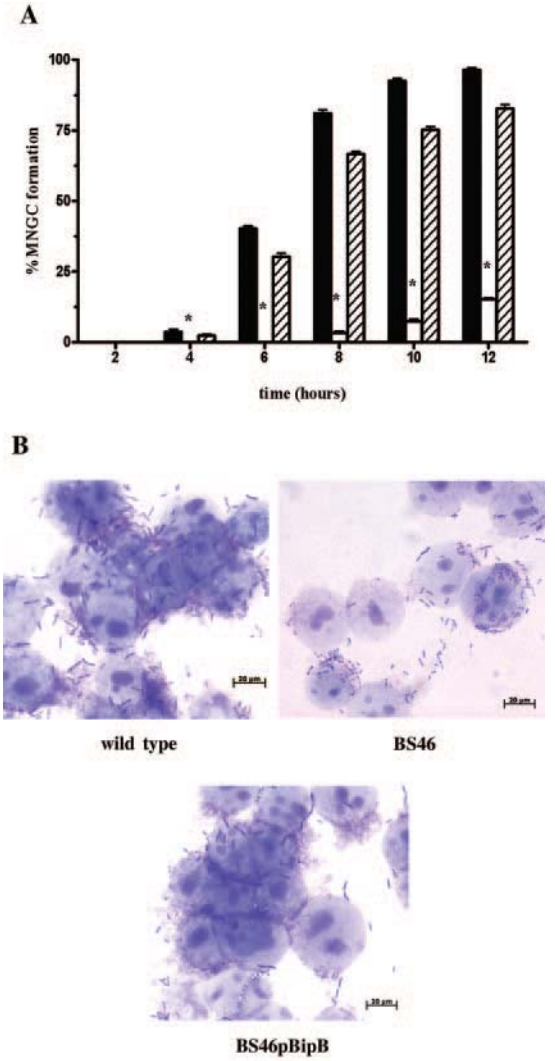

FIG. 2. MNGC formation of *B. pseudomallei*. (A) The percentages of MNGC formation of J774A.1 cells infected with *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars) were determined every 2 h. Asterisks indicate significant differences ($P < 0.05$, *t* test) between the wild type and BS46 at 4 h ($P = 0.0142$) and 6 to 12 h ($P < 0.0001$) and between BS46 and BS46pBipB at 4 h ($P = 0.0155$) and 6 to 12 h ($P < 0.0001$). Percentage of MNGC formation was determined by the following equation: MNGC formation = (number of nuclei within multinucleated giant cells/total number of nuclei counted) × 100. Error bars represent standard errors of the means for experiments performed in triplicate. (B) Giemsa staining of MNGC formation of J774A.1 cells infected with wild type, BS46, or BS46pBipB. Bars, 20 μm.

The polar *bipB* mutant is defective in MNGC formation. To investigate the potential role of BipB in MNGC formation, *B. pseudomallei* K96243 (wild type), BS46 (*bipB*::pSSB-1), and BS46pBipB (BS46 harboring pBipB) were used to infect J774A.1 murine macrophage-like cells as described previously (14). At different times after initiation of the challenge, the infected cells were fixed, Giemsa stained, and evaluated for MNGC formation. Figure 2 shows that BipB protein plays a role in *B. pseudomallei*-induced MNGC formation. At 12 h

postinfection (Fig. 2A), wild-type bacteria induced extensive MNGC formation (96.46%), while BS46 did not (15.12%). The formation of MNGC was restored in a complementation assay using strain BS46pBipB (82.7%). Figure 2B shows that MNGC loaded with numerous bacilli could be readily observed at 6 h after infection with wild-type bacteria but that this was abolished in the *bipB* mutant BS46. However, this defective phenotype was transcomplemented by reintroduction of the plasmid-born *bipB* gene. However, when the observation period was extended to 24 h, formation of MNGC in BS46-infected macrophage did occur but was still significantly less than the wild-type strain. Thus, BipB is necessary for optimal MNGC induction, but BipB-independent fusion can also occur, albeit at a reduced efficiency.

The mechanism for the MNGC formation is still unknown, and to our knowledge, this altered phenotype has not been observed in other intracellular bacteria that possess the TTSS. Based on the *Salmonella* SipB-induced fusion events in vitro (10) and those that would be transient in vivo (9), we hypothesize that BipB may have membrane fusion activity as well. It may act in concert with other proteins to induce fusion of host cell membranes. A combination of biochemistry, cell biology, and proteomics will be required to unveil the detailed pathways of MNGC formation.

The polar *bipB* mutant is defective in cell-to-cell spread and invasion into epithelial cells. The observation of MNGC led us to look closely at cell-to-cell spread of infected host cells by using a plaque assay previously described (14). HeLa cells were infected with *B. pseudomallei* and overlaid with an agarose medium containing kanamycin (250 μg/ml). To enhance visualization, plaques were overlaid with agarose containing an additional 0.01% neutral red and observed 4 h later. Figure 3A demonstrates that plaque-forming efficiencies for *B. pseudomallei* wild type (2.66) and BS46pBipB (0.68) were significantly higher than that for BS46 (0.2). It is possible that only partial complementation in BS46pBipB could have resulted from a polar effect that disrupted downstream *bipC* and *bipD* genes also participating in cell-to-cell spreading. This hypothesis is supported by a previous report, from Stevens et al. (20), that a *bipD* mutant exhibited an inability to escape from endocytic vacuoles, a requirement for cell-to-cell spread. If so, it would indicate that BipB works cooperatively with BipC and BipD in a manner similar to that of SipABCD in *Salmonella* (4).

The strategies that intracellular bacteria, i.e., *Listeria* sp. and *Shigella* sp., use to spread from cell to cell via interepithelial protrusion are quite similar (8). The process depends on the efficiency of bacterial invasion into the epithelial cytosol, protrusion formation, and the lysis of the double-membrane-bound protrusion vacuole to release bacteria into the adjacent cell. To investigate whether defective cell-to-cell spread (as detected by plaque assay) was due to an invasion defect, invasion efficiency was determined by using human respiratory epithelial cell line A549 challenged with *B. pseudomallei* as described earlier. This cell line was chosen because it is more susceptible to invasion than HeLa cells. Intracellular bacteria were counted after lysing of infected cells. Invasion efficiency of BS46 was severely restricted (0.09%) when compared to that of the wild type (0.39%), but invasion efficiency was restored to nearly normal levels in BS46pBipB (0.28%) (Fig. 3B). These data correlated with those for the *bipD* mutant that exhibited

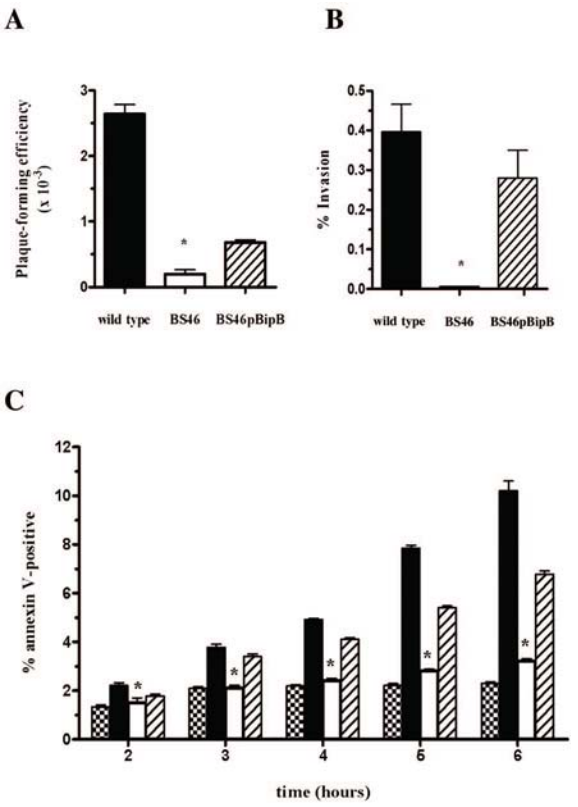

FIG. 3. Plaque formations, invasion, and apoptosis induction. (A) Plaque formations of HeLa cells by *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars). Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 ($P = 0.0001$) and between BS46 and BS46pBipB ($P = 0.0031$). Plaque-forming efficiency was determined by the following equation: plaque-forming efficiency = number of plaques/bacterial CFU added per well. Error bars represent standard errors of the means for experiments performed in triplicate. (B) Invasion of A549 cells by *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), and BS46pBipB (BS46 harboring pBipB; striped bars) strains. Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 ($P = 0.0050$) and between BS46 and BS46pBipB ($P = 0.0173$). Percent invasion was determined by the following equation: invasion = (number of intracellular bacteria postinfection/number of CFU added) × 100. Error bars represent standard errors of the means for experiments performed in triplicate. (C) Effect of *bipB* mutation on induction of apoptosis. J774A.1 cells were infected with *B. pseudomallei* K96243 (wild type; solid bars), BS46 (*bipB*::pSSB-1; open bars), BS46pBipB (BS46 harboring pBipB; striped bars), and uninfected cells (checkered bars). The percentages of J774A.1 cells stained fluorescein isothiocyanate positive and propidium iodide negative by flow cytometry were analyzed. Asterisks indicate significant differences ($P < 0.05$, *t* test) between wild type and BS46 at 2 h ($P = 0.0123$), 3 h ($P = 0.0004$), and 4 to 6 h ($P < 0.0001$) and between BS46 and BS46pBipB at 2 h ($P = 0.1064$), 3 h ($P = 0.0006$), and 4 to 6 h ($P < 0.0001$). Error bars represent standard errors of the means for experiments performed in triplicate.

impaired entry into nonphagocytic host cells (18). In this scenario, we believe that several effector proteins, such as BopE, that contribute to invasion (18) would not be delivered into the host cell cytoplasm, even though it was expressed. This proposed mechanism is based on the study of *Salmonella* in which inactivation of *sip* genes resulted in impaired invasion effi-

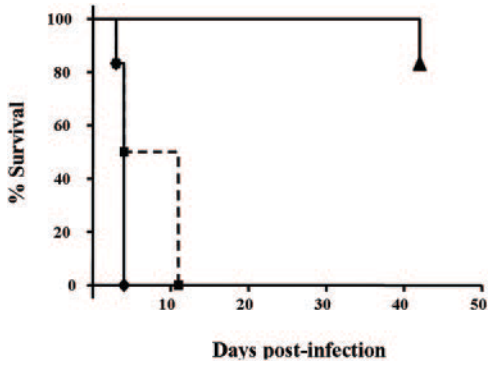

FIG. 4. Survival of BALB/c mice (six mice per group) inoculated intranasally with 10^3 CFU of *B. pseudomallei* K96243 (■) or BS46 (▲) or BS46pBipB (◆). Mice were observed daily, and percent survival was plotted against time.

ciency due to the lack of translocation of effector proteins, such as SopE, into host cells (4, 13, 24). In addition to invasion, BipB may play a role in other steps involved in cell-to-cell spreading. Further experiments are required to investigate this possibility.

The polar *bipB* mutant is defective in induction of apoptosis. *B. pseudomallei* can induce apoptotic death in infected macrophages (14). To determine the role of BipB in this process, J774A.1 cells were infected with *B. pseudomallei* strains. At different time intervals, the supernatant and cells were collected to quantify the apoptosis level by using an annexin V-fluorescein isothiocyanate detection kit (BD Biosciences, CA). At 6 h postinfection (Fig. 3C), cells infected with wild-type *B. pseudomallei* yielded significantly higher numbers of positive cells (10.20%) than those infected with BS46 (3.21%). Infection with BS46pBipB restored cytotoxicity (6.77%). These data indicated that BipB was required for efficient induction of apoptosis in host cells, although a low level of apoptosis may occur via a BipB-independent mechanism, since the level of apoptosis in uninfected cells is 2.3%. This is the first report identifying a *B. pseudomallei* virulence factor that mediates apoptosis. Interestingly, this finding joins a growing list of bacteria, including *Pseudomonas aeruginosa*, *Yersinia* sp., *Salmonella* sp., and *Shigella flexneri*, that kill host cells via apoptotic death through a type III secretion-mediated mechanism. In *Salmonella* and *Shigella*, SipB and IpaB have been shown to induce macrophage apoptotic death by activating caspase-1 (11, 25). Here, we also expect that apoptosis induced by *B. pseudomallei* will involve BipB interaction with the caspase pathway (14).

Effect of *bipB* mutation on virulence of *B. pseudomallei* in vivo. The finding that BipB is important in induction of MNGC, plaque formation, bacterial invasion, and killing of phagocytic cells in vitro led to the hypothesis that a mutant unable to produce this protein could be less virulent than the wild-type strain in vivo. We therefore assayed the virulence of the *bipB* mutant in a pulmonary model of melioidosis in BALB/c mice as previously described (19). *B. pseudomallei* strains were administered via the intranasal route. Viable counts were performed to confirm the inoculation dose, and the mice were monitored twice daily for signs of infection. There was a significant difference in percentage survival (the P value was <0.05, as determined by a log rank test) for mice infected with wild-type *B. pseudomallei* versus mice infected with BS46 (Fig. 4). All mice given the wild-type strain died within 5 to 11 days, whereas five of six mice infected with the *bipB* mutant survived until day 42 (termination of experiment). To confirm that attenuation resulted from the inactivation of *bipB*, we also challenged mice with strain BS46pBipB, and all died by day 4 postchallenge (Fig. 4), which was not significantly different from the wild-type strain. These observations indicated that a functional *bipB* gene was required for full virulence of *B. pseudomallei* in mice. This result is supported by previous reports (19, 21) that TTSS3/Bsa plays an important role for maximal virulence in all of its animal hosts.

Delivery of virulence-associated effector proteins into eukaryotic cells requires a set of translocator proteins. The translocons are components of oligomeric protein channels that insert themselves into the eukaryotic cell membrane to form a pore which effector proteins can pass through to gain access to the cytosolic host targets (5, 16). We have shown here that BipB translocator plays a critical role in the intracellular lifestyle of *B. pseudomallei* (i.e., MNGC formation, invasion of nonphagocytic cells, and induction of apoptotic death). We hypothesize that the *bipB* mutant is unable to deliver the effector proteins into the host cell cytoplasm and was thus impaired in invasion efficiency and ability to induce apoptosis. However, it is also possible that BipB acts as an effector protein to induce apoptotic death. Deletion of BipB clearly also reduces the efficiency of MNGC formation; however, the relationship between BipB protein and the fusion process is still under investigation. In vivo, BipB was required for full virulence of *B. pseudomallei* in mice, thus further confirming the importance of BipB for virulence in murine models of melioidosis.

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