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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.

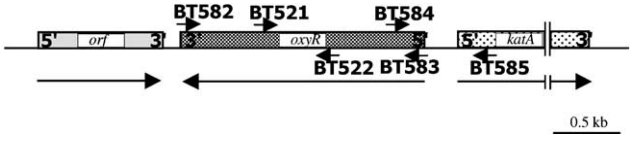

Fig. 1. Gene and transcriptional organization of the *oxyR* locus. The physical map of *A. tumefaciens oxyR*, small arrows indicate the positions of primers and the long arrows indicate the direction of transcription.

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*,

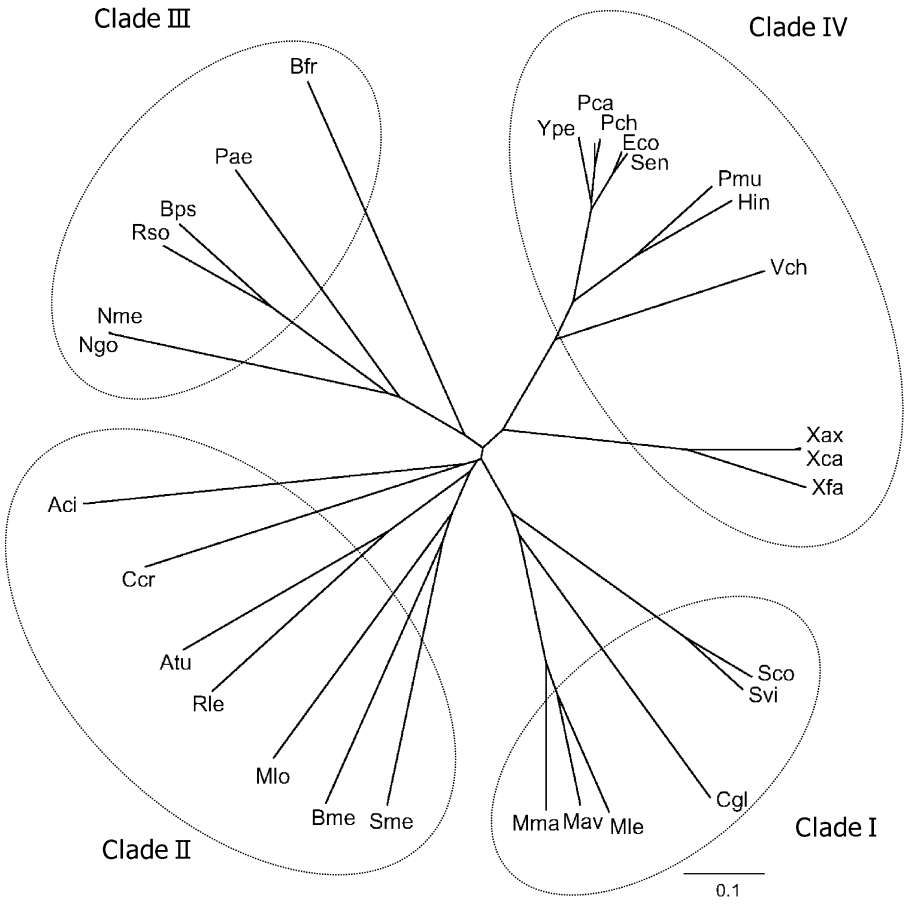

Fig. 2. Phylogenetic tree constructed from OxyR sequences from various microorganisms. Aci, *Acinetobacter* sp. CAA86928; Atu, *Agrobacterium tumefaciens* AAK88806; Bfr, *Bacteroides fragilis* AAG02620; Bme, *Brucella melitensis* biovar Abortus AAD00508; Bps, *Burkholderia pseudomallei* AAK72465; Ccr, *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).

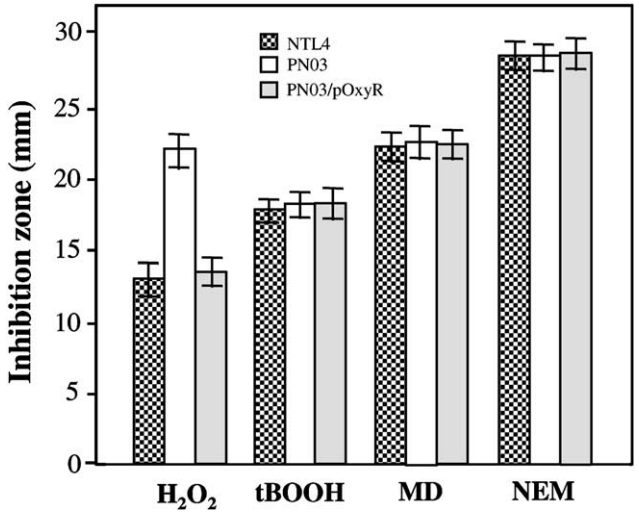

Fig. 3. Determination of the levels of resistance to killing concentrations of oxidants in the *A. tumefaciens* PN03, PN03 harboring pOxyR, and the parental strain NTL4. The resistance levels to oxidants of the *A. tumefaciens* PN03, the *oxyR* mutant complemented with *oxyR* on the expression vector (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

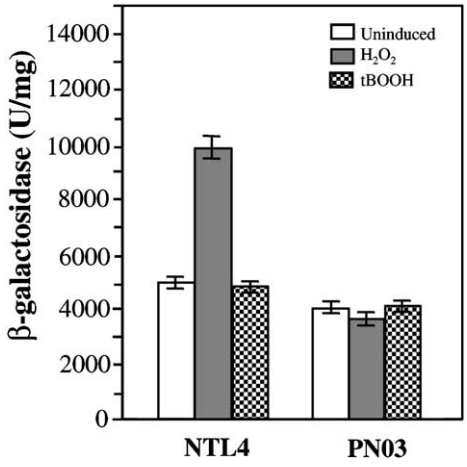

Fig. 4. In vivo *katA* promoter characterization. β-Galactosidase activity of *A. tumefaciens* NTL4 and the PN03 harboring pP_{katA}. Cells were harvested from cultures that were either uninduced or induced with either H_2O_2 or tBOOH. Values are means and SD of four replicates.

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

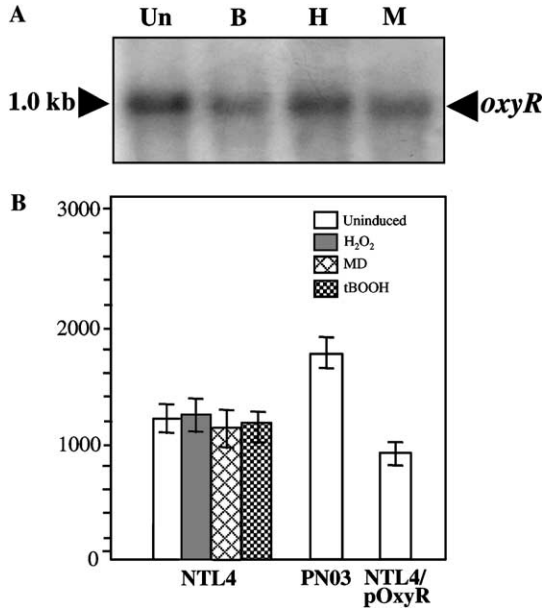

Fig. 5. In vivo regulation of the *oxyR* promoter. In (A) Northern blot analysis of *oxyR* in response to oxidant pre-treatments. Total RNA was prepared from *A. tumefaciens* induced with tBOOH (B), H_2O_2 (H), menadione (M), and an uninduced culture (Un) was separated and probed with *katA* as described 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.

Acknowledgments

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