Contribution of OxyR Towards Differential Sensitivity to Antioxidants in *Xanthomonas oryzae* pathovars *oryzae* and *oryzicola*

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Accepted 12 June 2018.

OxyR and SoxR are two transcriptional regulators in response to oxidative stress in most bacteria, and SoxR has been reported to be activated by the endogenous redox-cycling compound phenazine in phenazine-producing organisms. However, which transcriptional regulator is activated in pathogens treated with the antibiotic phenazine-1-carboxylic acid (PCA) has not been determined. In this study, we found that PCA treatment activated OxyR rather than SoxR in the phytopathogenic bacteria *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. We also found that *X. oryzae* pv. *oryzae* was much more sensitive to PCA and H₂O₂ and had a defective antioxidant system (i.e., less of total antioxidant capacity and total catalase activity than *X. oryzae* pv. *oryzicola*, although *X. oryzae* pvs. *oryzae* and *oryzicola* are very closely related). Based on KEGG sequences, OxyR differs in 10 amino acids in *X. oryzae* pv. *oryzae* versus *X. oryzae* pv. *oryzicola*. By exchanging OxyR between *X. oryzae* pvs. *oryzae* and *oryzicola*, we elucidated that OxyR contributed to the differences in antioxidant capacity, total catalase activity, and sensitivity to PCA and H₂O₂. We also found that OxyR affected *X. oryzae* pvs. *oryzae* and *oryzicola* growth in a nutrient-poor medium, virulence on host plants (rice), and the hypersensitive response on nonhost plants (*Nicotiana benthamiana*). Thus, OxyR is a critical regulator that relates to the differences in antioxidative stress between *X. oryzae* pvs. *oryzae* and *oryzicola* and contributes to the differences in survival of them against oxidative stress.

Xanthomonas oryzae pv. *oryzae* (the causal agent of rice bacterial leaf blight) and *X. oryzae* pv. *oryzicola* (the causal agent of rice bacterial leaf streak) are two gram-negative phytopathogenic bacteria (Swings et al. 1990). Similar to many other plant pathogens, *X. oryzae* pvs. *oryzae* and *oryzicola* must overcome oxidative stress induced by oxidants in the environments or generated by plant cells during infection

(Aznar et al. 2015; Breitenbach et al. 2015; Fones and Preston 2012). Phenazine-1-carboxylic acid (PCA), a secondary metabolite secreted by *Pseudomonas* spp. and several plant-associated bacteria (*Pectobacterium*, *Streptomyces*, *Burkholderia*, *Brevibacterium*, and others) (Jaaffar et al. 2017; Morales et al. 2010; Saleh et al. 2009; Thomashow et al. 1990), is found to be effective against *X. oryzae* pvs. *oryzae* and *oryzicola* by stimulating the formation of reactive oxygen species (ROS) in cells in the presence of oxygen (Pan et al. 2017; Wang and Newman 2008; Xu et al. 2015). ROS such as hydrogen peroxide (H₂O₂), the superoxide anion radical (O₂^{•-}), and the hydroxyl radical (OH[•]) (Imlay 2015a,b) can damage nucleic acids, proteins, and lipids, ultimately resulting in cell death (Cabiscol et al. 2000). In order to avoid ROS-induced damage, bacteria have evolved several defense mechanisms. In prokaryotes, the primary defense mechanism involves scavenging enzymes systems, including catalase (CAT), alkyl hydroperoxide reductase (Ahp) subunits CF (AhpCF), and thioredoxin (Trx), which scavenge H₂O₂; and superoxide dismutase (SOD), which scavenges O₂^{•-} (Cooke et al. 2003; Vázquez-Torres 2012; Xia et al. 2017).

Two key transcriptional regulators, SoxR and OxyR, have been well known to regulate the enzymes in the antioxidant systems in response to oxidative stress (Sporer et al. 2017; Storz et al. 1990). SoxR is activated in response to O₂^{•-} and, in turn, includes the transcription of target genes, including SOD (Pomposiello and Demple 2001; Seo et al. 2015; Singh et al. 2013). In contrast, OxyR is activated in response to H₂O₂ and, in turn, activates CAT, Trx, and AhpCF (Imlay 2015b; Seo et al. 2015). In phenazine-producing organisms, SoxR and OxyR have been researched widely. It is reported that SoxR can be directly activated by phenazines in *Pseudomonas aeruginosa* (Dietrich et al. 2008). PCA can alter the transcript levels of an efflux pump gene through SoxR in *P. aeruginosa* M18 (Du et al. 2015). OxyR is also important for defense against oxidative stress in *P. aeruginosa* (Vinckx et al. 2010). However, there have been few reports concerning the responses of SoxR or OxyR in phytopathogenic bacteria *X. oryzae* pvs. *oryzae* and *oryzicola* against PCA.

As two pathovars of *X. oryzae*, *oryzae* and *oryzicola* are very similar in the size of genome and gene content (Seo et al. 2008), and showed almost similar sensitivity to streptomycin (Xu et al. 2010). However, *X. oryzae* pv. *oryzae* is found to be much more sensitive than *X. oryzae* pv. *oryzicola* to PCA (Xu et al. 2015). This may be caused by different CAT activities in both bacteria as noted in our previous study (Pan et al. 2017; Xu et al. 2015) but the detailed mechanism needs to be exposed. Interestingly,

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Funding: This research was supported by the Key Program of National Natural Science Foundation of China (31730072) and the Fundamental Research Funds for the Central Universities (KYTZ201604).

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

CAT is regulated by OxyR in most bacteria, including *X. oryzae* pv. *oryzae* (Heo et al. 2009; Kong et al. 2004; Seo et al. 2015; Tseng et al. 2003; Yu et al. 2016), which raises the possibility that OxyR in phytopathogenic bacteria *X. oryzae* pvs. *oryzae* and *oryzicola* may be involved in regulating the enzymes in the antioxidant systems in response to PCA.

In this study, we deleted and exchanged the gene *oxyR*, which encodes the transcriptional regulator OxyR in *X. oryzae* pvs. *oryzae* and *oryzicola*, and investigated the roles of OxyR in these two bacteria in response to PCA. We also revealed the differences in OxyR involved in the differences of the two bacteria in antioxidant capacity, total CAT activity, and sensitivity to H₂O₂. These results should help us shed light on the different oxidative stress response in *X. oryzae* pvs. *oryzae* and *oryzicola* and contribute to understanding the roles of OxyR in physiology and pathogenesis in these two bacteria.

RESULTS

Sensitivity of *X. oryzae* pvs. *oryzae* and *oryzicola* to phenazines, bismethiazol, captan, and streptomycin on *X. oryzae* pvs. *oryzae* and *oryzicola*.

In the sensitivity tests, we found that *X. oryzae* pv. *oryzicola* was much less sensitive than *X. oryzae* pv. *oryzae* to the phenazines PCA, phenazine (PHZ), and 1-hydroxyphenazine (1-OH-PHZ) (Table 1). However, *X. oryzae* pvs. *oryzae* and *oryzicola* did not differ in sensitivity to bismethiazol or captan and differed only to a small (but statistically significant, $P < 0.05$) degree in sensitivity to the antibiotic streptomycin (Table 1). The concentration of PCA that resulted in 50% inhibition of bacterial cell growth (EC₅₀) values of bismethiazol against *X. oryzae* pvs. *oryzae* and *oryzicola* were 12.83 ± 1.34 and 16.27 ± 1.06 µg/ml, respectively, and those of captan were 0.063 ± 0.0169 and 0.091 ± 0.0092 µg/ml, respectively.

PCA activates OxyR and induces gene expression of CAT, Trx, and AhpCF in *X. oryzae* pvs. *oryzae* and *oryzicola*.

In an earlier study, PCA was found to activate SoxR in phenazine-producing pathogens but induced the gene expression of CAT (*catB* and *katE*) in *X. oryzae* pvs. *oryzae* and *oryzicola* with PCA treatment (Du et al. 2015; Pan et al. 2017). RNA sequencing (RNA-seq) also showed that the transcript levels of *oxyR*, *ankB* (encoding AnkB, which interacts with CatB), *catB* (encoding CAT CatB), *katE* (encoding CAT KatE), *trxA* (encoding Trx), and *ahpC* (encoding AhpC) were all upregulated in *X. oryzae* pv. *oryzae* ZJ173 with PCA treatment (Table 2). To further determine which transcriptional regulator was activated by PCA, and to determine whether the genes encoding other antioxidant enzymes were also induced by PCA in these two bacteria, quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed. In the presence of PCA, the expression of *oxyR*, *ankB*, *catB*, *trxA*, *ahpC*, and *ahpF* (encoding AhpF) increased in *X. oryzae* pv. *oryzae* ZJ173 (Fig. 1A). In contrast, the gene expression of *oxyR*, *ankB*, and *catB* was not induced or slightly induced in *X. oryzae* pv. *oryzicola* RS105 with the same PCA treatment (Fig. 1B). Interestingly, higher concentrations of PCA induced the expression of *ankB*, *catB*, *katE*, and *trxA* in RS105, and the highest test concentration induced the expression of *oxyR* (Fig. 1C). In *X. oryzae* pv. *oryzae*, PCA only induced the expression of *sodM1*. In *X. oryzae* pv. *oryzicola*, PCA did not induce the expression of *soxR* or the genes encoding SOD.

The antioxidant abilities of *X. oryzae* pvs. *oryzae* and *oryzicola*.

RNA-seq data indicated that the transcript levels of *oxyR* and the genes (*ankB*, *catB*, *katE*, and *ahpC*) in the antioxidant

system regulated by OxyR were higher in *X. oryzae* pv. *oryzicola* than in *X. oryzae* pv. *oryzae* (Table 2). Thus, we suspected that antioxidant ability is much stronger in *X. oryzae* pv. *oryzicola* than in *X. oryzae* pv. *oryzae*. To test this, we compared H₂O₂ sensitivity, total antioxidant capacity, and CAT activity. The results indicated that *X. oryzae* pv. *oryzae* was more sensitive to H₂O₂ than *X. oryzae* pv. *oryzicola* (Fig. 2A). Similarly, total antioxidant capacity and CAT activity were greater in *X. oryzae* pv. *oryzicola* than that in *X. oryzae* pv. *oryzae* (Fig. 2D and E).

Sequence analysis, deletion, replacement, and complementation of *oxyR*

in *X. oryzae* pvs. *oryzae* and *oryzicola*.

Consistent with the finding that CAT, Trx, and Ahp are regulated by OxyR (Seo et al. 2015), the transcript levels of these genes were much higher in *X. oryzae* pv. *oryzicola* than in *X. oryzae* pv. *oryzae* according to RNA-seq (Table 2). We then aligned amino acids encoded by *oxyR* in *X. oryzae* pvs. *oryzae* and *oryzicola* and found 10 different sites (Supplementary Fig. S1). To further investigate whether the differences in amino acids may contribute to the differences in antioxidant ability, H₂O₂ sensitivity, and PHZ sensitivity, we generated *oxyR* gene deletion mutants, replacement mutants, and complementation mutants in *X. oryzae* pvs. *oryzae* and *oryzicola*. The *oxyR* deletion mutants for *X. oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105, which were designated ΔZ (*oxyR*), and ΔR (*oxyR*), respectively; the wild-type ZJ173 when the *oxyR* gene of ZJ173 was replaced with the *oxyR* gene of RS105, which was designated as ZJ173-*oxyR*^{RS105}; and the wild-type RS105 when the *oxyR* gene of RS105 was replaced with the *oxyR* gene of ZJ173, which was designated as RS105-*oxyR*^{ZJ173}, were confirmed by PCR amplification. The replacement mutants were also sequenced.

Determination of gene expression in the antioxidant system, total antioxidant capacity, and CAT activity in *oxyR* mutants.

qRT-PCR was used to assess the expression of genes in the antioxidant system in the *oxyR* mutants. The results indicated that the expression of *catB*, *ankB*, and *trxA* was significantly decreased in the *oxyR* deletion mutants of both strains (Fig. 2B and C). The expression of genes encoding AhpCF (*ahpC* and *ahpF*) was greater in the deletion mutants than in the wild-type strains. The expression of the genes encoding CAT (*katE*) was greatly enhanced in ΔR (*oxyR*) but was not changed in ΔZ (*oxyR*) (Fig. 2B and C). Interestingly, the expression of all genes tested except *srpA* and *ahpF* was

Table 1. Sensitivity of *Xanthomonas oryzae* pv. *oryzae* (ZJ173) and *X. oryzae* pv. *oryzicola* (RS105) to phenazines (phenazine-1-carboxylic acid [PCA], phenazine [PHZ], and 1-hydroxyphenazine [1-OH-PHZ]), bismethiazol, streptomycin, and captan

Pesticides	EC ₅₀ (µg/ml) ^a		Ratio (RS105/ZJ173)
	ZJ173	RS105	
PCA	0.21 ± 0.032 a	14.56 ± 1.89 b	69
PHZ	0.0025 ± 0.0011 a	2.31 ± 0.78 b	924
1-OH-PHZ	0.0062 ± 0.0028 a	1.91 ± 0.54 b	308
Bismethiazol	12.83 ± 1.34 a	16.27 ± 1.06 a	1.27
Streptomycin	0.48 ± 0.15 a	1.76 ± 0.33 b	3.67
Captan	0.063 ± 0.0169 a	0.091 ± 0.0092 a	1.44

^a EC₅₀ = the concentration of PCA that resulted in 50% inhibition of bacterial cell growth. Values are means ± standard error of three experiments. Means in a row with different letters are significantly different at $P < 0.05$.

significantly greater than in the wild-type *X. oryzae* pv. *oryzae* ZJ173 when the *oxyR* gene of ZJ173 was replaced with the *oxyR* gene of RS105 (ZJ173-*oxyR*^{RS105}) (Fig. 2B). In contrast, the expression of *ankB*, *catB*, *katE*, and *trxA* was significantly lower but the expression of *ahpC* was significantly greater than in the wild-type strain RS105 when the *oxyR* gene of RS105 was replaced with the *oxyR* gene of ZJ173 (RS105-*oxyR*^{ZJ173}) (Fig. 2C).

We then measured the total antioxidant capacity and CAT activity in the *oxyR* mutants. The deletion of *oxyR* significantly reduced the total antioxidant capacity (Fig. 2D) and CAT activity (Fig. 2E) of both deletion mutants. When the *oxyR* gene of *X. oryzae* pv. *oryzae* ZJ173 was replaced with the *oxyR* gene of *X. oryzae* pv. *oryzicola* RS105 (ZJ173-*oxyR*^{RS105}), total antioxidant capacity and CAT activity were significantly greater than in the wild type. In contrast, replacement of the *oxyR* gene of RS105 with the *oxyR* gene of ZJ173 (RS105-*oxyR*^{ZJ173}) did not restore the total antioxidant capacity and CAT activity to the levels in the wild-type RS105 (Fig. 2D and E).

Sensitivities of OxyR mutants to H₂O₂ and PCA.

The deletion mutants ΔZ (*oxyR*) and ΔR (*oxyR*) were more sensitive than their wild types (*X. oryzae* pv. *oryzae* ZJ173

and *X. oryzae* pv. *oryzicola* RS105, respectively) to H₂O₂ (Fig. 3A). The replacement mutant ZJ173-*oxyR*^{RS105} was more resistant than ZJ173 to H₂O₂, and the sensitivity to 0.1 mM H₂O₂ was similar for ZJ173-*oxyR*^{RS105} and RS105. In contrast, the replacement mutant RS105-*oxyR*^{ZJ173} was more sensitive than RS105 to H₂O₂, and its sensitivity to 0.05 mM H₂O₂ was intermediate between that of ΔR (*oxyR*) and RS105.

The deletion mutants ΔZ (*oxyR*) and ΔR (*oxyR*) were more sensitive than their wild types to PCA (Fig. 3B; Table 3). The replacement mutant RS105-*oxyR*^{ZJ173} was also more sensitive to PCA than *X. oryzae* pv. *oryzicola* RS105. However, the sensitivity to PCA did not significantly differ between the replacement mutant ZJ173-*oxyR*^{RS105} and *X. oryzae* pv. *oryzae* ZJ173 (i.e., replacement of its *oxyR* gene with that from RS105 did not increase its resistance to PCA).

OxyR affects ROS accumulation in *X. oryzae* pvs. *oryzae* and *oryzicola* in response to PCA.

In our previous study, PCA treatment significantly increased ROS levels in *X. oryzae* pv. *oryzae* (Xu et al. 2015). To further investigate whether ROS scavenging ability in *X. oryzae* pvs. *oryzae* and *oryzicola* is related to OxyR, we quantified ROS accumulation in the *oxyR* mutants after treatment with PCA. In

Table 2. Genes upregulated or downregulated in both *Xanthomonas oryzae* pv. *oryzae* (ZJ173) treated with phenazine-1-carboxylic acid (PCA) at 4 µg/ml and *X. oryzae* pv. *oryzicola* (RS105) relative to *X. oryzae* pv. *oryzae* ZJ173^z

Gene ID	Gene name	Fold change (ZJ173 PCA vs. ZJ173)	Fold change (RS105 vs. ZJ173)	Annotation
YP_198679.1	<i>trxA</i>	13.96	NA	Thioredoxin
YP_199041.6	<i>hemB</i>	3.29	2.06	Δ-Aminolevulinic acid dehydratase
YP_199056.1	<i>catB</i>	11.13	21.04	Catalase
YP_199057.1	<i>ankB</i>	18.76	27.23	Ankyrin-like protein
YP_199106.1	<i>zwf</i>	2.28	2.23	Glucose-6-phosphate 1-dehydrogenase
YP_199125.1	<i>pcaC</i>	2.34	2.38	4-Carboxymuconolactone decarboxylase
YP_199278.1	<i>cioA</i>	2.84	10.29	Cyanide insensitive terminal oxidase
YP_199326.1	<i>motB</i>	2.59	9.79	Flagellar motor protein
YP_199499.1	<i>PnuC</i>	2.84	4.08	Nicotinamide mononucleotide transporter
YP_199617.1	<i>cirA</i>	0.47	0.11	Colicin I receptor
YP_199994.1	<i>iucA</i>	0.43	0.20	Iron transporter
YP_200043.1	<i>ynhD</i>	0.38	0.48	ABC transporter ATP-binding protein
YP_200045.1	...	0.32	0.43	Predicted transcriptional regulator
YP_200092.1	<i>VgrG</i>	0.46	0.30	VGR-related protein
YP_200105.1	<i>cheR</i>	10.57	92.02	Response regulator for chemotaxis
YP_200106.1	<i>vieA</i>	4.78	58.37	Response regulator
YP_200108.1	<i>mcp</i>	2.33	68.91	Chemotaxis protein
YP_200109.1	<i>cheA</i>	2.68	32.02	Chemotaxis histidine protein kinase
YP_200359.1	...	3.70	29.78	Hypothetical protein
YP_200386.1	<i>pilH</i>	0.46	0.45	Twitching motility two-component system response regulator
YP_200408.1	<i>bga</i>	0.25	0.25	β-Galactosidase
YP_200409.1	...	0.31	0.36	Putative α-1,2-mannosidase
YP_200411.1	<i>EmrA</i>	0.37	0.0012	multidrug resistance efflux pump
YP_200413.1	<i>tfus2961</i>	0.30	0.0012	hypothetical protein
YP_200422.1	<i>fucA1</i>	0.20	0.20	α-L-fucosidase
YP_200423.1	<i>iroN</i>	0.25	0.25	TonB-dependent receptor
YP_200444.1	<i>BtuB</i>	0.50	0.47	Outer membrane cobalamin receptor protein
YP_200505.1	...	2.12	3.48	Conserved hypothetical protein
YP_200521.1	...	0.48	0.49	Serine protease
YP_200540.1	<i>araJ</i>	2.20	2.20	MFS transporter
YP_200541.1	...	18.51	2.12	Conserved hypothetical protein
YP_200597.1	<i>CcmA</i>	0.40	0.0014	ABC-type multidrug transport system, ATPase component
YP_200619.1	<i>fasD</i>	2.15	2.60	Outer membrane usher protein FasD
YP_200711.1	...	0.35	0.43	N-acyl-D-glucosamine 2-epimerase
YP_200714.1	<i>UbiE</i>	2.36	2.72	Methylase involved in ubiquinone/menaquinone biosynthesis
YP_200991.6	<i>bglS</i>	0.41	0.27	β-Glucosidase

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^z Data shown in bold indicate the genes involved in the antioxidant system. NA = not available.

response to PCA, ROS accumulation was greater in *oxyR* deletion mutants ΔZ (*oxyR*) and ΔR (*oxyR*) than in their respective wild types (Fig. 4). ROS accumulation was lower for ZJ173-*oxyR*^{RS105} than for its wild type and was higher for RS105-*oxyR*^{ZJ173} than for its wild type (i.e., the exchange of *oxyR* genes reduced ROS accumulation in *X. oryzae* pv. *oryzae* ZJ173 and increased ROS accumulation in *X. oryzae* pv. *oryzicola* RS105).

OxyR is required for growth and full virulence in a host (rice) and contributes to the hypersensitive response in a nonhost (*Nicotiana benthamiana*).

To determine whether OxyR is involved in growth, we measured the growth rate of the *oxyR* mutants in a minimal (MMX) medium and in a nutrient-rich (NB) medium. In MMX medium, ΔZ (*oxyR*) could not grow (Fig. 5A), while ΔR (*oxyR*) grew slowly (Fig. 5B). In MMX medium, ZJ173-*oxyR*^{RS105} grew slightly faster than the wild-type strain *X. oryzae* pv. *oryzae* ZJ173 (Fig. 5A) but RS105-*oxyR*^{ZJ173} grew slower than the wild-type strain *X. oryzae* pv. *oryzicola* RS105 (Fig. 5B). The growth of both *oxyR* complementation mutants was similar to that of their respective wild types in MMX medium. In NB medium, the growth rate of ΔZ (*oxyR*) was slightly slower than that of the wild-type strain ZJ173 (Fig. 5C), and the growth rates

of *X. oryzae* pv. *oryzicola* *oxyR* mutants did not differ from that of the wild-type strain *X. oryzae* pv. *oryzicola* RS105.

The earliest observable responses to plant pathogens include an oxidative burst followed by the accumulation of ROS (Wojtaszek 1997). Because OxyR has been demonstrated to play a critical role in response to ROS which produced by the interaction of plant and bacterial pathogens (Ishiga and Ichinose 2016), we performed virulence, bacterial number, and hypersensitive response (HR) assays to determine whether OxyR is important in host and nonhost interactions. These assays included the mutants and wild types of *X. oryzae* pvs. *oryzae* and *oryzicola* and host rice plants and nonhost *Nicotiana benthamiana* plants. On rice, both deletion mutants ΔZ (*oxyR*) and ΔR (*oxyR*) were less virulent than their respective wild types (Fig. 5D and E). The virulence on rice was greater for ZJ173-*oxyR*^{RS105} than for the wild-type strain (i.e., replacement of the *oxyR* gene of *X. oryzae* pv. *oryzae* ZJ173 with the *oxyR* gene of *X. oryzae* pv. *oryzicola* RS105 resulted in a substantial increase in virulence) (Fig. 5D). In contrast, the virulence on rice was not restored in RS105-*oxyR*^{ZJ173} (Fig. 5E). At 1 day postinoculation (dpi) and 4 dpi, the bacterial numbers of ΔZ (*oxyR*), ΔR (*oxyR*), and RS105-*oxyR*^{ZJ173} were significantly reduced compared with their respective wild-type strains. At 4 dpi, the bacterial numbers of ZJ173-*oxyR*^{RS105} were significantly increased in comparison

Table 2. (Continued from previous page)

Gene ID	Gene name	Fold change (ZJ173 PCA vs. ZJ173)	Fold change (RS105 vs. ZJ173)	Annotation
YP_201207.1	<i>cheV</i>	6.53	73.85	Chemotaxis protein
YP_201250.1	<i>fliP</i>	0.46	0.45	Flagellar biosynthetic protein
YP_201251.1	<i>fliQ</i>	0.40	0.18	Flagellar biosynthesis
YP_201255.1	...	2.57	17.11	GGDEF family protein (diguanylate cyclase)
YP_201377.1	<i>acvB</i>	0.37	0.30	Virulence protein
YP_201413.1	<i>gabD</i>	2.36	2.07	Succinate-semialdehyde dehydrogenase
YP_201622.1	...	3.25	73.32	Mg-protoporphyrin IX monomethyl ester oxidative cyclase
YP_201632.1	<i>RfbX</i>	3.60	7.07	Membrane protein involved in the export of O-antigen and teichoic acid
YP_201634.1	...	5.46	26.72	Hypothetical protein
YP_201639.1	...	5.82	32.72	Cytochrome C
YP_201648.1	<i>cycK</i>	2.81	17.10	C-type cytochrome biogenesis membrane protein
YP_201758.1	<i>btuB</i>	4.39	21.27	Pseudogene
YP_201776.1	<i>dapA</i>	2.45	2.41	Dihydroxydipicolinate synthase
YP_201777.1	<i>HcaD</i>	2.10	2.33	Oxidoreductase
YP_201959.1	<i>oar</i>	2.26	3.92	Oar protein
YP_202062.6	<i>kaiE</i>	6.71	2.51	Catalase
YP_202150.1	...	0.48	0.24	Hypothetical protein
YP_202284.1	<i>ahpC</i>	3.84	3.98	Alkyl hydroperoxide reductase
YP_202286.1	<i>oxyR</i>	2.39	7.03	Oxidative stress transcriptional regulator
YP_202298.1	...	0.40	0.15	Two-component system regulatory protein
YP_202435.1	<i>ppc</i>	2.67	3.31	Phosphoenolpyruvate carboxylase
YP_202449.1	<i>metF</i>	3.31	2.84	5,10-Methylenetetrahydrofolate reductase
YP_202503.1	...	2.21	2.22	Conserved hypothetical protein
YP_202732.1	...	3.88	14.45	Conserved hypothetical protein
YP_202735.1	...	2.25	3.90	Conserved hypothetical protein
YP_202753.1	<i>yiaA</i>	0.44	0.025	Membrane protein
YP_202973.1	...	2.26	141.39	Conserved hypothetical protein
YP_203016.1	<i>accC</i>	0.30	0.13	Biotin carboxylase
YP_203017.1	<i>accD</i>	0.32	0.12	Acetyl-coenzyme A carboxylase carboxyl transferase
YP_203056.6	<i>xylA</i>	0.28	0.21	Xylose isomerase
YP_203096.1	...	0.46	0.36	Conserved hypothetical protein
YP_203167.6	...	2.24	4.33	Quinone oxidoreductase
YP_203168.1	<i>mocA</i>	2.45	3.21	Rhizopine catabolism protein mocA
YP_203174.1	...	0.36	0.00090	RTS β protein (L-fuconate dehydratase)
YP_203175.1	<i>MhpD</i>	0.42	0.00073	2-Keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway)
YP_203176.1	<i>ucpA</i>	0.46	0.0015	Oxidoreductase
YP_203178.1	<i>Tas</i>	0.37	0.0016	Oxidoreductase

with the wild-type strain ZJ173 (Fig. 5F and G). In the HR assay, the HR was stronger for the deletion mutants ΔZ (*oxyR*) and ΔR (*oxyR*) than for their respective wild types (Fig. 5H). The HR in *N. benthamiana* was less for ZJ173-*oxyR*^{RS105} but greater for RS105-*oxyR*^{ZJ173} relative to their respective wild types (Fig. 5H).

DISCUSSION

As noted above, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are important bacterial pathogens of rice (Lang et al.

2010; Niño-Liu et al. 2006; Ou 1985). The two bacteria are very closely related (i.e., they not only infect the same host but also have genomes of similar size, gene content, and percent G+C) (Seo et al. 2008). In the current study, however, PCA and also PHZ and 1-PHZ-OH were much more effective against *X. oryzae* pv. *oryzae* than *X. oryzae* pv. *oryzicola* (Table 1). *X. oryzae* pvs. *oryzae* and *oryzicola* did not greatly differ in their sensitivity to other commonly used bactericides such as bismethiazol and streptomycin and fungicide captan. The results indicated that the differences in sensitivity to phenazines in *X. oryzae* pvs. *oryzae* and *oryzicola* may be specific. It is still unclear why *X. oryzae* pv. *oryzae* has specific sensitivity to phenazines. In this study, we identified a transcriptional regulator, OxyR, in *X. oryzae* pvs. *oryzae* and *oryzicola*, that relates to the differences in total antioxidant capacity, CAT activity, and sensitivity to H₂O₂. Moreover, our study also revealed that OxyR affects the sensitivity to PCA and participates in the pathogenicity of *X. oryzae* pvs. *oryzae* and *oryzicola* on host rice and HR on nonhost *N. benthamiana*.

PCA is well known as a redox-active compound that affects the endogenous redox balance in phenazine-producing organisms and in non-phenazine-producing pathogens; the change in redox balance resulting from PCA treatment causes the target pathogens to produce ROS, which can result in cell death (Okegbe et al. 2017; Xu et al. 2015). Unlike endogenous pyocyanin, which activates SoxR in a phenazine-producing strain of *P. aeruginosa* (Dietrich et al. 2008), PCA treatment did not activate SoxR in *X. oryzae* pvs. *oryzae* and *oryzicola* but activated OxyR and the genes typically involved in detoxification of H₂O₂ in both bacteria in the current study (Fig. 1; Table 2). ROS accumulates in *X. oryzae* pv. *oryzae* treated with PCA (Xu et al. 2015), and levels of ROS accumulation in the current study were higher in *X. oryzae* pv. *oryzae* than in *X. oryzae* pv. *oryzicola* (Fig. 4). In addition, RNA-seq indicated that transcript levels of the relevant genes were higher in *X. oryzae* pv. *oryzicola* than in *X. oryzae* pv. *oryzae* (Table 2). These results suggested that antioxidant ability may be much weaker in *X. oryzae* pv. *oryzae* than in *X. oryzae* pv. *oryzicola*. In support of this inference, we found that *X. oryzae* pv. *oryzae* was more sensitive than *X. oryzae* pv. *oryzicola* to exogenous H₂O₂ (Fig. 2A), and that total antioxidant capacity and CAT activity were lower in *X. oryzae* pv. *oryzae* than in *X. oryzae* pv. *oryzicola* (Fig. 2D and E). The current results further provide strong evidence that the ROS scavenging machinery is much less effective in *X. oryzae* pv. *oryzae* than in *X. oryzae* pv. *oryzicola*, such that *X. oryzae* pv. *oryzae* is much more sensitive to H₂O₂ and other redox compounds than *X. oryzae* pv. *oryzicola*.

OxyR is widely distributed in bacteria and is directly oxidized by H₂O₂; once oxidized, it binds to DNA and thereby regulates gene expression so as to reduce H₂O₂ levels (Aslund et al. 1999; Choi et al. 2001; Ieva et al. 2008; Loprasert et al. 2000). In the current study, the genes induced in *X. oryzae* pv. *oryzae* by PCA are also regulated by OxyR (Liu et al. 2016; Yu et al. 2016). Based on KEGG sequences, OxyR in *X. oryzae* pvs. *oryzae* and *oryzicola* differs in 10 amino acids. Given these results and the differences in antioxidant system and CAT activity, we suspected that OxyR might be involved in regulating the expression of enzymes in the antioxidant system in response to PCA, and that this regulation may reduce sensitivity to PCA and may be a possible cause of the difference in the antioxidant systems of *X. oryzae* pv. *oryzae* versus *X. oryzae* pv. *oryzicola*. As expected, the expression was significantly decreased for *ankB*, *catB*, and *trxA* but was increased for *ahpC* and *ahpF* in the *oxyR* deletion mutants of both *X. oryzae* pvs. *oryzae* and *oryzicola*. In addition, the expression of *oxyR*, *ankB*, *catB*, *katE*, *trxA*, and *ahpC* was significantly increased when replacing the *oxyR* gene in

Fig. 1. Expression of *oxyR*, *soxR* and the genes involved in antioxidant systems in **A**, *Xanthomonas oryzae* pv. *oryzae* ZJ173 and **B** and **C**, *X. oryzae* pv. *oryzicola* strain RS105 in response to phenazine-1-carboxylic acid (PCA). Tested strains were grown in nutrient-rich medium to an optical density at 600 nm of 0.2, and PCA was added to a final concentration of 1, 2, or 4 µg/ml for ZJ173 (A) and RS105 (B), and to a final concentration of 8, 16, or 32 µg/ml for RS105 (C). The amount of RNA in acetone was used as the control and was set at 1.0. Values are means $\pm$ standard deviation from three biological replicates and asterisks (*) indicate *P* value < 0.01 (*t* test).

X. oryzae pv. *oryzae* ZJ173 with the *oxyR* gene from *X. oryzae* pv. *oryzicola* RS105 (Fig. 2B). The changes of the gene expression were further demonstrated to influence the total antioxidant capacity in the *oxyR* mutants (Fig. 2B and C). Combined with our previous report that CatB is the major CAT in *X. oryzae* pvs. *oryzae* and *oryzicola* and helps protect *X. oryzae* pvs. *oryzae* and *oryzicola* under H₂O₂ and PCA stresses (Pan et al. 2017), the current results show that CAT activity was dramatically reduced when we deleted *oxyR* but was significantly increased in ZJ173-*oxyR*^{RS105}, when the *oxyR* gene in *X. oryzae* pv. *oryzae* was replaced with the *oxyR*

gene from RS105. In contrast, replacement of the *oxyR* gene in *X. oryzae* pv. *oryzicola* with the *oxyR* gene from *X. oryzae* pv. *oryzae* ZJ173 did not restore the total antioxidant capacity and CAT activity to the levels of the wild-type strain RS105. These results indicated that differences in OxyR between *X. oryzae* pvs. *oryzae* and *oryzicola* contribute to the differences in the antioxidant abilities of *X. oryzae* pv. *oryzae* versus *X. oryzae* pv. *oryzicola*.

Sensitivity of ZJ173-*oxyR*^{RS105} to exogenous H₂O₂ was substantially reduced compared with the wild-type strain *X. oryzae* pv. *oryzae* ZJ173, and was close to the wild-type strain *X. oryzae*

Fig. 2. A, H₂O₂ sensitivity of *Xanthomonas oryzae* pv. *oryzae* ZJ173 and *X. oryzae* pv. *oryzicola* RS105. Gene expression of *oxyR* and genes regulated by it involved in antioxidant systems in B, *X. oryzae* pv. *oryzae* strains and C, *X. oryzae* pv. *oryzicola* strains; D, total antioxidant capacity; and E, total catalase activity of *X. oryzae* pv. *oryzae* (ZJ173), *X. oryzae* pv. *oryzicola* (RS105), and their *oxyR* mutants. The one-, three-, and ninefold dilutions (1×, 3× and 9×) of the bacterial suspension were spotted onto nutrient-rich (NB) medium containing 0, 0.0125, 0.025, 0.05, and 0.1 mM H₂O₂. Strains were grown and treated in NB medium. Values are means ± standard deviation (SD) of three biological replicates, and asterisks (*) indicate *P* value < 0.05 (*t* test). For B and C, expression was assessed by quantitative reverse-transcription polymerase chain reaction. Relative mRNA levels of tested genes in ZJ173 and RS105 when treated with acetone were used as control and were set at 1. For D and E, total antioxidant capacity and total catalase activity was measured from total protein extract. Values are means ± SD of three biological replicates, and asterisks (*) indicate *P* value < 0.05 (*t* test).

pv. *oryzicola* RS105. However, sensitivity of RS105-*oxyR*^{ZJ173} to exogenous H₂O₂ was much greater than the wild-type strain *X. oryzae* pv. *oryzicola* RS105. Combined with the results above, it suggested that the ability of OxyR to induce gene expression in antioxidant systems may be stronger in *X. oryzae* pv. *oryzicola* than *X. oryzae* pv. *oryzae*, which contribute to higher total antioxidant ability and total CAT activity in it and, thus, ultimately increased the bacterium's ability to defend against oxidative stress. The results also indicated that OxyR could be a key protein contributing to the different sensitivities of *X. oryzae* pvs. *oryzae* and *oryzicola* to H₂O₂ that has never been reported before. However, it is noteworthy that the differences in target gene promoters and DNA binding ability and stability of OxyR may also contribute to the difference in the sensitivity of *X. oryzae* pvs. *oryzae* and *oryzicola*. When *oxyR* was absent or was obtained from *X. oryzae* pv. *oryzae* ZJ173, the ability to scavenge ROS that had accumulated in response to PCA

treatment was decreased, so that sensitivity to PCA was increased in the mutants ΔZ (*oxyR*), ΔR (*oxyR*), and RS105-*oxyR*^{ZJ173}. Although we expected that the mutant ZJ173-*oxyR*^{RS105} would have increased resistance to PCA, that was not the case (Fig. 3B; Table 3). The antioxidant capacity was increased but the levels of ROS remained high, even though the levels were lower than in the wild-type strain ZJ173 (Fig. 4). On the one hand, this suggested that OxyR is necessary for protecting *X. oryzae* pvs. *oryzae* and *oryzicola* from PCA stress. However, on the other hand, it may not be possible to increase the resistance to PCA of *X. oryzae* pv. *oryzae* though exchanging OxyR of *X. oryzae* pv. *oryzicola*. As noted earlier, phenazines are thought to mainly act as electron acceptors that disturb redox homeostasis (Price-Whelan et al. 2006; Rabaey et al. 2005; Yano et al. 2011). The mechanism of resistance to PCA may be much more complicated than we thought and needs to be further investigated in the future. Unexpectedly, the *oxyR* mutants of *X. oryzae* pvs.

Fig. 3. A, H₂O₂ sensitivity and **B**, phenazine-1-carboxylic acid (PCA) sensitivity of *Xanthomonas oryzae* pv. *oryzae* ZJ173 (wild type), *X. oryzae* pv. *oryzicola* RS105 (wild type), and their *oxyR* mutants on nutrient-rich (NB) medium plates. The one-, three-, and ninefold dilutions (1×, 3× and 9×) of the bacterial suspension were spotted onto NB medium. For A, NB medium contained 0, 0.0125, 0.025, 0.05, and 0.1 mM H₂O₂. For B, NB medium contained PCA at 0, 0.2, and 0.4 µg/ml for ZJ173 and PCA at 0, 16, and 32 µg/ml for RS105.

oryzae and *oryzicola* showed no obvious difference from their wild-type strains in the presence of paraquat (the superoxide generator) and heavy metals (Cu^{2+} and Fe^{3+}) (Supplementary Fig. S3). A similar phenomenon has been reported before in *Ralstonia solanacearum* and *P. aeruginosa*. In *R. solanacearum*, the *oxyR* deletion mutant was more sensitive to H_2O_2 but grew better than the wild-type strain in the presence of paraquat (Flores-Cruz and Allen 2011). In *P. aeruginosa*, the *oxyR* deletion mutant didn't differ in growth on casamino acid plates containing 50 μM FeCl_3 (Vinckx et al. 2008). We also found that *soxR* deletion mutants of *X. oryzae* pvs. *oryzae* and *oryzicola* weren't more sensitive to H_2O_2 than their wild-type strains (data not shown). These results suggested that the antioxidant system mediated by OxyR is not involved in the scavenging of oxidative stress induced by paraquat and heavy metals in *X. oryzae* pvs. *oryzae* and *oryzicola*.

Loss of OxyR was previously reported to affect the growth of several species of bacteria (Chung et al. 2015; Maciver and Hansen 1996; Vinckx et al. 2008). In our study, the growth of *oxyR* deletion mutants of *X. oryzae* pvs. *oryzae* and *oryzicola* was blocked or reduced in MMX medium but was not significantly reduced in NB medium. This was not surprising because prolonged starvation may disturb the cellular ROS homeostasis, and restoring the homeostasis may be difficult or impossible for *oxyR*-defective mutants. ROS is also an important component of immune responses of plants against pathogen infection (Dubbs and Mongkolsuk 2012; Orozco-Cárdenas et al. 2001).

Table 3. Phenazine-1-carboxylic acid (PCA) sensitivity of *Xanthomonas oryzae* pv. *oryzae* (ZJ173) and *X. oryzae* pv. *oryzicola* (RS105)

Strain	EC ₅₀ ($\mu\text{g/ml}$) ^z
ZJ173	0.21 ± 0.032 a
$\Delta\text{Z}/\text{oxyR}$	0.098 ± 0.014 b
$\Delta\text{Z}/\text{oxyR}(\text{oxyR})$	0.19 ± 0.011 a
ZJ173- <i>oxyR</i> ^{RS105}	0.23 ± 0.020 a
RS105	14.56 ± 1.89 a
$\Delta\text{R}/\text{oxyR}$	3.82 ± 0.53 b
$\Delta\text{R}/\text{oxyR}(\text{oxyR})$	13.69 ± 1.77 a
RS105- <i>oxyR</i> ^{ZJ173}	4.47 ± 0.38 b

^z EC₅₀ = the concentration of PCA that resulted in 50% inhibition of bacterial cell growth. Values are means ± standard error of three experiments. Means with different letters are significantly different at $P < 0.05$. Bold indicates wild-type strains.

The reduced virulence and bacterial numbers of ΔZ (*oxyR*), ΔR (*oxyR*), and RS105-*oxyR*^{ZJ173} and the enhanced virulence and bacterial numbers of ZJ173-*oxyR*^{RS105} indicate that OxyR in *X. oryzae* pvs. *oryzae* and *oryzicola* might modulate the levels of ROS generated during bacterial infection. OxyR was also confirmed to be required for the pathogenicity of DC3000 on *Arabidopsis* (Ishiga and Ichinose 2016). In addition, our results showed that OxyR in *X. oryzae* pvs. *oryzae* and *oryzicola* was associated with the HR in the nonhost *N. benthamiana*. Plants can exploit the vulnerability of bacteria to ROS by generating ROS as part of the oxidative burst and thereby initiating an important defense response (Apel and Hirt 2004; Lamb and Dixon 1997). The HR response of *N. benthamiana* to RS105 and ZJ173-*oxyR*^{RS105} was relatively weak because these bacteria have a powerful antioxidation system that can effectively scavenge the ROS generated by the plant. Conversely, the HR response of *N. benthamiana* to ZJ173, ΔZ (*oxyR*), ΔR (*oxyR*), and RS105-*oxyR*^{ZJ173} was strong.

In conclusion, our results indicated that *X. oryzae* pv. *oryzicola* has a stronger antioxidant system than *X. oryzae* pv. *oryzae* and accumulates lower levels of ROS in response to redox compounds, which is proposed in Figure 6. As a consequence, *X. oryzae* pv. *oryzae* is more sensitive than *X. oryzae* pv. *oryzicola* to redox compounds. Our findings also highlight the significance of OxyR, which relates to the difference in antioxidant ability between *X. oryzae* pvs. *oryzae* and *oryzicola* and the difference in their sensitivity to PCA. Although OxyR in *X. oryzae* pvs. *oryzae* and *oryzicola* differs in 10 amino acids, which amino acids are responsible for the observed difference in OxyR activity remains to be determined. Our results also showed that OxyR is important for their virulence and for their induction of HR in host and nonhost plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and bactericides.

Bacterial strains, plasmids, and primers used in this study are listed in Table 4 and Supplementary Table S1. Strains ZJ173 of *X. oryzae* pv. *oryzae* and RS105 of *X. oryzae* pv. *oryzicola* are commonly used by researchers in China and are used as the parent strains in this study. *X. oryzae* pvs. *oryzae* and *oryzicola* wild-type strains, *oxyR* mutants, and their complemented

Fig. 4. Reactive oxygen species (ROS) accumulation in **A**, *Xanthomonas oryzae* pv. *oryzae* strains and **B**, *X. oryzae* pv. *oryzicola* strains when stimulated with phenazine-1-carboxylic acid (PCA) (0.5, 1, 2, and 4 $\mu\text{g/ml}$ for *X. oryzae* pv. *oryzae* strains and 4, 8, 16, and 32 $\mu\text{g/ml}$ for *X. oryzae* pv. *oryzicola* strains). Acetone was used as solvent control. Relative fluorescence units were the ROS accumulation in strains after 4 h at the excitation wavelength of 488 nm and the emission wavelength of 525 nm. Relative fluorescence units in *X. oryzae* pv. *oryzae* ZJ173 or *X. oryzae* pv. *oryzicola* RS105 at the same concentration of PCA were used as control. Values are the means ± standard deviation of three replicates, and asterisks (*) indicate P value < 0.05 (t test).

strains were cultured in NB medium (beef extract at 3 g/liter, polypeptone at 5 g/liter, yeast extract at 1 g/liter, and sucrose at 10 g/liter), with shaking at 175 rpm and 28°C. NB agar (NA) was used as a solid medium. MMX used for determination of growth rate contained trisodium citrate at 1 g/liter, glucose at 5 g/liter, (NH₄)SO₄ at 2 g/liter, MgSO₄·7H₂O at 0.2 g/liter, K₂HPO₄ at 4 g/liter, and KH₂PO₄ at 6 g/liter. *Escherichia coli* DH5 α (Vazyme) was grown in Luria-Bertani (LB) (NaCl at 10 g/liter, typtone at 10 g/liter, and yeast extract at 5 g/liter) or on LB agar plates containing kanamycin at 50 μ g/ml and 37°C.

PCA (98%; Shanghai Nongle Biological Products Co., Ltd.) was dissolved in acetone. The 99% PHZ and 95% 1-OH-PHZ were provided by TCI (Shanghai) Development Co., Ltd. and

were dissolved in alcohol. Bismethiazol (98%; Longwan Agricultural Chemical Co., Ltd.) was dissolved in N,N-dimethylformamide. Streptomycin (98%; Sinopharm Chemical Reagent Co., Ltd.) was dissolved in water. Captan (95%) was stored in our laboratory and was dissolved in acetone.

Phenazines, paraquat, H₂O₂, and heavy metals sensitivity assays.

The sensitivities of *X. oryzae* pvs. *oryzae* and *oryzicola* to phenazines, paraquat, H₂O₂, and heavy metals (Cu²⁺ and Fe³⁺) were determined as previously described (Pan et al. 2017). The strains were grown in NB medium with shaking (175 rpm) at 28°C to an optical density (OD) of 1.0 (10⁹ CFU/ml). A 2- μ l

Fig. 5. Growth rate of **A**, *Xanthomonas oryzae* pv. *oryzae* ZJ173 (wild type) and its *oxyR* mutants; **B**, *X. oryzae* pv. *oryzicola* RS105 (wild type) and its *oxyR* mutants in a nutrient-poor (MMX) medium; and **C**, *X. oryzae* pv. *oryzae* ZJ173 (wild type), *X. oryzae* pv. *oryzicola* RS105 (wild type), and their *oxyR* mutants in a nutrient-rich (NB) medium, as indicated by optical density at 600 nm (OD₆₀₀). OD₆₀₀ was measured every 12 h in MMX medium and every 2 h in NB medium. Values are means $\pm$ standard deviation (SD) of three biological replicates, and asterisks (*) indicate *P* value < 0.05 (*t* test). Pathogenicity of **D**, *X. oryzae* pv. *oryzae* strains and **E**, *X. oryzae* pv. *oryzicola* strains in rice IR24. Bar charts show lesion lengths. Three replicates were used for each treatment, and the experiment was repeated three times. Bacterial numbers of **F**, *X. oryzae* pv. *oryzae* strains and **G**, *X. oryzae* pv. *oryzicola* strains in rice IR24. Three replicates were used for each treatment, and the experiment was repeated twice. Values are the means $\pm$ SD of three replicates, and asterisks (*) indicate *P* value < 0.05 (*t* test). Hypersensitive response assay of **F**, *X. oryzae* pv. *oryzae* ZJ173 (wild type); **G**, *X. oryzae* pv. *oryzicola* RS105 (wild type); and **H**, their *oxyR* mutants in *Nicotiana benthamiana*. Tested strains were infiltrated in to the leaves of *N. benthamiana* by using a needleless syringe. Necrosis was indicated by a dotted circle.

volume of bacterial suspension was spotted on NA plates containing different concentrations of phenazines, paraquat, H_2O_2 , CuSO_4 , or FeCl_3 . The final concentrations of PCA were 0.2 and 0.4 $\mu\text{g/ml}$ for *X. oryzae* pv. *oryzae* and 16 and 32 $\mu\text{g/ml}$ for *X. oryzae* pv. *oryzicola*. The concentrations of paraquat and H_2O_2 were 0.0125, 0.025, 0.05, and 0.1 mM for both bacteria. The concentrations of Cu^{2+} were 0.1, 0.2, and 0.4 mM for *X. oryzae* pv. *oryzae* and 0.02, 0.04, and 0.06 mM for *X. oryzae* pv. *oryzicola*. The concentrations of Fe^{3+} were 0.2, 0.4, and 0.6 mM for *X. oryzae* pv. *oryzae* and 0.1, 0.2, and 0.4 mM for *X. oryzae* pv. *oryzicola*. The plates were incubated at 28°C for 3 days (*X. oryzae* pv. *oryzicola*) or 4 days (*X. oryzae* pv. *oryzae*). The EC_{50} of PCA against *X. oryzae* pvs. *oryzae* and *oryzicola* was measured as follows. Bacterial cultures (OD at 600 nm [OD_{600}] = 1.0) were adjusted to OD_{600} = 0.2 with fresh NB medium, and 120 μl of the diluted bacterial suspensions and 100 μl of PCA were subsequently added into 25 ml of fresh NB medium. The concentrations of PCA were 0, 0.0125, 0.025, 0.05, 0.1, 0.2, or 0.4 $\mu\text{g/ml}$ for *X. oryzae* pv. *oryzae* strains and 0, 1, 2, 4, 8, 16, or 32 $\mu\text{g/ml}$ for *X. oryzae* pv. *oryzicola* strains. The final acetone concentration was 0.4% (vol/vol). Three replicates were conducted for each treatment. The OD_{600} values were measured after 36 and 24 h of incubation (28°C with shaking at 175 rpm) for *X. oryzae* pvs. *oryzae* and *oryzicola*, respectively. The EC_{50} values were assessed by the Data Processing System (Hangzhou Reifeng Information Technology Ltd.). The experiments were performed three times.

Real-time qRT-PCR and RNA-Seq.

RNA isolation and qRT-PCR were performed as previously described (Pan et al. 2017). All strains were grown to an OD of

0.5 (5×10^8 CFU/ml), and a 2-ml volume of bacterial suspension was harvested by centrifugation ($8,000 \times g$ for 2 min at 4°C). Total RNA was extracted using the RNAPrep pure cell/bacteria kit (TIANGEN Biotech) according to the manufacturer's protocol. A 500-ng quantity of total RNA eliminated genomic DNA (gDNA) with 2 μl of 4 $\times$ gDNA wiper mix (42°C, 2 min). Then, the RNA was reverse transcribed to cDNA with 2 μl of 5 $\times$ qRT supermix II of the Hiscript Q RT supermix for qPCR kit (Vazyme). The cDNA was used as template of qRT-PCR which was conducted using the ChamQ SYBR qPCR Maser Mix kit (Vazyme) with a CFX Connect Real-Time System (Bio-Rad Laboratories). Gene expression of *oxyR* (XOO3647/XOC_0957), *soxR* (XOO1257/XOC_1405), *ankB* (XOO0418/XOC_4324), *catB* (XOO0417/XOC_4325), *katE* (XOO3423/XOC_1265), *srpA* (XOO0447/XOC_4290), *katG* (XOC_3410), *trxA* (XOO0040/XOC_0180), *ahpC* (XOO3645/XOC_0959), *ahpF* (XOO3646/XOC_0958), *sodB* (XOO3208/XOC_2909), *sodC2* (XOO4481/XOC_0298), *sodM1* (XOO2712/XOC_2116), *sodM2* (XOO2828/XOC_2312), and *yojM* (XOO4482/XOC_0299) was assessed. 16S ribosomal RNA was used as endogenous control. RNA-seq was conducted by Novogene Bioinformatics Technology. RNA sample testing, sequencing, and data analysis were performed according to a previous study (Liang et al. 2018). RNA-seq profiles of PCA-treated (4 $\mu\text{g/ml}$) and acetone-treated *X. oryzae* pv. *oryzae* and acetone-treated *X. oryzae* pv. *oryzicola* are shown in Supplementary Fig. S2.

Generation of *oxyR* mutants.

Xoo3647 (*oxyR* in *X. oryzae* pv. *oryzae*) and Xoc_0957 (*oxyR* in *X. oryzae* pv. *oryzicola*) were deleted by nonmarker

Fig. 6. Model showing different responses to oxidative stress in *Xanthomonas oryzae* pv. *oryzae* versus *X. oryzae* pv. *oryzicola*. Reactive oxygen species (ROS) are generated by oxidation of phenazine-1-carboxylic acid (PCA) (PCA_{red} = reduction of PCA and PCA_{ox} = oxidation of PCA) (Wang and Newman 2008). Here, we take hydrogen peroxide (H_2O_2) as an example. OxyR senses oxidative stress induced by PCA treatment or plants during their infection and, in turn, activates the antioxidant systems to remove H_2O_2 in the two bacteria. The antioxidant systems regulated by OxyR are much greater in *X. oryzae* pv. *oryzicola* (right circle) than in *X. oryzae* pv. *oryzae* (left circle). Ultimately, less H_2O_2 is accumulated in the bacterial cells of *X. oryzae* pv. *oryzicola*.

homologous recombination, as previously described (Qian et al. 2013). In brief, an upstream and a downstream fragment of *oxyR* in ZJ173 or RS105 were amplified by PCR using the primer pairs ZoxyR-1F/1R and ZoxyR-2F/2R or RoxyR-1F/1R and RoxyR-2F/2R, respectively, and the fragments were then cloned into the suicide vector pK18mobSacB. The recombinant plasmid pK18ZOR or pK18ROR was introduced into ZJ173 or RS105, respectively, by electroporation. Transformants were isolated on NA medium without sucrose containing kanamycin at 20 µg/ml. After they were incubated in 25 ml of NB medium without sucrose for 7 to 9 h at 28°C, and the transformants were spread on NA medium containing sucrose at 100 µg/ml. The positive colonies were confirmed by PCR using primer pairs ZoxyR-1F/2R and RoxyR-1F/2R, respectively. Plasmid pUFR034 was used to construct replacement and complemented mutants. A 1,119-bp fragment carrying the full length of *oxyR* with its promoter region was amplified from the gDNA of ZJ173 or RS105 with primer pair Z/oxyRH-F/R, respectively. The fragment was then fused to pUFR034. To construct complemented mutants, the recombinant plasmid pUFRZOR or pUFRROR was then introduced into the *oxyR* deletion mutants of ZJ173 or RS105, respectively. To construct replacement mutants, the recombinant plasmid pUFRROR or pUFRZOR was introduced into *oxyR* deletion mutants of ZJ173 or RS105, respectively. The complemented and replacement mutants were all obtained using the electroporation method; they were screened on NA plates containing kanamycin at 20 µg/ml, and were then confirmed by PCR using primer pair Z/oxyRH-F/R.

Total antioxidant capacity assay.

The tested strains were grown at 28°C overnight in NB medium (OD₆₀₀ = 1.0). Bacterial cells were collected from 2 ml of culture suspension by centrifugation (4,000 × *g* for 2 min) and were resuspended in 500 µl of precooling phosphate-buffered saline (1 liter: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM NaH₂PO₄; pH 7.4). The cells were subjected to ultrasonication for 40 s (amplitude 15%, ultrasound 1 s, pause 3 s). The supernatant was then collected by centrifugation (12,000 × *g* for 5 min at 4°C), and the protein

concentration was determined with the BCA Protein Concentration Determination Kit (Beyotime). Total antioxidant capacity was then determined with the Total antioxidant capacity assay kit (Beyotime). Each treatment was represented by three replicates, and the experiment was conducted three times. The results were analyzed with SPSS 20.0 (independent-sample *t* tests).

CAT activity assay.

CAT activity was assessed as previously described (Pan et al. 2017). In brief, bacterial cells were treated with 200 µl of Western and IP Cell Lysis Liquid (Beyotime). The protein concentration in the supernatant was determined with the BCA Protein Concentration Determination Kit (Beyotime), and the total CAT activity was determined with the Catalase Test Kit (Beyotime), according to the manufacturer's protocol. The sampling volume of the supernatant was 40 µl for ZJ173, Δ*ZoxyR*, Δ*RoxyR*, and RS105-*oxyR*^{ZJ173}; 10 µl for RS105; and 20 µl for ZJ173-*oxyR*^{RS105}. The experiment was performed three times, and each independent experiment included three replicates. The results were analyzed with SPSS 20.0 (independent-sample *t* tests).

ROS accumulation assay.

ROS accumulation was quantified according to the protocol of an ROS assay kit (Beyotime), as previously described (Xu et al. 2015). The nonionic, nonpolar 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used to detect ROS (Wang and Joseph 1999). In brief, bacterial cells were collected from 15 ml of culture suspension (OD₆₀₀ = 1.0) by centrifugation (5,000 × *g* for 2 min). The cells were then resuspended in 8 ml of DCFH-DA solution and incubated for 20 min at 28°C. After they were washed three times, the cells were resuspended in 6 ml of fresh XOM medium (1 liter: 4.5 g of KH₂PO₄, 1 g of (NH₄)₂SO₄, 1 mM MgSO₄, 10.5 g of K₂HPO₄, 20 mM succinic acid, and 0.15% casein hydrolysate). The preparation was then divided into seven samples of 700 µl per sample. Each sample was then treated with PCA before 200 µl of the suspension was added to the wells of black microplates. The final concentrations of PCA were 0.5, 1, 2, and 4 µg/ml for ZJ173 and 4, 8, 16, and 32 µg/ml

Table 4. Strains and plasmids used in this study

Strains, plasmids, and primers	Characteristics ^z	Source
Strains		
<i>Escherichia coli</i> DH5α	φ80 <i>lacZ</i> ΔM15, Δ(<i>lacZYA-argF</i>)U169. <i>recA1</i>	Vazyme, Nanjing, China
ZJ173	Rif ^R , wild-type strain of <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	Laboratory collection
Δ <i>ZoxyR</i>	Rif ^R , deletion of <i>oxyR</i> in wild-type strain ZJ173	This study
Δ <i>ZoxyR</i> (<i>oxyR</i>)	Rif ^R , Km ^R , complemented strain of Δ <i>ZoxyR</i>	This study
RS105	Rif ^R , wild-type strain of <i>X. oryzae</i> pv. <i>oryzicola</i>	Laboratory collection
Δ <i>RoxyR</i>	Rif ^R , deletion of <i>oxyR</i> in wild-type strain RS105	This study
Δ <i>RoxyR</i> (<i>oxyR</i>)	Rif ^R , Km ^R , complemented strain of Δ <i>RoxyR</i>	This study
Plasmids		
pK18 <i>mobsacB</i>	Km ^R , allelic exchange suicide vector, <i>sacB</i> oriT(RP4)	Laboratory collection
pK18ZOR	Km ^R , a 2,094-bp fusion fragment of ZoxyR-1, ZoxyR-2gene ligated in pK18 <i>mobsacB</i>	This study
pK18ROR	Km ^R , a 2,269-bp fusion fragment of RoxyR-1, RoxyR-2gene ligated in pK18 <i>mobsacB</i>	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
PUFRZOR	Km ^R , a 1,119-bp fusion fragment of ZoxyR-H gene in ZJ173 ligated in pUFR034	This study
PUFRROR	Km ^R , a 1,119-bp fusion fragment of RoxyR-H gene in RS105 ligated in pUFR034	This study

^z Rif^R, Km^R, and Nm^R = resistance to rifampicin, kanamycin, and neomycin, respectively.

for RS105. The acetone concentration was 0.4% (vol/vol) in all cases. ROS levels were determined by fluorescence, which was assessed with a fluorescence microplate reader every 20 min during a 4-h period. The excitation wavelength was 488 nm and the emission wavelength was 525 nm. Each sample was assessed three times, and three independent experiments were performed.

Growth rate assay.

The tested strains were grown at 28°C overnight in NB medium (OD₆₀₀ = 1.0). Bacterial cells were collected from 2 ml of culture suspension by centrifugation (5,000 × g for 2 min) and were resuspended in 2 ml of sterile water. A 2-ml volume of cell suspension was then added to 100 ml of NB medium or MMX medium. After the cell suspension was incubated for 12 h, the bacterial growth dynamics were determined by measuring OD₆₀₀ every 2 h in NB medium and every 12 h in MMX medium.

HR assay.

The tested strains were grown at 28°C overnight in NB medium. After the culture suspension was adjusted to an OD₆₀₀ of 0.5 (5 × 10⁸ CFU/ml), it was infiltrated into the leaves of *N. benthamiana* by using a needleless syringe. The HR was assessed after 40 to 48 h of infiltration. Each strain was assayed three times, and three independent experiments were performed.

Pathogenicity assay and determination of bacterial numbers in rice leaves.

Pathogenicity assay was conducted as previously described (Pan et al. 2017). In brief, the tested strains were grown to an OD₆₀₀ of 1.0 (10⁹ CFU/ml). *X. oryzae* pvs. *oryzae* and *oryzicola* strains were inoculated into leaves of 6- and 8-week-old rice plants (IR24, a susceptible cultivar), respectively. The *X. oryzae* pv. *oryzae* suspension was inoculated into leaves that had been cut with sterile scissors, and the *X. oryzae* pv. *oryzicola* suspension was inoculated into leaves that had been pierced with sterile needles. Lesion lengths were scored at 12 dpi. For each experiment, three leaves on each of three plants were inoculated with each strain, and three independent experiments were performed. The average numbers of bacterial CFU per square centimeter in rice leaves inoculated with *X. oryzae* pvs. *oryzae* and *oryzicola* strains were determined immediately after inoculation and at 1 and 4 dpi. Then, 2 cm² of each sample from inoculation sites was cut and surface sterilized in 5% H₂O₂ for 30 s. Sterilized samples were ground in 1 ml of sterile water and serially diluted, and CFU numbers were determined after spreading and incubation. Each sample was cut three leaves. Each leaf represented a biological replicate, and the experiment was performed twice.

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