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The Catalase-Peroxidase KatG Is Required for Virulence of *Xanthomonas campestris* pv. campestris in a Host Plant by Providing Protection against Low Levels of H_2O_2 ▽

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***Xanthomonas campestris* pv. campestris *katG* encodes a catalase-peroxidase that has a role in protecting the bacterium against micromolar concentrations of H_2O_2. A knockout mutation in *katG* that causes loss of catalase-peroxidase activity correlates with increased susceptibility to H_2O_2 and a superoxide generator and is avirulent in a plant model system. *katG* expression is induced by oxidants in an OxyR-dependent manner.**

Catalase-peroxidases (KatGs) are heme-containing enzymes that possess catalatic and substantial peroxidatic activities. KatGs belong to class I of the superfamily of bacterial, fungal, and plant peroxidases, and they are present in a variety of prokaryotes and some ascomycete fungi (21). These enzymes function as H_2O_2 scavengers, protecting cells from H_2O_2 toxicity. In addition, in *Mycobacterium tuberculosis*, KatG is also involved in the activation of the frontline antituberculosis drug isoniazid (32). A number of isoniazid-resistant strains are the consequence of mutations within *katG* (32). *katG* also has roles in bacterial pathogenicity (31).

In several enteric and soil bacteria, *katG* is a member of the OxyR regulon, and its expression is inducible by peroxides (13, 15). Reduced and oxidized OxyR regulons bind differently to the target promoters, and both repress or induce transcription of the target genes (28). We have been investigating the OxyR-mediated response to peroxide stress in *Xanthomonas campestris* (2, 5, 14, 17, 18). The expression of *ahpCF* and *katA*, which encode an alkyl hydroperoxide reductase and a monofunctional catalase, respectively, are governed by OxyR. H_2O_2, organic hydroperoxides, and superoxide generators are potent inducers for genes in the OxyR regulon (17). Analysis of the recently published genome sequence of *X. campestris* pv. campestris (7) revealed an additional gene annotated as Xcc1205, encoding a bifunctional catalase-peroxidase, KatG, in addition to *katA* and *katE*, encoding atypical and stationary-phase-dependent monofunctional catalases, respectively. In this study, the physiological roles of *X. campestris* pv. campestris *katG* in bacterial stress protection and its gene regulation were characterized. Furthermore, the role of *katG* in bacterial virulence was investigated.

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Role of *katG* in protection of *X. campestris* pv. campestris against H_2O_2. To evaluate the role of the putative *katG* gene annotated as Xcc1205 in the *X. campestris* pv. campestris genome (7), a *katG* knockout mutant was constructed by insertional inactivation using pKNOCK (1). The 300-bp EcoRI fragment of *katG* was cloned into pKNOCK-Km digested with EcoRI. The recombinant plasmid was subsequently transferred into *X. campestris* pv. campestris by electroporation, and recipient cells were selected by kanamycin resistance. The *X. campestris* pv. campestris *katG* mutant was verified by PCR and Southern blot analysis (data not shown). Next, the physiological role of the gene in oxidative stress protection was evaluated. The extent of resistance to H_2O_2 in the *katG* mutant and the *X. campestris* pv. campestris wild type was validated primarily using the plate sensitivity assay (23), in which bacterial cultures were serially diluted and plated on Silva Buddenhagen (SB) medium (17) containing 200 μM H_2O_2. Bacterial colonies capable of growing on H_2O_2-containing medium were counted after a 48-h incubation. The *katG* mutant was 10^4-fold less resistant to H_2O_2 than the isogenic wild-type strain (Fig. 1A).

We have previously observed that an *X. campestris* pv. phaseoli *katA* mutant is also less resistant to H_2O_2 (5). Nonetheless, the roles of each of these two catalase genes in protecting the bacteria from H_2O_2 have not been evaluated and compared. Therefore, an *X. campestris* pv. campestris *katA* knockout mutant was constructed using the pKNOCK system. A 200-bp *katA* (Xcc3949) DNA fragment was PCR amplified from *X. campestris* pv. campestris genomic DNA with BT2237 and BT2238 primers (Table 1). The resulting PCR products were cloned into pGEM-T Easy (Promega) before an EcoRI fragment was subcloned into pKNOCK-Gm. The resultant recombinant plasmid (pKNOCK-katA) was electroporated into *X. campestris* pv. campestris. The gentamicin-resistant transformants were screened for *katA* inactivation. The *katA* mutant was verified using both PCR and Southern analysis. A *katA katG* double mutant was also constructed by electroporating pKNOCK-katA into a *katG* mutant. Next, the levels of resistance to a low dose (200 μM) of H_2O_2 in *katG* and *katA*

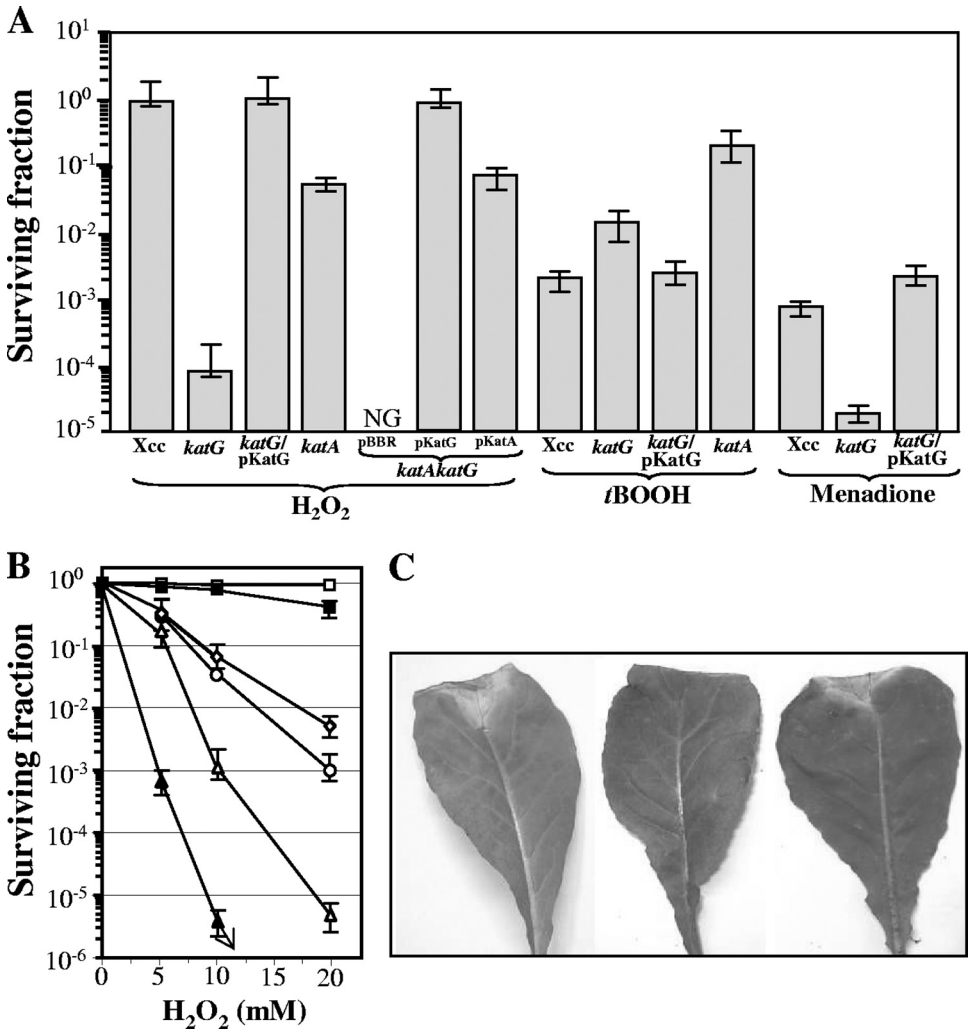

FIG. 1. Phenotypes of *X. campestris* pv. campestris strains. (A) H_2O_2 resistance levels were determined using the plate sensitivity assay (23). Serial dilutions of exponential-phase cultures of the *X. campestris* pv. campestris wild type (Xcc), the *katG* mutant and its complemented strain (*katG*/pKatG), the *katA* mutant, and a *katA katG* double mutant harboring the control plasmid pBBR1MCS4 (pBBR), pKatG, or pKatA were spotted on SB agar plates containing 200 μM H_2O_2, 150 μM *tert*-butyl hydroperoxide (*t*BOOH), or 200 μM menadione. Bacterial colonies surviving the treatment were counted after 48 h of incubation at 28°C and expressed as surviving fractions, which were calculated by dividing the number of CFU from plates containing H_2O_2 by the number of CFU from plates without an oxidant. NG indicates no surviving cell growth. (B) H_2O_2 resistance levels were determined using the H_2O_2 killing assay (5). One-milliliter aliquots of exponential-phase cultures of *X. campestris* pv. campestris (◇), the *katG* mutant (○), the *katA* mutant (△), and a *katA katG* double mutant harboring the vector control (▲), pKatA (□), or pKatG (■) were treated with 0, 5, 10, and 20 mM H_2O_2 for 30 min. Cells surviving the treatment were determined using viable cell counts. The surviving fraction was calculated by dividing the number of CFU from treated samples by the number of CFU from nontreated samples. (C) The virulence of *X. campestris* pv. campestris was determined on Chinese radish (*Raphanus sativus*), a compatible host plant, using the leaf-clipping method (9) with some modifications. Overnight cultures of *X. campestris* pv. campestris and the *katG* mutant and its complemented strain (*katG*/pKatG) in AB medium (a) supplemented with 0.1% (wt/vol) Casamino Acids were diluted to an optical density at 600 nm of 1.0 in fresh AB medium. The lesion lengths were measured at 14 days postinoculation. Experiments were repeated three times.

mutants and the parental strain were determined by the plate sensitivity assay. The results show that the *katG* mutant was roughly 500-fold less resistant to 200 μM of H_2O_2 than the *katA* mutant. The *katA* mutant was 50-fold less resistant to H_2O_2 than a wild-type strain (Fig. 1A). This suggests that *katG* has a principal role in protecting the *X. campestris* pv. campestris cells from a low dose of H_2O_2.

Next, we determined the levels of catalase in various *kat* mutants. The *katG* mutant had reduced total catalase activity (4.7 ± 0.5 U mg^{-1} protein) compared to that in an isogenic wild-type strain (6.0 ± 0.4 U mg^{-1} protein). The *katA* mutant had a drastically lower catalase activity (1.2 ± 0.3 U mg^{-1} protein). There are no direct correlations between resistance to 200 μM of H_2O_2 and total catalase activities in various strains; KatA is a major source of catalase activity in exponentially growing *X. campestris* pv. campestris cells. The *katG* mutant has 4-fold-higher levels of catalase and yet 500-fold less resistance to 200 μM H_2O_2 than the *katA* mutant, supporting the idea that KatG catalase-peroxidase has a primary role for bacterial survival under micromolar concentrations of H_2O_2. Generally, degradations of H_2O_2 by KatG catalase-peroxidase and KatA monofunctional catalase are two-step reactions. The

TABLE 1. List of primers used in this study

Primer	Sequence (5′→3′)	Purpose
BT1414	GCATCCGCGAGCGTTTTC	Reverse primer for *oxyR* fragment
BT1413	GTCGTGCCGCGACAGCGG	Forward primer for *oxyR* fragment
BT2237	GGCCAGGTCGTCCGGCTT	Reverse primer for *katA* fragment
BT2238	GAATCCACCCGCACGCTG	Forward primer for *katA* fragment
BT2239	TCTGCTTGCCACCGGACT	Reverse primer for *katG* fragment
BT2240	TGTGGCAGGACCCGATCC	Forward primer for *katG* fragment
BT2637	TTCGGCAAGCGCTGACGC	Forward primer for *katG* promoter
BT2652	CCGACGACGCTGTGGTTG	Reverse primer for *katG* promoter
BT2362	CTTCACTCAGGCCAGGTC	Reverse primer for full-length *katG*
BT2363	CCAGGTACCCTTCACCAC	Forward primer for full-length *katG*
BT2684	CGCAGCGTCTCGGTGACG	Forward primer for *ahpC* fragment
BT2685	AGTGGAAGACGCCGCTGA	Reverse primer for *ahpC* fragment
BT2781	GCCCGCACAAGCGGTGGAG	Forward primer for 16S ribosomal gene
BT2782	ACGTCATCCCCACCTTCCT	Reverse primer for 16S ribosomal gene

first step involves heme oxidation by H_2O_2 to form an oxyferryl species, compound I. The second reaction is a reduction of compound I by H_2O_2 to regenerate the resting state enzyme, water, and oxygen. While the apparent K_m values for H_2O_2 in the heme oxidation of both KatG catalase-peroxidases and KatA monofunctional catalases are somewhat similar (~ 300 μM), the apparent K_m values of KatGs for the reduction of compound I (2.4 to 4.5 mM) are much lower than those of KatA (38 to 200 mM) (26, 27). This reflects a higher affinity of KatG for H_2O_2 degradation.

Additionally, the *katA katG* double mutant was hypersensitive to H_2O_2. This mutant was unable to grow on medium containing 200 μM H_2O_2 (Fig. 1A). Nevertheless, the *katA katG* double mutant had no aerobic growth defects when grown in either liquid or solid enriched medium (SB). This is unlike a *Bradyrhizobium japonicum katG* mutant that has defects in aerobic growth, suggesting that the gene has a primary role in the detoxification of H_2O_2 generated from aerobic life (22). We have shown in *X. campestris* pv. phaseoli that an *ahpC* (alkyl hydroperoxide reductase) mutant has reduced ability to form colonies on an agar plate, but this ability could be rescued by the addition of a peroxide scavenger, 0.1% pyruvate, to the medium (19). In *Xanthomonas*, AhpC has a primary role in scavenging physiologically generated H_2O_2 analogous to that of *Escherichia coli* AhpC (25).

If catalatic activity is crucial for detoxification of high concentrations of H_2O_2, one would anticipate that a *katA* mutant is less resistant to treatment with a high concentration of H_2O_2 than a *katG* mutant. We performed additional experiments using the H_2O_2 killing assay (5). In this procedure, bacterial cultures are subjected to lethal doses of H_2O_2 (5, 10, and 20 mM) for 30 min. Cells surviving the treatments were determined by viable cell count. As illustrated in Fig. 1B, compared to the *X. campestris* pv. campestris wild type, the *katA* mutant was 10^3-fold less resistant to 20 mM H_2O_2 killing. The *katG* mutant was 500-fold more resistant to the same concentration of H_2O_2 than the *katA* mutant and only 5-fold less resistant than a wild-type strain. This supports the role of KatA as a major catalase responsible for protecting *X. campestris* pv. campestris against high levels of H_2O_2, whereas *katG* has only a minor role in the process (Fig. 1B). Additionally, the levels of resistance to high concentrations of H_2O_2 show a correlation with total catalase activities of the cells. As expected, the double mutant, which produced no detectable level of catalase activity, was the most sensitive to killing by a high concentration of H_2O_2 (Fig. 1B). These data support the hypothesis that the catalatic activity of KatA as well as that of KatG is responsible for the protection of *Xanthomonas* from killing by millimolar concentrations of H_2O_2.

We extended the investigation by assessing the ability of high levels of expression of either *katA* or *katG* from an expression vector to complement the H_2O_2-hypersensitive phenotype of the *katA katG* double mutant. The double mutant harboring pKatA or pKatG (pBBR1MCS4 expression vector [11] containing *X. campestris* pv. campestris *katA* or *katG* [full-length]) produced catalase activities of 195 ± 23 and 73 ± 8 U mg^{-1} protein, respectively. Although the *katA katG* double mutant harboring pKatA had more than twofold-higher total catalase activity, it failed to fully complement the H_2O_2-hypersensitive phenotype, as determined by the plate sensitivity assay using 200 μM H_2O_2 (Fig. 1A), but the strain was highly resistant to the high-concentration H_2O_2 killing treatment (Fig. 1B). On the other hand, a high-level expression of *katG* fully restored the H_2O_2-hypersensitive phenotype of the *katA katG* double mutant determined using both the 200 μM H_2O_2 plate sensitivity assay (Fig. 1A) and millimolar H_2O_2 killing treatments (Fig. 1B). The results further support the role of KatG in protection against micromolar concentrations of H_2O_2. In addition, the resistance to millimolar concentrations of H_2O_2 shows correlation with total catalase activity.

Taken together, the results of H_2O_2 resistance tests and the catalase levels indicate a departure from a current paradigm for the roles of different catalases. In *X. campestris*, both *katG* and *katA* have primary roles in protecting the bacteria against different levels of H_2O_2. The basis of the mechanism is the enzymes' different biochemical properties (bifunctional peroxidase/catalase versus monofunctional catalase) and not the activities of each enzyme.

The *katG* mutant has altered levels of resistance to other oxidants. The *katG* mutant showed a 10-fold increase in resistance to *tert*-butyl hydroperoxide compared with the wild-type strain, and the phenotype could be complemented by pKatG (Fig. 1A). Inactivation of *X. campestris* pv. phaseoli *katA* has been shown to produce a compensatory increase in *ahpC* expression along with enhanced bacterial tolerance to organic hydroperoxides (4). To test whether the level of *ahpC* expression is altered, an end-point reverse transcription (RT)-PCR was performed with RNA prepared from the *X. campestris* pv. campestris wild type, the *katG* mutant, and a *katG*-complemented strain by using *ahpC*-specific primers (BT2684 and

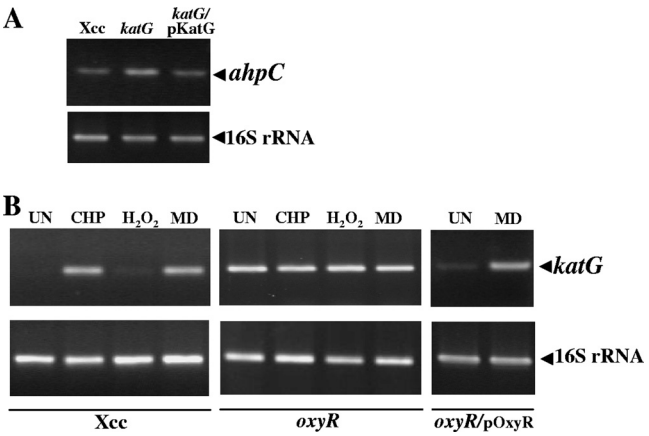

FIG. 2. Expression analysis of *ahpC* and *katG*. (A) The levels of *ahpC* transcripts in *X. campestris* pv. campestris (Xcc) and the *katG* mutant and its complemented strain (*katG*/pKatG) were measured using end-point RT-PCR with BT2684 and BT2685 primers and total RNA (5 μg) as the template. The RT reaction mixture was incubated at 25°C for 10 min and then at 42°C for 1 h. The cDNA products were PCR amplified under the following conditions: 95°C (for 30 s), 62°C (for 30 s), and 72°C (for 30 s) for 25 cycles. The amplified DNA products were separated on a 1.8% agarose gel. (B) *katG* expression in the *X. campestris* pv. campestris wild type and an *oxyR* mutant was analyzed by end-point RT-PCR with *katG*-specific primers (BT2239 and BT2240). Total RNAs (5 μg) extracted from exponentially growing cells either uninduced (UN) or induced with 100 μM H_2O_2, menadione (MD), or cumene hydroperoxide (CHP) were added to the RT reaction as described for panel A. The PCR was performed under the following conditions: 95°C (for 30 s), 58°C (for 30 s), and 72°C (for 30 s) for 30 cycles.

BT2685 [Table 1]). The level of *ahpC* transcripts in the *katG* mutant was higher than the level in either the *X. campestris* pv. campestris wild type or the *katG*-complemented strain (Fig. 2A). Thus, this compensatory increase in *ahpC* expression accounted for, at least in part, the enhanced resistance to organic hydroperoxide observed in the *katG* mutant. However, the precise mechanism responsible for this resistance is not yet clear. Likewise, inactivation of *ahpC* leads to compensatory upregulation of *katG* in *X. campestris* pv. phaseoli (19).

We also determined the resistance levels of a superoxide generator, menadione, in the *X. campestris* pv. campestris wild type and the *katG* mutant by using the plate sensitivity assay with 200 μM menadione. The results in Fig. 1A illustrate that the *katG* mutant was 100-fold less resistant to menadione than the wild-type strain. Furthermore, the phenotype could be complemented by pKatG. The superoxide anions formed are dismutated to yield H_2O_2 and other reactive oxygen species. Efficient degradation of H_2O_2 in part contributes to protection against menadione toxicity. Thus, impaired H_2O_2 detoxification in the *katG* mutant is likely responsible for its reduced menadione resistance phenotype.

The *X. campestris* pv. campestris *katG* mutant is avirulent. *X. campestris* pv. campestris is a causative agent of black rot disease in cruciferous crops. Oxidative stress is an important component of the plant defense response against microbes (12). Hence, we determined the effects of *katG* inactivation on the virulence of the bacteria on a host plant. The *X. campestris* pv. campestris wild type, the *katG* mutant, and the *katG*-complemented strain (*katG*/pKatG) were inoculated into Chinese radish (*Raphanus sativus*) leaves by using the leaf-clipping method (9). The results in Fig. 1C reveal that the *katG* mutant is avirulent on the radish, as shown by the lack of detectable lesions in all tests, while the *X. campestris* pv. campestris wild type and the *katG*-complemented strain (*katG*/pKatG) produced similarly sized lesions (10.7 ± 2.7 mm for *X. campestris* pv. campestris and 9.8 ± 2.4 mm for the *katG*/pKatG strain), and no significant differences in the length of lesions ($P > 0.05$ by paired *t* test) could be detected. The results suggest that *katG* is required for full virulence of *X. campestris* pv. campestris on the Chinese radish host plant. A study of the sugar beet plant defense response reveals that the levels of H_2O_2 in the oxidative burst of the phase I response peaked at 2 mM in elicited plant leaves (3). The phase II burst occurs at approximately 2 h postinoculation and peaks at 4 mM H_2O_2. These levels of H_2O_2 would have lethal effects on bacterial pathogens, including *Xanthomonas*. We speculate that the avirulent phenotype of the *katG* mutant is due in part to a reduction in the ability of the bacteria to cope with plant-generated H_2O_2. Our speculation is supported by the in vitro experiments that show hypersensitivity of the *katG* mutant to H_2O_2 (Fig. 1A and B). In addition, we found that the *X. campestris* pv. campestris *katA* mutant showed an avirulent phenotype on the Chinese radish host, indicating that monofunctional catalase, which contributes to the protection of millimolar levels of H_2O_2, also plays a crucial role in *Xanthomonas* pathogenicity (S. Mongkolsuk, S. Buranajitpakorn, and P. Vattanaviboon, unpublished data).

***katG* expression is regulated by OxyR.** The expression profile of *katG* in *X. campestris* pv. campestris was investigated using end-point RT-PCR. The experiments were performed using total RNAs extracted from untreated *X. campestris* pv. campestris cultures and cultures treated with various oxidants and *katG*-specific primers (BT2239 and BT2240 [Table 1]). Representative results demonstrate that the levels of *katG* transcripts were markedly increased in cells treated with a superoxide generator, menadione (100 μM), and to a lesser extent in those treated with organic hydroperoxides (100 μM *tert*-butyl or cumene hydroperoxides) or H_2O_2 (100 μM) (Fig. 2B). The *katG* expression profiles in response to oxidants resemble typical OxyR-dependent gene expression in *X. campestris* (5, 14, 17). The RT-PCR experiments were repeated in an *X. campestris* pv. campestris *oxyR* knockout mutant that was constructed using pKNOCK-Gm containing a 210-bp *oxyR* (Xcc0832) fragment amplified with BT1413 and BT1414 primers (Table 1). The oxidant's induction of *katG* expression was abolished in the *oxyR* mutant (Fig. 2B). This indicates that OxyR regulates the peroxide-inducible expression of *katG* and that the gene is a member of the OxyR regulon, as in several bacteria (6, 16, 20).

OxyR commonly regulates transcription of a target gene through binding to specific motif sequences located in close proximity to the −35 promoter region. Primer extension was performed to map the 5′ end of *katG* mRNA and to localize the promoter by using a labeled BT2652 primer (Table 1) and total RNA extracted from uninduced and menadione-induced *X. campestris* pv. campestris cultures. The transcription start site (+1) based on the primer extension product was mapped to the A located 21 nucleotides upstream of the ATG codon (Fig. 3A). The putative −35 and −10 elements were identified

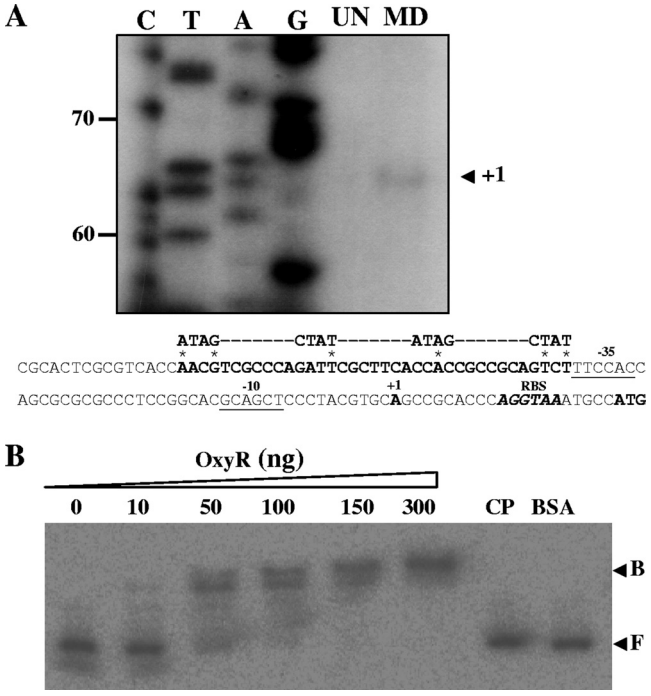

FIG. 3. Analysis of the *katG* promoter. (A) Primer extension was carried out as previously described (24). Reverse transcription was performed using the ^{32}P-labeled primer BT2652 and RNA extracted from the *X. campestris* pv. campestris wild type and the *oxyR* mutant either uninduced (UN) or induced with 100 μM menadione (MD). The extension products were separated on a 6% acrylamide-7 M urea sequencing gel. C, T, A, and G represent the DNA sequence ladder prepared using a PCR sequencing kit with labeled pUC/M13 forward primer and pGEM-3Zf (+) as the template (Applied Biosystems). Numbers to the left indicate fragment lengths. The arrowhead indicates the transcription start site (+1). Putative −35 and −10 elements are underlined, and the ribosome binding site is in italic font. The proposed OxyR binding site sequence is in boldface and correspondingly aligned with the *E. coli* consensus sequence for OxyR binding that is shown above the sequence line. The conserved bases are indicated by asterisks. The bold ATG represents the initiation codon. RBS indicates the putative ribosome binding site. (B) The gel mobility shift assay was done as described previously (8). Purified OxyR at the indicated concentrations was mixed with a ^{32}P-labeled 366-bp DNA fragment spanning the putative *katG* promoter that was prepared from PCR amplification using BT2637 and BT2652 primers in 25-μl binding reaction mixtures. The binding complexes were separated on a nondenaturing polyacrylamide gel. F and B indicate free and bound probes, respectively. CP represents the binding complexes that were treated with a cold probe. BSA indicates that bovine serum albumin was added to the binding reaction instead of purified OxyR.

as TTCCAC and GCAGCT, respectively, and were separated by a suboptimal distance of 20 nucleotides. Both motifs share low sequence identity to the proposed consensus σ^{70} binding sequences for *X. campestris* promoters that consist of the −35 element, TTGTNN, and the −10 element, T/AATNAA/T (10). The poor promoter sequence and structure of *katG* are a likely reason for a relatively low expression level of the gene. A putative OxyR binding site could be identified immediately upstream of the −35 element (Fig. 3A). This proposed binding site poorly matches (6 of 16) the consensus sequence of the *E. coli* OxyR binding site (ATAGN$_7$CTATN$_7$ATAGN$_7$CTAT) (28). This is quite surprising because we have shown that the putative *oxyR* binding sites in the *katA* and *ahpC* promoters in *X. campestris* pv. phaseoli highly match (10 of 16) the *E. coli* consensus sequence (5, 14). Nonetheless, in several microorganisms, the OxyR binding sequences of the target promoters are diverse and differ from the consensus sequence (20, 30).

We next tested the binding of *X. campestris* OxyR to the *katG* promoter fragment by using a gel mobility shift assay. Purified *X. campestris* pv. phaseoli OxyR (14), which shares 99% homology with *X. campestris* pv. campestris OxyR, was mixed with radioactively labeled *katG* promoter DNA fragments in the binding buffer. The results clearly illustrate that purified OxyR bound to the *katG* promoter fragment (Fig. 3B). The binding showed high specificity, as shown by competition experiments; only cold *katG* promoter DNA fragments could compete with the labeled probe in the binding reaction. The results support a notion that OxyR binds in the vicinity of the *katG* promoter and regulates its expression.

Conclusions. *X. campestris* pv. campestris has *katA*, *katG*, *katE*, and *ahpC* genes that are involved in the degradation of H_2O_2. A major puzzle in the overall bacterial peroxide stress response is whether these peroxide-scavenging genes are redundant or whether they serve disparate roles in the process. *katE* has no protective roles during the exponential phase of growth but is involved in protecting the bacteria from H_2O_2 in the stationary phase (29). The remaining three genes are regulated by OxyR and are presumably activated by similar concentrations of H_2O_2. Hence, the physiological roles of these genes could not be distinguished at the transcription activation level. Induction of three distinct types of peroxide-degrading enzymes enables the bacteria to detoxify H_2O_2 at a wide range of concentrations as well as other organic peroxides. AhpC is associated with the detoxification of low-concentration H_2O_2 generated from normal aerobic metabolism and organic hydroperoxides (25). At intermediate levels, KatG is better suited for detoxification of H_2O_2 at high micromolar levels. KatA, being the most abundant σ^{70} and also having a high apparent K_m, is responsible for protection against millimolar concentrations of H_2O_2.

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