CatB is Critical for Total Catalase Activity and Reduces Bactericidal Effects of Phenazine-1-Carboxylic Acid on *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*

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ABSTRACT

Rice bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae*, and rice bacterial leaf streak, caused by *X. oryzae* pv. *oryzicola*, are major diseases of rice. Phenazine-1-carboxylic acid (PCA) is a natural product that is isolated from *Pseudomonas* spp. and is used to control many important rice diseases in China. We previously reported that PCA disturbs the redox balance, which results in the accumulation of reactive oxygen species in *X. oryzae* pv. *oryzae*. In this study, we found that PCA significantly upregulated the transcript levels of *catB* and *katE*, which encode catalases, and that PCA sensitivity was reduced when *X. oryzae*

pvs. *oryzae* and *oryzicola* were cultured with exogenous catalase. Furthermore, *catB* deletion mutants of *X. oryzae* pvs. *oryzae* and *oryzicola* showed dramatically decreased total catalase activity, increased sensitivity to PCA, and reduced virulence in rice. In contrast, deletion mutants of *srpA* and *katG*, which also encode catalases, exhibited little change in PCA sensitivity. The results indicate that *catB* in both *X. oryzae* pvs. *oryzae* and *oryzicola* encodes a catalase that helps protect the bacteria against PCA-induced stress.

Additional keywords: total catalase activities.

Xanthomonas oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola*, the causal agents of rice bacterial leaf blight (BLB) and rice bacterial leaf streak (BLS), respectively, are widespread and important pathogens of rice. BLB and BLS can cause up to 75% yield loss in tropical regions of Asia (Ou 1985). Since 1955, a large number of antibiotics (penicillin, streptomycin, aureomycin, and others) have been used to control BLB and BLS but their application at high rates for long periods of time has resulted in phytotoxicity and in the development of resistance in *Xanthomonas* spp. (Arata et al. 1961). Therefore, novel bactericides are urgently needed for controlling BLB and BLS.

Phenazines are a large class of secondary metabolites produced by many bacterial species (Mavrodi et al. 2006; Zhou et al. 2016). Yoneyama et al. (1969) were the first to report that phenazines were effective in controlling BLB and had low phytotoxicity. Phenazine-1-carboxylic acid (PCA), a novel phenazine metabolite of *Pseudomonas aeruginosa* M18, was registered as “Shenqinmycin” in China against rice sheath blight. (Chen et al. 2012; Huang et al. 2016; Ye et al. 2010; Zhang et al. 2015). PCA was also used to control rice blast in Guangdong province of China (Zhou and Zhang 2016). In 2015, Xu et al. (2015) found that PCA was effective against not only various fungal plant pathogens but also against *X. oryzae* pv. *oryzicola* and especially against *X. oryzae* pv. *oryzae*. Moreover, PCA has already been used in field control of BLS in the rice-planting region of South China (Zhou et al. 2014).

The mechanism of action of phenazines is associated with their ability to undergo oxidation-reduction transformations that cause the accumulation of reactive oxygen species (ROS) in target cells

(Laursen and Nielsen 2004). ROS are a large group of highly reactive molecular forms of oxygen carrying unpaired electrons (Riley 1994). ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$). These are byproducts of cellular respiration or protein folding or are the end products of a number of metabolic reactions (Reczek and Chandel 2015; Toledano et al. 2010). Among these ROS species, O_2^- is the most active oxidant and can generate H_2O_2 in the presence of superoxide dismutase (Hertzberger et al. 2014; Wang et al. 2015). H_2O_2 is considered the most stable ROS species and can generate $OH^\bullet$ through the Fenton reaction (Lu et al. 2013; Pridmore et al. 2008).

Catalases are vital components of the detoxification pathways that convert H_2O_2 to water and molecular oxygen (Schouten et al. 2002). Catalases differ among bacterial species. Bacterial catalases have been classified into three types: monofunctional heme-containing catalases, bifunctional heme-containing catalase-peroxidases, and nonheme or Mn-containing catalases (Chelikani et al. 2004; Klotz et al. 1997). Each type of catalase has a different function at different phases of bacterial growth, and catalase levels differ depending on the nature of the environmental stress (Wang et al. 2013a). *X. oryzae* pv. *oryzae* has monofunctional heme-containing catalases, including three enzymes encoded by *catB*, *katE*, and *srpA*. *X. oryzae* pv. *oryzicola* has three genes that are homologous to the catalase genes of *X. oryzae* pv. *oryzae* and one other bifunctional heme-containing catalase-peroxidase encoded by *katG*.

Although the general mode of action of PCA against *X. oryzae* pv. *oryzae* has been described, the detailed interactions between PCA and the catalases of *X. oryzae* pvs. *oryzae* and *oryzicola* remain unclear (Xu et al. 2015). In this study, all of the catalases of *X. oryzae* pvs. *oryzae* and *oryzicola* were investigated under PCA-induced stress. For this purpose, we generated deletion mutants of all of the catalase genes of *X. oryzae* pvs. *oryzae* and *oryzicola* and determined the roles of these catalases in protecting the bacterial cells from PCA.

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and two supplementary tables are published online.

MATERIALS AND METHODS

Bacterial strains, plasmids, bactericides, and culture media. The bacterial strains and plasmids used in this study are listed in Table 1. *X. oryzae* pvs. *oryzae* and *oryzicola* strains were grown at 28°C in nutrient broth (NB) medium or on nutrient agar (NA) plates. NA medium consisted of beef extract at 3 g/liter, yeast extract at 1 g/liter, polypeptone at 5 g/liter, sucrose at 10 g/liter, and agar powder at 12 g/liter. NB medium contained the same components but lacked agar powder. Nutrient-limiting broth (MMX) medium (pH 7.0) was used as a minimal medium and was prepared with glucose at 5 g/liter, (NH₄)₂SO₄ at 2 g/liter, MgSO₄·7H₂O at 0.2 g/liter, K₂HPO₄ at 4 g/liter, KH₂PO₄ at 6 g/liter, and trisodium citrate at 1 g/liter. *Escherichia coli* DH5α was used for vector construction and was cultured in Luria-Bertani (LB) medium or on LB agar plates containing kanamycin at 50 µg/ml. PCA (98%) was provided by the Shanghai Nongle Biological Products Co., Ltd. and was dissolved in acetone as a stock solution.

Determination of transcript levels of catalase genes in *X. oryzae* pvs. *oryzae* and *oryzicola*. ZJ173 and RS105 were used as wild-type strains of *X. oryzae* pvs. *oryzae* and *oryzicola*, respectively. All wild-type strains and deletion mutants were cultured to different growth phases (as indicated by optical density at 600 nm [OD₆₀₀] values) in NB medium at 28°C. The bacterial cells were harvested by centrifugation (12,000 × *g* for 2 min at 4°C). Total RNA was extracted with the RNeasy pure cell/bacteria kit (TIANGEN Biotech) and was reverse transcribed into cDNA with the PrimeScript RT reagent Kit (TaKaRa) according to the instruction manual. Quantitative real-time polymerase chain reaction (qRT-PCR)

was conducted using SYBR Green supermix (Bio-Rad) with an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems). Transcript levels of *XOO0417* (*catB*, AAW73671), *XOO3423* (*katE*, AAW73701), and *XOO0447* (*srpA*, AAW76677) in wild-type and mutant *X. oryzae* pv. *oryzae* strains and *XOC_4325* (*catB*, AEQ98396), *XOC_1265* (*katE*, AEQ98362), *XOC_4290* (*srpA*, AEQ95453); and *XOC_3410* (*katG*, AEQ97503) in wild-type and mutant *X. oryzae* pv. *oryzicola* strains (gene names are according to the annotations of *X. oryzae* pv. *oryzae* strain KACC 10331 and *X. oryzae* pv. *oryzicola* strain BLS256) were assessed. 16S ribosomal RNA was used as the endogenous control. The primer sequences are listed in Supplementary Table S2.

Construction of a phylogenetic tree. The sequences of all catalases of *X. oryzae* pv. *oryzae* KACC 10331, *X. oryzae* pv. *oryzicola* BLS256, *X. campestris* pv. *campestris* ATCC 33913, and *X. axonopodis* pv. *citri* 306 were searched in the National Center for Biotechnology Information protein database (<http://www.ncbi.nlm.nih.gov/protein/>). A phylogenetic tree of all catalases aligned with CLUSTALW was constructed using the software MEGA 4.0 (Kumar et al. 2008).

Generation of mutants and complemented strains. To investigate the functions of catalase genes in *X. oryzae* pvs. *oryzae* and *oryzicola*, we generated all catalase deletion mutants via a nonmarker homologous recombination method (Yu et al. 2015). pK18*mobsacB* was used as a suicide vector in this study. The genomic DNA of *X. oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105 were used as the templates to amplify the upstream and downstream fragments of all catalase genes. The primers of all upstream and downstream fragments are listed in Supplementary Table S1. The recombinant plasmids listed in Table 1 were

TABLE 1. Strains and plasmids used in this study^z

Strains and plasmids	Characteristics	Source
Strains		
<i>Escherichia coli</i> DH5α	φ80 <i>lacZ</i> ΔM15, Δ(<i>lacZYA-argF</i>)U169. <i>recA</i> 1	Vazyme, Nanjing, China
ZJ173	Rif ^R , wild-type strain of <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	Laboratory collection
Δ <i>Z/catB</i>	Rif ^R , deletion of <i>catB</i> in wild-type strain ZJ173	This study
Δ <i>Z/katE</i>	Rif ^R , deletion of <i>katE</i> in wild-type strain ZJ173	This study
Δ <i>Z/srpA</i>	Rif ^R , deletion of <i>srpA</i> in wild-type strain ZJ173	This study
Δ <i>Z/catB/katE</i>	Rif ^R , double deletion of <i>catB</i> and <i>katE</i> in wild-type strain ZJ173	This study
Δ <i>Z/catB</i> (<i>catB</i>)	Rif ^R , Km ^R , complemented strain of Δ <i>Z/catB</i>	This study
Δ <i>Z/catB/katE</i> (<i>catB</i>)	Rif ^R , Km ^R , complemented strain of Δ <i>Z/catB/katE</i>	This study
RS105	Rif ^R , wild-type strain of <i>Xanthomonas oryzae</i> pv. <i>oryzicola</i>	This study
Δ <i>R/catB</i>	Rif ^R , deletion of <i>catB</i> in wild-type strain RS105	This study
Δ <i>R/katE</i>	Rif ^R , deletion of <i>katE</i> in wild-type strain RS105	This study
Δ <i>R/srpA</i>	Rif ^R , deletion of <i>srpA</i> in wild-type strain RS105	This study
Δ <i>R/katG</i>	Rif ^R , deletion of <i>katG</i> in wild-type strain RS105	This study
Δ <i>R/catB/katE</i>	Rif ^R , double deletion of <i>catB</i> and <i>katE</i> in wild-type strain RS105	This study
Δ <i>R/catB</i> (<i>catB</i>)	Rif ^R , Km ^R , complemented strain of Δ <i>R/catB</i>	This study
Δ <i>R/catB/katE</i> (<i>catB</i>)	Rif ^R , Km ^R , complemented strain of Δ <i>R/catB/katE</i>	This study
Plasmids		
pK18 <i>mobsacB</i>	Km ^R , allelic exchange suicide vector, <i>sacB</i> oriT(RP4)	Laboratory collection
pK18ZCB	Km ^R , a 1,598-bp fusion fragment of <i>ZcatB</i> -1, <i>ZcatB</i> -2gene ligated in pK18 <i>mobsacB</i>	This study
pK18ZKE	Km ^R , a 1,575-bp fusion fragment of <i>ZkatE</i> -1, <i>ZkatE</i> -2gene ligated in pK18 <i>mobsacB</i>	This study
pK18ZSA	Km ^R , a 1,520-bp fusion fragment of <i>ZsrpA</i> -1, <i>ZsrpA</i> -2gene ligated in pK18 <i>mobsacB</i>	This study
pK18RCB	Km ^R , a 1,841-bp fusion fragment of <i>RcatB</i> -1, <i>RcatB</i> -2gene ligated in pK18 <i>mobsacB</i>	This study
pK18RKE	Km ^R , a 1,584-bp fusion fragment of <i>RkatE</i> -1, <i>RkatE</i> -2gene ligated in pK18 <i>mobsacB</i>	This study
pK18RSA	Km ^R , a 1,414-bp fusion fragment of <i>RsrpA</i> -1, <i>RsrpA</i> -2gene ligated in pK18 <i>mobsacB</i>	This study
pK18RKG	Km ^R , a 1,332-bp fusion fragment of <i>RkatG</i> -1, <i>RkatG</i> -2gene ligated in pK18 <i>mobsacB</i>	This study
pHMI	Sp ^R , broad-host-range cosmid vector, pSa <i>ori</i>	Laboratory collection
pHMIZCB	Km ^R , <i>ZcatB</i> -H gene in ZJ173 ligated in pHMI	This study
pUFR034	<i>lncW</i> , Nm ^R , Km ^R , <i>Mob</i> ⁺ , <i>Mob</i> (<i>p</i>), <i>lacZ</i> α, PK2 replicon, cosmid	Laboratory collection
pUFRRCB	Km ^R , <i>RcatB</i> -H gene in RS105 ligated in pUFR034	This study

^z Rif^R, Km^R, Sp^R, and Nm^R indicate resistant to rifampicin, kanamycin, spectinomycin, and neomycin, respectively.

transformed into ZJ173 and RS105 competent cells using an electroporation method (Yu et al. 2015), and the cells were subsequently screened on plates of NA medium without sucrose containing kanamycin at 20 µg/ml. The colonies were transferred to 25 ml of NB medium without sucrose. After 7 h of incubation at 28°C, 120 µl of suspension was spread on plates of NA medium containing sucrose at 100 g/liter. The colonies were subsequently confirmed by PCR and Southern hybridization (Roche) using the primers listed in Supplementary Table S1. The confirmed mutants were used for further study. Complemented mutants were constructed using pHMI for *X. oryzae* pv. *oryzae* and pUFR034 for *X. oryzae* pv. *oryzicola*. All catalase genes (including predicted promoters) were amplified from the genomic DNA of strains ZJ173 and RS105 with the primers listed in Supplementary Table S1. Promoter prediction and electroporation were described in a previous study (Qian et al. 2013). The promoter was predicted with the website http://www.fruitfly.org/seq_tools/promoter.html.

Growth curves of deletion mutants in MMX medium and NB medium. Strains were cultured to midlogarithmic phase (OD₆₀₀ of approximately 1.0) in NB medium at 28°C with shaking at 175 rpm. Bacterial cells were collected by centrifugation (5,000 × g for 2 min) from 2 ml of culture suspension, and the cell pellets were resuspended in water. A 2-ml volume of cell suspension was then added to 100 ml of MMX medium or NB medium. Bacterial cells were grown at 28°C with shaking at 175 rpm. The OD₆₀₀ value was determined with an Eppendorf BioPhotometer Plus every 12 h in the MMX medium until the stationary stage. The OD₆₀₀ value was determined with same instrument every 4 h in NB medium until stationary stage.

PCA inhibition of *X. oryzae* pvs. *oryzae* and *oryzicola* as affected by addition of catalase. *X. oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105 were cultured to midlogarithmic phase (OD₆₀₀ of approximately 1.0) in NB medium at 28°C with shaking at 175 rpm. The OD₆₀₀ value of the bacterial suspension was adjusted to 0.1 with NB medium. A 100-µl volume of the bacterial suspension was added to 25 ml of NB medium, which was then supplemented with acetone or acetone containing PCA to achieve a final PCA concentration of 0.4 µg/ml in the ZJ173 culture or 16 µg/ml in the RS105 culture and a final acetone concentration of 0.4% (vol/vol). After that, 0, 10, 20, 40, or 80 U of catalase were added to the medium for ZJ173 and 0, 20, 40, 80, or 160 U of catalase were added to the medium for RS105. The cultures were kept at 28°C with shaking at 175 rpm, and OD₆₀₀ values were determined after 36 h. The effect of catalase on PCA inhibition of growth was expressed as a percentage of growth inhibition relative to a control that was not treated with PCA.

Determination of catalase activity. All wild-type strains and deletion mutants were cultured to early logarithmic phase (OD₆₀₀ of approximately 0.5) in NB medium. The bacterial cells were collected by centrifugation (8,000 × g for 2 min at 4°C) from 2 ml of bacterial cultures. The supernatant was removed, and 200 µl of Western and IP Cell Lysis Liquid (Beyotime) was added to resuspend the bacterial cell pellet. After 15 min of incubation, the supernatant was collected by centrifugation (8,000 × g for 2 min at 4°C). The protein concentrations were determined with the BCA Protein Concentration Determination Kit (Beyotime). Total catalase activities were then determined with the Catalase Test Kit (Beyotime).

Determination of H₂O₂ sensitivity. All wild-type strains and deletion mutants were cultured to midlogarithmic phase (OD₆₀₀ of approximately 1.0) in NB medium, and threefold- and ninefold-diluted suspensions were made with NB medium. NA plates containing 0, 0.1, or 0.25 mM H₂O₂ were also prepared. A 5-µl volume of the undiluted or diluted cultures of each strain was spotted onto NA plates (in triplicate), and the plates were kept for 36 h at 28°C (Lan et al. 2010). H₂O₂ sensitivity was assessed

based on colony growth. This experiment was repeated three times.

Determination of PCA sensitivity. All wild-type strains and deletion mutants were grown to midlogarithmic phase (OD₆₀₀ of approximately 1.0) in NB medium at 28°C with shaking at 175 rpm. The bacterial suspensions were diluted to OD₆₀₀ of approximately 0.25 by adding NB medium. A 120-µl volume of the diluted bacterial suspension was then added to 25 ml of fresh NB medium containing PCA at 0, 0.025, 0.05, 0.1, 0.2, 0.4, or 0.8 µg/ml (final acetone concentration 0.4% [vol/vol]) for *X. oryzae* pv. *oryzae* suspensions, and containing PCA at 0, 1, 2, 4, 8, 16, or 32 µg/ml (final acetone concentration 0.4% [vol/vol]) for *X. oryzae* pv. *oryzicola* suspensions. Three replicates for each concentration were used for each strain. Bacterial cells were grown at 28°C with shaking at 175 rpm for 36 h for *X. oryzae* pv. *oryzae* and 24 h for *X. oryzae* pv. *oryzicola* before OD₆₀₀ values were determined. For each strain, the average OD₆₀₀ values were used to calculate the PCA concentration that resulted in 50% inhibition of bacterial cell growth (EC₅₀). The EC₅₀ values were calculated with the Data Processing System computer program (Hangzhou Reifeng Information Technology Ltd.). Each experiment was done three times.

Virulence assays and determination of numbers of *X. oryzae* pvs. *oryzae* and *oryzicola* in rice plants. A pathogenicity assay was conducted in an illuminated growth incubator at 25°C with susceptible rice IR24. The bacterial suspensions were inoculated on rice according to a previous study (Yang and Bogdanove 2013), with slight modifications. *X. oryzae* pvs. *oryzae* and *oryzicola* strains were cultivated in NB medium at 28°C and 175 rpm to approximately 10⁹ CFU/ml for inoculation. Six-week-old rice leaves were clipped with sterile scissors that had been dipped in bacterial suspensions of *X. oryzae* pv. *oryzae* strains. Leaves of 8-week-old rice plants were inoculated with *X. oryzae* pv. *oryzicola* strains by piercing the leaves with a needle that had been dipped in the bacterial suspension. Two leaves in each of 10 replicate plants were inoculated for each strain. Lesion lengths were measured 10 days postinoculation (dpi). Each plant represented a biological replicate. The average number of bacterial CFU per square centimeter of rice leaf tissue was determined immediately after inoculation of each *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strain and at 3 dpi. Samples (2 cm²) from the inoculation sites of three leaves were cut and surface sterilized in 5% H₂O₂. Each leaf represented a biological replicate. The samples were ground in 1 ml of sterile water and CFU numbers were determined following plating of serially diluted samples.

Data analysis. All analyses were conducted using SPSS 20.0 (SPSS Inc.). Statistical differences of gene expression, catalase activity, virulence assays, and bacterial numbers in planta were detected with independent-sample *t* tests. Inhibition of growth by PCA and PCA sensitivity of *X. oryzae* pvs. *oryzae* and *oryzicola* were analyzed with one-way analysis of variance (Fisher's least significant difference test).

RESULTS

Transcript levels of all catalase genes in *X. oryzae* pvs. *oryzae* and *oryzicola* at different growth phases. Transcript levels of catalase genes in *X. oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105 differed among genes and growth phases in NB medium (Fig. 1). When the OD₆₀₀ value increased to approximately 1 for *X. oryzae* pv. *oryzae*, the transcript levels increased twofold for *katE* but had not significantly changed for *srpA* (Fig. 1A). Surprisingly, the transcript levels of *catB* in *X. oryzae* pv. *oryzae* were the highest at OD₆₀₀ of approximately 0.2 and thereafter rapidly decreased (Fig. 1A). In *X. oryzae* pv. *oryzicola*, the transcript levels of *catB* and *katE* were lowest at OD₆₀₀ of approximately 0.2 and then increased 1.2- to 3.0-fold at later growth

phases (Fig. 1B). Transcript levels of *srpA* and *katG* in *X. oryzae* pv. *oryzicola* were the lowest at OD₆₀₀ of approximately 0.4 (Fig. 1B).

Effect of exogenous PCA on transcript levels of catalase genes in *X. oryzae* pvs. *oryzae* and *oryzicola*. qRT-PCR revealed that, in *X. oryzae* pv. *oryzae* ZJ173, *catB* and *katE* were 13.8- and 4.2-fold upregulated, respectively, in response to exogenous PCA (Fig. 2A). In *X. oryzae* pv. *oryzicola* RS105, *catB* and *katE* were 5.5- and 2.1-fold upregulated, respectively, in response to exogenous PCA (Fig. 2B). In contrast, transcript levels of *srpA* were unchanged by exogenous PCA in both strains (Fig. 2A and B), and transcript levels of *katG* were unchanged in *X. oryzae* pv. *oryzicola* (Fig. 2B).

PCA inhibition and catalase transcript levels of *X. oryzae* pvs. *oryzae* and *oryzicola* as affected by exogenous catalase. PCA inhibition of growth in culture of *X. oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105 was reduced by addition of catalase (Fig. 3A and B). In addition, the transcript levels of *catB* and *katE* induced by PCA treatment were reduced by addition of catalase (Fig. 3C and D).

Sequence analysis, deletion, and complementation of catalase genes in *X. oryzae* pvs. *oryzae* and *oryzicola*. The catalase family consists of *catB* (XOO0417), *katE* (XOO3423), and *srpA* (XOO0447) in *X. oryzae* pv. *oryzae* and *catB* (XOC_4325), *katE* (XOC_1265), *srpA* (XOC_4290), and *katG* (XOC_3410) in

X. oryzae pv. *oryzicola* (gene numbers are according to the annotations of *X. oryzae* pv. *oryzae* KACC 10331 and *X. oryzae* pv. *oryzicola* BLS256, respectively). *catB*, *katE*, and *srpA* are highly conserved in *X. oryzae* pvs. *oryzae* and *oryzicola* (Supplementary Fig. S1). These genes are present at different locations in the genome of *X. oryzae* pvs. *oryzae* and *oryzicola* (<http://www.genome.jp/kegg/>).

To investigate the functions of catalase genes in *X. oryzae* pvs. *oryzae* and *oryzicola* under PCA-induced stress, we generated targeted deletion mutants by a nonmarker homologous recombination method in the background of strains ZJ173 and RS105, respectively. Deletion strains were verified by PCR using different primer combinations (Supplementary Fig. S2A). Southern blot analyses with 500- to 800-bp DNA fragment probes confirmed the deletion mutants (Supplementary Fig. S2B). In addition, qRT-PCR analysis of RNA extracted from the mutants showed that they had completely lost expression of the target transcripts (Fig. 4). $\Delta Z/catB$ and $\Delta Z/catB/katE$ mutants of *X. oryzae* pv. *oryzae* ZJ173 and $\Delta R/catB$ and $\Delta R/catB/katE$ mutants of *X. oryzae* pv. *oryzicola* RS105 were complemented with plasmids pHMI and pUFR034 carrying the open reading frames of *catB* and its predicted promoter of ZJ173 and RS105, respectively. Complementation of the mutants was confirmed by PCR (Supplementary Fig. S2C).

Fig. 1. Transcript levels of all catalase genes at different growth phases in **A**, *Xanthomonas oryzae* pv. *oryzae* strain ZJ173 and **B**, *X. oryzae* pv. *oryzicola* strain RS105 as determined by quantitative real-time polymerase chain reaction analysis. RNA extracts were obtained from nutrient broth cultures at optical density at 600 nm (OD₆₀₀) = 0.2, 0.4, 0.6, 0.8, and 1.0 and 28°C. Columns and vertical bars indicate the means and standard errors of three independent experiments. Each independent experiment included three biological replicates and three technical replicates. Similar results were obtained from three independent experiments. Asterisks indicate a significant difference relative to the expression at OD₆₀₀ of approximately 0.2 in independent-sample *t* tests (*P* < 0.05).

Fig. 2. Transcript levels of catalase genes in **A**, *Xanthomonas oryzae* pv. *oryzae* strain ZJ173 and **B**, *X. oryzae* pv. *oryzicola* strain RS105 in response to exogenous phenazine-1-carboxylic acid (PCA) (1 µg/ml for ZJ173 and 64 µg/ml for RS105) as determined by quantitative real-time polymerase chain reaction analysis. RNA extracts were obtained from nutrient broth cultures at 28°C containing PCA + acetone, acetone alone, or neither (control). Columns and vertical bars indicate the means and standard errors of three independent experiments. Each independent experiment included three biological replicates and three technical replicates. Similar results were obtained from three independent experiments. Asterisks indicate a significant difference relative to control in independent-sample *t* tests (*P* < 0.05).

Growth of catalase deletion and complemented mutants.

The growth rates of all mutants were determined in MMX and NB media. The *X. oryzae* pv. *oryzae* ZJ173 mutants $\Delta Z/catB$ and $\Delta Z/catB/katE$ could not grow in MMX (Fig. 5A). The *X. oryzae* pv. *oryzicola* RS105 mutant $\Delta R/catB/katE$ could not grow in MMX medium, and the $\Delta R/catB$ mutant grew slowly in this medium (Fig. 5B). The other deletion mutants of both strains grew normally in MMX medium. If growth of deletion mutants in MMX medium was reduced, it was restored by complementation (Fig. 5A and B). All catalase deletion mutants exhibited a growth rate similar to that of the wild-type strains in NB medium (Fig. 5C).

Transcript levels of catalase genes in *X. oryzae* pvs. *oryzae* and *oryzicola* deletion mutants. As expected, expression of the catalase genes was not detected in mutant strains deleted in the corresponding genes (Fig. 4). Deletion of a catalase gene caused transcriptional changes of other catalase genes in NB medium. *catB* deletion increased *katE* expression levels by 8.6-fold in *X. oryzae* pv. *oryzae* ZJ173 and by 3.9-fold in *X. oryzae* pv. *oryzicola* RS105. In *X. oryzae* pv. *oryzae* ZJ173 mutant $\Delta Z/katE$, *catB* transcription was upregulated by 1.5-fold, whereas *srpA* transcription was downregulated by 1.2-fold. In *X. oryzae* pv. *oryzicola* RS105 mutant $\Delta R/katE$, *catB* and *srpA* transcriptions were upregulated by 1.4- and 1.3-fold, respectively, whereas *katG* transcription was downregulated by 2-fold. In *X. oryzae* pv. *oryzae* ZJ173 mutant $\Delta Z/srpA$ and *X. oryzae* pv. *oryzicola* RS105 mutant $\Delta R/srpA$, *catB* transcription was slightly upregulated, whereas *katE* and *katG* transcription was slightly downregulated. *katG* deletion in *X. oryzae* pv. *oryzicola* RS105 had little influence on other genes.

katE transcription was upregulated by 2.2-fold in *X. oryzae* pv. *oryzae* ZJ173 mutant $\Delta Z/catB/katE$ compared with *X. oryzae* pv. *oryzae* ZJ173 and by 1.7-fold in *X. oryzae* pv. *oryzicola* RS105 mutant $\Delta R/catB/katE$ compared with *X. oryzae* pv. *oryzicola* RS105.

Catalase activity in mutants and complemented strains of *X. oryzae* pvs. *oryzae* and *oryzicola*. Catalase activity was significantly lower in *X. oryzae* pv. *oryzae* ZJ173 mutants $\Delta Z/catB$ and $\Delta Z/catB/katE$ than in the wild-type strain (Fig. 6A). Similarly, catalase activity was significantly lower in *X. oryzae* pv. *oryzicola* RS105 mutants $\Delta R/catB$ and $\Delta R/catB/katE$ than in wild-type strain RS105 (Fig. 6B). In contrast, the catalase activity of the other deletion and complemented mutants did not significantly differ from the activity in the corresponding wild-type strains.

Effects of catalase genes on *X. oryzae* pvs. *oryzae* and *oryzicola* sensitivity to H_2O_2 or PCA. $\Delta Z/catB$ and $\Delta Z/catB/katE$ mutants were more sensitive to H_2O_2 than wild-type *X. oryzae* pv. *oryzae* ZJ173 (Fig. 7A). As expected, tolerance to H_2O_2 was partially restored in the complemented mutants $\Delta Z/catB$ (*catB*) and $\Delta Z/catB/katE$ (*catB*). Similarly, $\Delta R/catB$ and $\Delta R/catB/katE$ mutants were more sensitive to H_2O_2 than wild-type *X. oryzae* pv. *oryzicola* RS105. Colony growth of mutants $\Delta R/catB$ and $\Delta R/catB/katE$ was also inhibited when H_2O_2 concentration reached 0.25 mM (Fig. 7B).

To further verify whether the disruption of catalase genes increases PCA sensitivity in *X. oryzae* pvs. *oryzae* and *oryzicola*, we performed bacterial sensitivity tests. EC_{50} values were significantly lower for all *catB* deletion mutants than for the wild-type strains of

Fig. 3. Phenazine-1-carboxylic acid (PCA) inhibition of growth of **A**, *Xanthomonas oryzae* pv. *oryzae* strain ZJ173 and **B**, *X. oryzae* pv. *oryzicola* strain RS105 as affected by exogenous catalase. Strains were grown in nutrient broth containing a PCA concentration that inhibited growth by 65% (0.4 μg/ml for ZJ173 and 16 μg/ml for RS105) and that also contained the indicated units of catalase. After 36 h, reduction in PCA inhibition by catalase addition was assessed based on OD_{600} values. Columns and vertical bars indicate the means and standard errors for three biological replicates. Statistically significant differences are noted as different letters according to analysis of Fisher's least significant difference test ($P < 0.05$). Transcript levels of catalase genes in **C**, *X. oryzae* pv. *oryzae* strain ZJ173 treated with PCA at 0.4 μg/ml and **D**, *X. oryzae* pv. *oryzicola* strain RS105 treated with PCA at 16 μg/ml in response to exogenous catalase as determined by quantitative real-time polymerase chain reaction analysis. Columns and vertical bars indicate the means and standard errors of three independent experiments. Each independent experiment included three biological replicates and three technical replicates. Similar results were obtained from three independent experiments. Asterisks indicate a significant difference from 0.4 μg/ml PCA in C and 16 μg/ml in D without any exogenous catalase in independent-sample *t* tests ($P < 0.05$).

Fig. 4. Relative transcript levels of catalase genes in **A**, *Xanthomonas oryzae* pv. *oryzae* ZJ173 and **B**, *X. oryzae* pv. *oryzicola* strain RS105 and their catalase deletion mutants, as determined by quantitative real-time polymerase chain reaction analysis. NB = nutrient broth. Columns and vertical bars indicate the means and standard errors of three independent experiments. Each independent experiment included three biological replicates and three technical replicates. Similar results were obtained from three independent experiments. Asterisks indicate a significant difference relative to the corresponding wild-type strains in independent-sample *t* tests ($P < 0.05$).

Fig. 5. Growth of wild-type strains, catalase deletion mutants, and complemented mutants of *Xanthomonas oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105 in a nutrient-poor medium (MMX) and a nutrient-rich medium (NB), as indicated by optical density at 600 nm (OD₆₀₀). **A**, Growth rate in MMX of the *X. oryzae* pv. *oryzae* ZJ173; the catalase deletion mutants $\Delta Z/catB$, $\Delta Z/katE$, $\Delta Z/srpA$, and $\Delta Z/catB/katE$; and their complemented mutants $\Delta Z/catB(catB)$ and $\Delta Z/catB/katE(catB)$. **B**, Growth rate in MMX of the *X. oryzae* pv. *oryzicola* RS105; the catalase deletion mutants $\Delta R/catB$, $\Delta R/katE$, $\Delta R/srpA$, $\Delta R/katG$, and $\Delta R/catB/katE$; and their complemented mutants $\Delta R/catB(catB)$ and $\Delta R/catB/katE(catB)$. **C**, Growth rate in NB of *X. oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105, their catalase deletion mutants, and their complemented mutants.

Fig. 6. Total catalase activity from total crude protein extract of **A**, *Xanthomonas oryzae* pv. *oryzae* ZJ173 and **B**, *X. oryzae* pv. *oryzicola* RS105, and their catalase deletion and complemented mutants. Columns and vertical bars indicate the means and standard errors of three independent experiments. Each independent experiment included three biological replicates and three technical replicates. Similar results were obtained from three independent experiments. Asterisks indicate a significant difference relative to the corresponding wild-type strains in independent-sample *t* tests ($P < 0.05$).

X. oryzae pvs. *oryzicola* and *oryzae* (ZJ173 and RS105, respectively) (Table 2). In contrast, EC_{50} values of mutants impaired in other catalase genes were slightly lower but did not significantly differ from the EC_{50} values of the corresponding wild-type strains.

Virulence of *X. oryzae* pvs. *oryzae* and *oryzicola* catalase mutants and complemented strains on rice. Relative to the virulence of *X. oryzae* pv. *oryzae* ZJ173, the virulence of mutants $\Delta Z/catB$ and $\Delta Z/catB/katE$ was significantly reduced, whereas the other mutants of ZJ173 were not affected in this feature under tested conditions (Fig. 8A and B). Similarly, the virulence of the *X. oryzae* pv. *oryzicola* RS105 mutants $\Delta R/catB$ and $\Delta R/catB/katE$ was dramatically reduced, whereas the other mutants of RS105 were not affected in virulence under tested conditions (Fig. 8C and D).

At 3 dpi, bacterial numbers of mutants $\Delta Z/catB$ and $\Delta Z/catB/katE$ were significantly lower than those of wild-type ZJ173 and the complemented strains (Fig. 8E). Similarly, at 3 dpi, bacterial numbers of mutants $\Delta R/catB$ and $\Delta R/catB/katE$ were also lower than those of wild-type RS105 and the complemented mutants (Fig. 8F).

TABLE 2. Sensitivity of *Xanthomonas oryzae* pvs. *oryzae* and *oryzicola* strains to phenazine-1-carboxylic acid

Strain	EC_{50} ($\mu\text{g/ml}$) ^z
ZJ173	0.181 ± 0.0155 a
$\Delta Z/catB$	0.132 ± 0.0349 b
$\Delta Z/katE$	0.179 ± 0.0120 a
$\Delta Z/srpA$	0.156 ± 0.0158 a
$\Delta Z/catB/katE$	0.133 ± 0.0412 b
$\Delta Z/catB(catB)$	0.168 ± 0.0212 a
$\Delta Z/catB/katE(catB)$	0.162 ± 0.0158 a
RS105	13.112 ± 0.192 a
$\Delta R/catB$	8.342 ± 0.581 b
$\Delta R/katE$	10.881 ± 1.081 a
$\Delta R/srpA$	10.212 ± 1.234 a
$\Delta R/katG$	10.325 ± 0.788 a
$\Delta R/catB/katE$	8.280 ± 1.024 b
$\Delta R/catB(catB)$	12.451 ± 1.564 a
$\Delta R/catB/katE(catB)$	11.032 ± 1.111 a

^z Values are means ± standard errors of three biological independent experiments. Similar results were obtained from three biological independent experiments. Means in a column followed by different letters are significantly different according to Fisher's least significant difference test ($P < 0.05$).

Fig. 7. Effects of catalase genes on H_2O_2 sensitivity of **A**, *Xanthomonas oryzae* pv. *oryzae* ZJ173 and **B**, *X. oryzae* pv. *oryzicola* RS105, and their catalase deletion and complemented mutants in nutrient broth (NB) medium. On the left side, 1x, 3x, and 9x refer to the fold-dilution of the bacterial suspensions that were added to NB medium.

The other mutants did not differ in bacterial numbers from their corresponding wild-type strains at 3 dpi.

DISCUSSION

Phenazine derivatives of redox-active compounds from *P. aeruginosa* were previously reported to alter the oxidative metabolism or to increase the formation of ROS in *E. coli* (Hassan and Fridovich 1979). PCA, a secondary metabolite of *P. aeruginosa*, increased oxidant accumulation and altered gene expression, including the expression of genes encoding extracellular catalase in human airway epithelial cells (Look et al. 2005). Xu et al. (2015) also found that PCA increased the accumulation of ROS and reduced catalase activity in *X. oryzae* pv. *oryzae*. However, a detailed understanding of the antioxidative functions of catalases in *X. oryzae* pvs. *oryzae* and *oryzicola* has not been well documented.

Most bacteria have various catalases that are encoded by different genes and that function at different growth phases or under different environmental conditions, suggesting that they may be independent of each other and may differ in phylogeny, structure, and function (Switala et al. 1990; Wang et al. 2013a). The results of the current study indicate that *catB* is a critical catalase gene in *X. oryzae* pvs. *oryzae* and *oryzicola*. Deletion of *catB* resulted in increased sensitivity to H_2O_2 and PCA and reduced virulence in both bacteria.

The transcript levels of *catB* and *katE* in *X. oryzae* pvs. *oryzae* and *oryzicola* differed greatly depending on growth phase (Fig. 1), indicating that *catB* and *katE* encode the catalases that are largely responsible for H_2O_2 scavenging during the growth of *X. oryzae* pvs. *oryzae* and *oryzicola*. The expression of *catB* was down-regulated in *X. oryzae* pv. *oryzae* but was upregulated in *X. oryzae* pv. *oryzicola* after the lag phase, which may explain why total catalase activity was lower for *X. oryzae* pv. *oryzae* than for *X. oryzae* pv. *oryzicola* (Fig. 6). We also found that *X. oryzae* pv. *oryzicola* was less sensitive to PCA than *X. oryzae* pv. *oryzae*, which suggested that catalase activity may be closely related to PCA sensitivity. In response to exogenous PCA, expression of *catB* and *katE* was significantly increased in *X. oryzae* pvs. *oryzae* and *oryzicola* (Fig. 2). Moreover, other oxidative stress agents such as clofazimine and paraquat could also increase the expression of *catB* and *katE* in *X. oryzae* pvs. *oryzae* and *oryzicola* (Supplementary Fig. S3). We inferred that PCA increased the intracellular ROS levels and thereby induced expression of genes involved in cell detoxification, which partially agrees with previous report regarding the mechanism by which PCA inhibits *Phellius noxius* (Huang et al. 2016). In support of this inference, addition of catalase to the growth medium reduced PCA sensitivity and suppressed the increased expression of catalase in response to PCA in *X. oryzae* pvs. *oryzae* and *oryzicola* (Fig. 3).

Fig. 8. Virulence of *Xanthomonas oryzae* pv. *oryzae* ZJ173, *X. oryzae* pv. *oryzicola* RS105, and their catalase deletion and complemented mutants on IR24 rice. **A** and **C**, Representative images of rice leaves inoculated with the indicated strains at 10 days postinoculation (dpi). **B** and **D**, Length of lesions caused by all tested strains on rice leaves at 10 dpi. Columns and vertical bars indicate the means and standard errors of 10 biological replicates. Asterisks indicate relative to the corresponding wild-type strains in independent-sample *t* tests ($P < 0.05$). **E** and **F**, Bacterial numbers in leaves at 0 and 3 dpi. In **B**, **D**, **E** and **F**, columns and vertical bars indicate the means and standard errors of three biological replicates. Asterisks indicate relative to the corresponding wild-type strains in independent-sample *t* tests ($P < 0.05$).

The functions of catalases differ among organisms. *katA* is the major catalase gene induced in *P. aeruginosa* in response to H₂O₂ (Heo et al. 2010). Catalase KatE performs compensatory roles in H₂O₂ metabolism in *Brucella abortus* (Steele et al. 2010). Catalases CatA and CatB are active at different stages of the *Aspergillus nidulans* life cycle (Kawasaki et al. 1997). In our study, we found that *catB* was more important than *katE* in *X. oryzae* pvs. *oryzae* and *oryzicola*. Deletion of *catB* reduced the growth rate, while double deletion of *catB* and *katE* completely inhibited the growth of *X. oryzae* pvs. *oryzae* and *oryzicola* in MMX medium (Fig. 5). These results suggested that catalase activity influences the growth of *X. oryzae* pvs. *oryzae* and *oryzicola* only under adverse conditions. Therefore, we used NB medium in subsequent experiments to minimize the effects of the mutations on growth.

Knockout of *catB* caused transcript levels of *katE* to be upregulated in *X. oryzae* pvs. *oryzae* and *oryzicola*. Relative to the wild-type strains, however, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* mutants $\Delta Z/katE$ and $\Delta R/katE$, respectively, showed no significant change in catalase activity or sensitivity to H₂O₂ or PCA, suggesting that, in these bacteria, *katE* is not the major gene responsible for catalase activity, which is consistent with a previous finding for *X. oryzae* pv. *oryzae* strain PXO99 (Wang et al. 2013b). We also found that the double deletion mutants of *X. oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105, $\Delta Z/catB/katE$ and $\Delta R/catB/katE$, respectively, did not significantly differ in catalase activity or sensitivity to H₂O₂ or PCA compared with the mutants impaired only in *catB*, further confirming that *katE* is not the major gene responsible for catalase activity in these bacteria. The combined results concerning the growth of mutants $\Delta Z/catB$, $\Delta R/catB$, $\Delta Z/catB/katE$, and $\Delta R/catB/katE$ in MMX medium suggest that *catB* may act as a repressor of *katE* and that *katE* could help compensate for loss of *catB* in growth; however, this remains to be determined.

In a previous study, H₂O₂ toxicity was increased in *Shewanella oneidensis* when *katB*, a heme-containing catalase involved in H₂O₂ degradation, was inactivated (Wu et al. 2015). In our study, inactivation of *catB* caused a 72% reduction in total catalase activity in *X. oryzae* pv. *oryzae* and an 83% reduction in *X. oryzae* pv. *oryzicola* (Fig. 6). Inactivation of *catB* increased the sensitivity of *X. oryzae* pvs. *oryzae* and *oryzicola* to H₂O₂ (Fig. 7) and PCA (Table 2), indicating that *catB* rather than *katE* protects against H₂O₂ and PCA in these bacteria. These results are consistent with a previous report showing that expression of *catB* from *Deinococcus radiodurans* in *E. coli* UM2 restored resistance to low concentrations of H₂O₂ (Zheng et al. 2000). Complementation of mutants $\Delta Z/catB/katE$ and $\Delta R/catB/katE$ with corresponding *catB* genes from strains ZJ173 and RS105, respectively, restored the wild-type phenotypes, confirming that *catB* is responsible for most of the catalase activity that reduces oxidative stress in *X. oryzae* pvs. *oryzae* and *oryzicola*.

Virulence and bacterial numbers were reduced in mutants $\Delta Z/catB$ and $\Delta R/catB$ relative to the wild-type strains, and were further reduced in $\Delta Z/catB/katE$ and $\Delta R/catB/katE$ (Fig. 8). These results indicate that *catB* is critical for the virulence and proliferation of *X. oryzae* pvs. *oryzae* and *oryzicola* in rice leaves. In citrus, the monofunctional catalase KatE of *Xanthomonas axonopodis* pv. *citri* is required for full virulence (Tondo et al. 2010). Virulence of *X. oryzae* pvs. *oryzae* and *oryzicola* may be partially dependent on growth because the reduced virulence of the mutants was associated with their reduced growth in rice leaves. Another key factor associated with the reduced virulence of $\Delta Z/catB$, $\Delta R/catB$, $\Delta Z/catB/katE$, and $\Delta R/catB/katE$ could be ROS intolerance caused by the deregulation of the detoxification system. Without *catB*, catalase activity is likely reduced, making the mutants more susceptible to the ROS burst.

In summary, we found that *catB* plays a crucial role in the pathogenicity of *X. oryzae* pvs. *oryzae* and *oryzicola* and in their ability to survive oxidative stress. The results also provide insight into how PCA affects *X. oryzae* pvs. *oryzae* and *oryzicola*. Finally,

our results indicate that *X. oryzae* pvs. *oryzae* and *oryzicola* differ greatly in catalase activity and sensitivity to PCA and H₂O₂.

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