Overexpression of *oxyR* Increases Phenazine-1-Carboxylic Acid Biosynthesis via Small RNA *phrS* in the Rhizobacterium Strain *Pseudomonas* PA1201

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Phenazine-1-carboxylic acid (PCA) is the primary active component in the newly registered, commercial biopesticide Shenqinmycin and is produced during fermentation by the engineered rhizobacterium strain *Pseudomonas* PA1201. Both *phz1* and *phz2* gene clusters contribute to PCA biosynthesis. In this study, we evaluated the role of OxyR in the regulation of PCA biosynthesis in PA1201. We first showed a functional link between *oxyR* expression and PCA biosynthesis. Deletion of *oxyR* and overexpression of *oxyR* both increase PCA biosynthesis. The molecular mechanisms underlying OxyR regulation of PCA production were investigated using several approaches. OxyR acts divergently in *phz1* and *phz2*. Overexpression of *oxyR* activated the expression of *phz1* and *phz1*-dependent PCA production. However, overexpression of *oxyR* had little effect on *phz2*-dependent PCA biosynthesis, while deletion of *oxyR* promoted *phz2*-dependent PCA production and exerted a negative effect on *phz2* expression. Further, OxyR directly bound to the *phz2* promoter region. In addition, the regulation of PCA biosynthesis by OxyR was associated with quorum sensing (QS) systems. Overexpression of OxyR positively regulated *pqs* QS system. Finally, transcriptomic analysis and subsequent genetic analysis revealed the small RNA *phrS* plays a key role in OxyR-dependent PCA accumulation. Specifically, OxyR directly binds to the *phrS* promoter region to positively regulate *phrS* expression wherein PhrS regulates the PCA positive regulator MvfR in order to control PCA biosynthesis.

Keywords: OxyR, phenazine-1-carboxylic acid, *phrS*, *Pseudomonas aeruginosa*, quorum sensing

Phenazines are nitrogen-containing, redox active compounds with diverse bioactivities and have long been known for their antifungal properties (Mazzola et al. 1992; Lau et al. 2004). Most of these compounds are secondary metabolites that are produced by various bacterial species, including fluorescent pseudomonads, streptomycetes, and archaeal *Methanosarcina* species (Blankenfeldt 2013; Pierson and Pierson 2006, 2010; Zhang et al. 2013). For example, phenazine-1-carboxylic acid (PCA) is produced by plant growth-promoting rhizosphere pseudomonads and has immense potential in agricultural and medicinal applications (Cezairliyan et al. 2013; Hu et al. 2005). Given its broad-spectrum antifungal activities, PCA has been used as the main active ingredient in an environmentally friendly fungicide registered under the name Shenqinmycin (product number PD20110315) by the Ministry of Agriculture of China in 2011. PCA is currently produced from a genetically engineered plant growth-promoting rhizobacterium, *P. aeruginosa* M18, and is used to manufacture Shenqinmycin solutions in China via catalytic fermentation. The 1% Shenqinmycin solutions have been shown to prevent nine types of rice, wheat, and vegetable diseases caused by *Rhizoctonia solani* and *Fusarium oxysporum* (Du et al. 2015; Su et al. 2010; Zhou et al. 2010).

During PCA biosynthesis, chorismic acid is converted to PCA by enzymes encoded by the biosynthetic operon *phzABCDEFGH*. Interestingly, *P. aeruginosa* contains two copies of this operon that are highly conserved, *phzA₁B₁C₁D₁E₁F₁G₁* (*phz1*) and *phzA₂B₂C₂D₂E₂F₂G₂* (*phz2*) (Cui et al. 2016; Li et al. 2011; Mentel et al. 2009; Recinos et al. 2012; Sun et al. 2016). In addition, the genes encoding these enzymes are controlled by complex regulatory networks that respond to environmental cues (Higgins et al. 2018). These networks comprise multiple components including quorum-sensing (QS) systems, two-component systems, small noncoding RNAs, and several specific and global transcriptional regulators (Balasubramanian et al. 2013; Sakhtah et al. 2013; Sonnleitner et al. 2009; Williams and Cámara 2009).

Numerous efforts have been made to maximize the PCA fermentation titer of strain M18, including altering fermentation

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conditions like temperature (Huang et al. 2009) and nutrient levels (Li et al. 2008) in addition to genetic engineering (Zhou et al. 2010). Nevertheless, strain M18 is still limited by unstable fermentation titers and high production costs, thereby rendering it a suboptimal strain for PCA production (Zhou et al. 2016). To overcome these limitations, the novel strain *P. aeruginosa* PA1201 was isolated from rice rhizospheres and was shown to produce high levels of PCA (Zhou et al. 2015). Further, deletion of the PCA modification genes *phzS*, *phzM*, and *phzH* generated a strain called MSH, which produces PCA as its sole phenazine (Jin et al. 2015). Thus, the MSH strain exhibits tremendous potential in studying the regulatory mechanism of PCA biosynthesis.

To further enhance PCA biosynthesis, it is critical to elucidate the biosynthetic pathways and regulatory networks that control PCA biosynthesis. We previously showed that both the *rhl* QS system and the *pqs* QS system were indispensable for PCA biosynthesis (Sun et al. 2016). The transcriptional regulator RsaL is a potent repressor of PCA biosynthesis that negatively regulates *phz1* expression and positively regulates *phz2* expression via multiple mechanisms (Sun et al. 2017). Further, the antiactivator QslA negatively regulates PCA biosynthesis by interacting with MvfR (Fang et al. 2018). Accordingly, the deletion of *rsaL* and *qslA* results in a significant increase in the PCA fermentation titer (Fang et al. 2018; Sun et al. 2017).

PA1201 contains a gene encoding the LysR-type transcriptional regulator OxyR, which is involved in H₂O₂ sensing and the regulation of antioxidant defense systems in many bacterial species (Imlay 2015). For example, OxyR was shown to activate the transcription of antioxidant genes in the human opportunistic pathogen *P. aeruginosa* PAO1, including *kata* (catalase A), *katB* (catalase B), and *ahpB/CF* (alkyl hydroperoxide reductases B/CF), which can protect bacterial cells from exogenous reactive oxygen species (ROS) produced from the human immune system (Wei et al. 2012). In addition to its role in oxidative stress protection, the upregulation of antioxidant enzymes in response to OxyR activation contributes to *P. aeruginosa* virulence by conferring increased resistance to human phagocytic cells (Lau et al. 2005) and the secretion of exotoxins (Melstrom et al. 2007). However, the role of *oxyR* in PA1201 is unknown.

The antifungal activity of PCA is generally attributed to its ability to generate ROS that interferes with the redox homeostasis of target pathogens, subsequently leading to cell death (Xu et al. 2015). Because PA1201 produces PCA, it has likely developed tolerance toward redox-active PCA via multiple mechanisms that can defend against the oxidative damage from ROS (Imlay 2015; Xu et al. 2015). Indeed, redox-active phenazines have been suggested to directly activate expression of antioxidant-dependent genes in phenazine-producing bacteria rather than acting on the ROS themselves (Dietrich et al. 2006; Gu and Imlay 2011). In addition to its role as a biological control agent of plant diseases, PCA is involved in the pathogenesis and virulence of *P. aeruginosa* via its action as an intercellular signaling molecule that facilitates biofilm formation (Okegbe et al. 2012; Wang et al. 2011). Given the paradoxical role of PCA in *P. aeruginosa*, we hypothesized that OxyR plays a key role in the regulation of PCA biosynthesis.

RESULTS

oxyR is responsible for H₂O₂ tolerance.

A homologous gene of PAM18_5464 in MSH encoded OxyR. OxyR is a LysR-type transcriptional regulator that constitutes an N-terminal helix-turn-helix DNA-binding domain and a C-terminal regulatory domain of the PBP2 superfamily. The gene is conserved among other *Pseudomonas* strains (Supplementary

Fig. S1A). Considering the well-known role of *oxyR* and PCA in bacterial antioxidant activities, the timing of *oxyR* expression and PCA biosynthesis during PA1201 growth was first evaluated in pigment-producing medium (PPM), which exhibits higher PCA production (Jin et al. 2015; Zhou et al. 2015). *oxyR* expression levels were determined by quantitative reverse transcription (qRT)-PCR and PCA production was analyzed by high performance liquid chromatography (HPLC), as previously described (Sun et al. 2016). Increases in both PCA production and *oxyR* expression were observed in parallel with cell growth (Fig. 1A), suggesting a functional link between *oxyR* expression and PCA biosynthesis. To then evaluate the effects of OxyR on H₂O₂ resistance of PA1201, an *oxyR* in-frame deletion strain was constructed for strain MSH (hereafter designated as $\Delta oxyR$), and an *oxyR* overexpression strain was constructed in the $\Delta oxyR$ mutant via a pBBR-1-MCS-derivative multicopy plasmid [hereafter $\Delta oxyR(oxyR)$]. The tolerance of the two strains, $\Delta oxyR$ and $\Delta oxyR(oxyR)$, to H₂O₂ was evaluated on PPM agar plates containing 0.1 mM H₂O₂. Compared with the parental strain MSH, $\Delta oxyR$ was more sensitive to H₂O₂, while $\Delta oxyR(oxyR)$ could tolerate a higher concentration of H₂O₂ (Fig. 1B). Thus, a positive regulatory role of OxyR was implicated in the mitigation of oxidative stress in PA1201.

Overexpression of *oxyR* significantly increased PCA production.

To evaluate the effects of *oxyR* on PCA biosynthesis, the aforementioned MSH strains were subjected to PCA production assays when grown in PPM medium. No significant differences were observed in the growth rates of all the strains (Supplementary Fig. S2A). Deletion of *oxyR* or *oxyR* overexpression in the MSH strain had little effect on PCA biosynthesis 12 h after inoculation (Fig. 2A). However, the PCA levels in the $\Delta oxyR$ cultures 24 and 48 h after inoculation were slightly higher than in the MSH cultures. The slightly increased production of PCA

Fig. 1. The expression level of *oxyR* is associated with phenazine-1-carboxylic acid (PCA) biosynthesis and H₂O₂ resistance in MSH. **A**, Bacterial growth over time, PCA concentrations and *oxyR* levels. The datapoints are averages for three technical repeats. **B**, Spot-inoculated, serially diluted MSH-derived bacterial suspensions on Luria Bertani agar plates that were supplemented with 0.1 mM H₂O₂.

could be restored to the levels exhibited by strain MSH by chromosomal integration of a single copy of *oxyR* at the *attB* locus ($\Delta oxyR::oxyR$) (Fig. 2A). Surprisingly, the *oxyR* overexpression strain $\Delta oxyR(oxyR)$ produced much higher levels of PCA than the MSH and $\Delta oxyR::oxyR$ strains 24 and 48 h after inoculation. A 2.4-fold increase in PCA production by strain $\Delta oxyR(oxyR)$ was observed when compared against strain MSH 48 h after inoculation. To further confirm the effects of *oxyR* overexpression on PCA biosynthesis, the above strains were grown in Luria Bertani (LB) medium and PCA biosynthesis was determined. Consistent with the above observations, the PCA production of strain $\Delta oxyR(oxyR)$ was also significantly elevated compared with strains MSH and $\Delta oxyR$ when grown in LB medium (Fig. 2B). Thus, overexpression of *oxyR* could be introduced into strain MSH to improve PCA production. The molecular mechanisms underlying this process were then investigated in further detail.

Effects of *oxyR* on *phz1*-dependent PCA production and *phz1* expression.

We previously observed that the two nearly identical PCA biosynthetic operons, *phz1* and *phz2*, differentially contribute to PCA synthesis in MSH (Sun et al. 2016). Thus, constructed $\Delta phz2$ and $\Delta phz1$ strains could be used to analyze *phz1*-dependent and *phz2*-dependent PCA biosynthesis, respectively (Sun et al. 2017). To elucidate the regulatory effect of OxyR on the *phz1* operons, we generated a $\Delta phz2\Delta oxyR$ strain and the corresponding complementation strain, $\Delta phz2\Delta oxyR(oxyR)$.

Fig. 2. Overexpression of *oxyR* significantly increases phenazine-1-carboxylic acid (PCA) biosynthesis. **A**, PCA production of MSH-derived strains in pigment-producing medium (PPM). **B**, PCA production of MSH-derived strains in Luria Bertani (LB) medium. Data are averages for three technical repeats. Statistically significant differences are indicated by one ($P \leq 0.05$) or two asterisks ($P \leq 0.01$).

oxyR deletion in $\Delta phz2$ did not significantly affect bacterial growth in PPM medium (Supplementary Fig. S2C). PCA levels in the $\Delta phz2\Delta oxyR$ cultures 12 to 48 h after inoculation were not significantly different from those in the $\Delta phz2$ cultures (Fig. 3A). In contrast, PCA levels in the $\Delta phz2\Delta oxyR(oxyR)$ cultures 36 and 48 h after inoculation were significantly higher than those in the $\Delta phz2$ and $\Delta phz2\Delta oxyR$ cultures (Fig. 3A). Strain $\Delta phz2\Delta oxyR(oxyR)$ produced 10.7 and 12.2 mg of PCA per liter at 36 and 48 h, respectively. In contrast, only 7.0 and 7.6 mg of PCA per liter were produced by strain $\Delta phz2$ at 36 and 48 h, respectively. (Fig. 3A). These differences correspond to an approximately 1.6-fold increase in PCA production in strain $\Delta phz2\Delta oxyR(oxyR)$ as compared with strain $\Delta phz2$. Thus, deletion of *oxyR* has little effect on *phz1*-dependent PCA biosynthesis, while overexpression of *oxyR* promotes *phz1*-dependent PCA biosynthesis during later growth stages.

To further verify the regulatory effect of OxyR on the *phz1* operon, the previously constructed fusion reporter gene $P_{phz1-lacZ}$ (Sun et al. 2016) was integrated into strain $\Delta phz2\Delta oxyR$ to generate the reporter strain $\Delta phz2\Delta oxyR::P_{phz1-lacZ}$. The β -galactosidase (GUS) activity was then determined, to evaluate *phz1*-dependent transcriptional activity. GUS activity indicated that the deletion of *oxyR* had little effect on *phz1*-dependent activity. However, overexpression of *oxyR* significantly increased *phz1*-dependent GUS activity (Fig. 3B). These results further demonstrated that overexpression of *oxyR* activates *phz1*-dependent PCA production and exerts a positive effect on *phz1* expression at the transcriptional level.

Effects of *oxyR* on *phz2*-dependent PCA production and *phz2* expression.

The *phz2* gene cluster makes a greater contribution to the PCA production (Sun et al. 2016). To elucidate the regulatory effect of OxyR on the *phz2* operon, a $\Delta phz1\Delta oxyR$ was generated in addition to the corresponding complementation strain $\Delta phz1\Delta oxyR(oxyR)$. Deletion of *oxyR* in the strain $\Delta phz1$ had no effect on PCA biosynthesis at 12 and 24 h. However, it led to significant 1.15- to 1.2-fold increases in PCA levels at 36 and 48 h after inoculation into PPM medium. Specifically, $\Delta phz1\Delta oxyR$ produced 103 and 115 mg of PCA per liter at 36 and 48 h, respectively. In contrast, only 89.5 and 96 mg of PCA per liter were produced by strain $\Delta phz1$ at 36 and 48 h, respectively (Fig. 3C). Overexpression of *oxyR* in strain $\Delta phz1$ had little effect on PCA biosynthesis (Fig. 3C). Thus, overexpression of *oxyR* has little effect on *phz2*-dependent PCA biosynthesis, while deletion of *oxyR* promotes *phz2*-dependent PCA biosynthesis during later growth stages.

To further verify the regulatory effects of OxyR on the *phz2* operon, the previously constructed fusion reporter gene $P_{phz2-lacZ}$ (Sun et al. 2016) was integrated into strain $\Delta phz1\Delta oxyR$ to generate the reporter strain $\Delta phz1\Delta oxyR::P_{phz2-lacZ}$. GUS was again used to evaluate *phz2*-dependent transcriptional activity. Overexpression of *oxyR* had little effect on *phz2*-dependent activity, while deletion of *oxyR* significantly increased *phz2*-dependent activity (Fig. 3D). Thus, deletion of *oxyR* promotes *phz2*-dependent PCA production and exerts a negative effect on *phz2* expression at the transcriptional level.

OxyR directly binds to the *phz2* promoter region but not to the *phz1* promoter region.

Considering that OxyR is a transcriptional factor, we hypothesized that OxyR directly bound one or both *phz1* and *phz2* to regulate PCA biosynthesis. An electrophoretic mobility shift assay (EMSA) was used to further explore this possibility. *oxyR* from *P. aeruginosa* MSH was first cloned into the pET28a vector and was expressed in the heterologous host *Escherichia coli* BL21 (DE3, pLysS). The expressed OxyR-His₆ fusion

protein was then purified by Ni²⁺-affinity chromatography (Fig. 4A). Second, biotin-labeled DNA probes corresponding to a DNA fragment encompassing the 339-bp region upstream of

Fig. 3. Effects of *oxyR* deletion and overexpression on *phz1*/*phz2*-dependent phenazine-1-carboxylic acid (PCA) biosynthesis and *phz1*/*phz2*-dependent β -galactosidase (GUS) activities. **A**, *phz1*-dependent PCA biosynthesis. **B**, *phz1*-dependent GUS activities. **C**, *phz2*-dependent PCA biosynthesis. **D**, *phz2*-dependent GUS activities. Data are averages for three technical repeats. Statistically significant differences are indicated by one ($P \leq 0.05$) or two asterisks ($P \leq 0.01$).

the *phz2* translational start site (i.e., P_{phz2}) and a DNA fragment encompassing the 368-bp region upstream of the *phz1* translational start site (i.e., P_{phz1}), respectively, were produced by PCR amplification. EMSA was performed with purified OxyR and a DNA probe as previously described (Sun et al. 2017). Purified OxyR (0.45 to 1.2 mg) formed a stable complex with 10 ng of labeled P_{phz2} probes. The binding was hampered by the addition of 2,000 ng of unlabeled P_{phz2} (Fig. 4B). These results thus suggest a direct interaction between OxyR and the promoter region of *phz2* that negatively regulates *phz2* expression. In contrast, purified OxyR was not able to form stable complexes with the promoter region P_{phz1} (Fig. 4C), suggesting an indirect regulatory effect of OxyR on *phz1* expression.

OxyR overexpression positively regulates the biosynthesis of QS signals 2-heptyl-4-hydroxyquinoline (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS). QS systems play a global regulatory role in PCA synthesis by MSH (Sun et al. 2016). Consequently, we evaluated whether QS systems are involved in the OxyR-mediated regulation of the two *phz* clusters. The abundances of two types of acyl homoserine lactone QS signal molecules, *N*-3-oxo-dodecanoyl homoserine lactone and *N*-butanoyl-homoserine lactone (i.e., 3-oxo-C12-HSL and C4-HSL, respectively) were qualitatively determined using the biosensor strains *Agrobacterium tumefaciens* CF11 and *Chromobacterium violaceum* 026 (CV026). Deletion or overexpression of *oxyR* had no significant effects on 3-oxo-C12-HSL and C4-HSL biosynthesis (Supplementary Fig. S3A and B). To further verify the above results, both 3-oxo-C12-HSL and C4-HSL were extracted from the cultures 12 and 36 h after inoculation and were measured, using ultra-performance liquid chromatographic time of flight mass spectrometry (UPLC-TOF-MS), as previously described (Sun

Fig. 4. OxyR directly binds to the *phz2* promoter region but not to the *phz1* promoter region. **A**, OxyR protein purification. Lane 1, total proteins and lane 2, purified OxyR proteins. **B**, Electrophoretic mobility shift assay (EMSA) results using purified OxyR protein and Cy5-labeled P_{phz2} as a probe. **C**, EMSA results using purified OxyR protein and Cy5-labeled P_{phz1} as a probe.

et al. 2016, 2017). The quantitative analysis indicated that deletion or overexpression of *oxyR* did not significantly affect production of 3-oxo-C12-HSL and C4-HSL (Supplementary Fig. S3C and D). Furthermore, the expression levels of *lasR* and *rhlR* that encode the regulators of the *las* and *rhl* QS systems were also investigated using the reporter strains $\Delta oxyR::PrhlR-lacZ$, $\Delta oxyR::PrhlR-lacZ(oxyR)$, $\Delta oxyR::PlasR-lacZ$, and $\Delta oxyR::PlasR-lacZ(oxyR)$. *lasR* and *rhlR* promoter-dependent GUS activity did not result in significant changes at any of the three

evaluated timepoints (Supplementary Fig. S4E and F). These results thus suggest that neither the *las* system nor the *rhl* system are involved in the regulation of OxyR-activated PCA production.

The *pqs* QS system in MSH comprises two major signal molecules, HHQ and PQS (Sun et al. 2016). Thus, the HHQ and PQS concentrations in the cultures of *oxyR* deletion and overexpression strains were measured using previously established UPLC-TOF-MS assays (Sun et al. 2016). Endogenous PQS levels were much higher than HHQ levels in MSH when grown in PPM medium (Fig. 5). No significant differences were observed in HHQ and PQS levels among MSH, $\Delta oxyR$, and $\Delta oxyR(oxyR)$ cultures when grown in PPM for 24 h. Thus, deletion of *oxyR* or overexpression of *oxyR* did not significantly affect the production of HHQ and PQS 24 h after inoculation. The levels of HHQ among all three strains were much lower in the PPM cultures 48 h after inoculation compared with those 24 h after inoculation. Deletion of *oxyR* did not significantly affect the production of both HHQ and PQS. However, overexpression of *oxyR* significantly increased HHQ production from 0.17 to 4.1 μ M 48 h after inoculation (Fig. 5A). In addition, PQS production 48 h after inoculation was higher than that at 24 h after inoculation. Deletion of *oxyR* in strain MSH had no significant effect on PQS production, although overexpression of *oxyR* in strain $\Delta oxyR$ significantly increased PQS levels from 30 to 61 μ M 48 h after inoculation (Fig. 5B).

To further verify the effects of *oxyR* on the PQS system, qRT-PCR was used to assess the transcriptional activity of *mvfR*, which is a key regulator of PQS signal biosynthesis. Deletion of *oxyR* had little effect on transcriptional expression of *mvfR* 24 and 48 h after inoculation compared with the parental strain MSH (Fig. 5C). Likewise, overexpression of *oxyR* did not significantly affect *mvfR* expression 24 h after inoculation but significantly increased *mvfR* expression 48 h after inoculation (Fig. 5C). These results suggest the involvement of the *pqs* system in the regulation of OxyR-activated PCA accumulation during later growth stages.

RNA-Seq analysis of the *oxyR*-overexpression strain.

To further identify the regulatory effect of OxyR on PCA production and the *pqs* QS system, the gene expression profiles of *oxyR* overexpression strains were evaluated and compared with those of the MSH strain. Total RNA was extracted from the cell cultures of $\Delta oxyR(oxyR)$ and MSH 24 h after inoculation, followed by RNA-Seq-based transcriptomic analysis. The distributions of gene coverage in the $\Delta oxyR$ and $\Delta oxyR(oxyR)$ strains are shown in Supplementary Figure S5A and B. Differentially expressed genes, based on the $\log_2$ ratio of $\Delta oxyR(oxyR)$ /MSH, were then selected for further analysis. Among these, 68 genes were repressed by OxyR while 101 genes were activated (Supplementary Fig. S4C). The differentially expressed genes could be divided into three categories (Table 1): i) oxidative stress response genes, including *recG*, *katB*, and *katE*; ii) virulence-related genes, especially including phenazine biosynthesis genes like *phz1*; and iii) putative transcriptional regulators and hypothetical proteins. Thus, the *oxyR* regulon identified by transcriptomic analysis is generally consistent with the above phenotypic analysis.

The small RNA *phrS* plays a key role in OxyR-dependent PCA accumulation.

Transcriptional regulators were targeted among the OxyR-regulated genes for further investigation (Table 1). Regulator proteins would typically act downstream of OxyR to mediate regulation of PCA biosynthesis. To identify a hypothetical regulator, regulator genes were individually deleted in strain $\Delta oxyR(oxyR)$. The PCA production of the resultant mutants

Fig. 5. Effects of *oxyR* overexpression on 2-heptyl-4-hydroxyquinoline (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS) production in addition to *mvfR* levels. **A**, HHQ levels at 12 to 48 h after inoculation, as determined by ultra-performance liquid chromatographic time of flight-mass spectrometry (UPLC-TOF-MS). **B**, PQS levels at 12 to 48 h after inoculation, as determined by UPLC-TOF-MS. **C**, Relative *mvfR* expression levels, as determined by quantitative reverse transcription PCR. Data are averages for three technical repeats. Statistically significant differences are indicated by an asterisk ($P \leq 0.05$).

were compared against parental strain $\Delta oxyR(oxyR)$ at 24 and 48 h after inoculation into PPM medium. Only deletion of PAM18_1661 led to a significant decrease in PCA levels at 24 and 48 h after inoculation (Fig. 6B). Deletion of other genes either had no significant effect on PCA production or only increased PCA production 48 h after inoculation (Supplementary Fig. S5).

PAM18_1661 (hereafter referred to as 1661) encodes a hypothetical protein that is only 37 amino acids in length. To further verify its role in OxyR-mediated PCA production, 1661 and 1661 with its promoter region (hereafter 1661p) were overexpressed via the cosmid vector pLARF3 in strain $\Delta oxyR(oxyR)\Delta 1661$. Surprisingly, overexpression of 1661 or 1661p was unable to restore PCA production levels to that of strain $\Delta oxyR(oxyR)\Delta 1661$ (Fig. 6B). Further examination of the PAM18_1661 locus revealed that the coding region of the small RNA *phrS* overlapped with the coding sequences of PAM18_1661. Both *phrS* and 1661 are located in the same transcribing orientation on the chromosome (Fig. 6A). Indeed, overexpression of *phrS* in strain $\Delta oxyR(oxyR)\Delta 1661$ could fully restore PCA production. However, overexpression of *phrS* lacking 39 bp (i.e., *phrS*-39) in strain $\Delta oxyR(oxyR)\Delta 1661$ and the overexpression part of *phrS* (i.e., 1661 and 1661p) all failed to restore PCA production (Fig. 6B). Thus, the small RNA *phrS* rather than PAM18_1661 likely plays a key role in increased PCA production via OxyR overexpression.

To further verify the role of *phrS* in PCA production, it was deleted in the $\Delta oxyR$ and $\Delta oxyR(oxyR)$ strains. The PCA production of the resultant strains $\Delta oxyR\Delta phrS$ and $\Delta oxyR(oxyR)\Delta phrS$ were then analyzed in PPM medium. Deletion of *phrS* in strain $\Delta oxyR$ did not significantly affect PCA production, but deletion of *phrS* halted the increase of PCA production in the *oxyR* overexpression strain (Fig. 6C).

OxyR directly binds to the *phrS* promoter region to positively regulate *phrS* expression.

To verify the potential role of *phrS* in OxyR-dependent PCA accumulation, we evaluated OxyR-dependent *phrS* expression via the generation of two reporter strains $\Delta oxyR::PphrS-lacZ$ and $\Delta oxyR(oxyR)::PphrS-lacZ$. The transcriptional activity of

phrS was then monitored by measuring GUS activity. The *phrS* promoter-dependent GUS activity in $\Delta oxyR(oxyR)::PphrS-lacZ$ was significantly higher than in the $\Delta oxyR::PphrS-lacZ$ cultures 24 to 48 h after inoculation (Fig. 7A).

The transcriptional activator OxyR has been shown to bind to a conserved site (ATTGATCTCGCTTAT) in the *P. aeruginosa* promoter regions of *ahpC* and *katA* (Wei et al. 2012). We identified a putative OxyR binding site, ATTGATCCAGGT CAA, in the promoter region of *phrS* that shares 67% nucleotide identity with the identified OxyR-binding site in the *phrS* promoter region (Fig. 7B). We therefore investigated whether OxyR directly binds to the *phrS* promoter to regulate its expression, by preparing a Cy5-labeled 369-bp DNA fragment comprising the region upstream of the *phrS* translational start site (termed P_{phrS}). EMSA was then conducted with purified OxyR protein and the P_{phrS} probe and indicated that OxyR could form stable complexes with the *phrS* promoter region (Fig. 7C). Further, point mutations of GAT to a CCC motif in the putative OxyR binding site of the *phrS* promoter region halted the formation of the OxyR- P_{phrS} complex (Supplementary Fig. S6).

Fig. 6. The small RNA *phrS* is involved in the regulation of OxyR-dependent phenazine-1-carboxylic acid (PCA) production. **A**, Genomic loci of PAM18_1661 and *phrS* in the PA1201 chromosome. **B**, Only *phrS* complemented the PAM18_1661 deletion mutant to restore PCA production. **C**, Deletion of *phrS* in the *oxyR* overexpression strain $\Delta oxyR(oxyR)$ significantly decreased PCA production. Data are averages for two technical repeats. Statistically significant differences are indicated by one ($P \leq 0.05$) or two asterisks ($P \leq 0.01$).

Point mutation of base-pairing residues in *phrS* significantly affected *oxyR*-dependent PCA production.

phrS has been identified as an activator of PqsR (also termed MvfR) synthesis, which is one of the key QS regulators in *P. aeruginosa* PAO1 (Sonnleitner et al. 2011). *phrS* acts by base-pairing with an upstream open reading frame (uof)-pqsR transcript. In addition, the expression of *phrS* requires the oxygen-responsive regulator ANR (Sonnleitner et al. 2011). Given the direct binding of the *phrS* promoter by OxyR (Fig. 7C) and the involvement of both genes in PCA production (Fig. 6B), we hypothesized that PhrS might use the same base-pairing mechanism to mediate OxyR-dependent PCA production in MSH. To evaluate this hypothesis, point mutations were introduced into the base-pairing region of *phrS* in the chromosomes of the $\Delta oxyR$ and $\Delta oxyR(oxyR)$ strains (Fig. 8A). The resultant strains, $\Delta oxyR::phrSm$ and $\Delta oxyR(oxyR)::phrSm$, were subjected to PCA production assays in PPM medium at 24 and 48 h after inoculation (Fig. 8B). PCA levels in the $\Delta oxyR::phrSm$ cultures were not significantly different from those of $\Delta oxyR$ (Fig. 8B). In contrast, PCA levels in the $\Delta oxyR(oxyR)::phrSm$ cultures were significantly lower than those for $\Delta oxyR(oxyR)$ (Fig. 8B). Thus, the mutated *phrS* less efficiently

mediated PCA production by *oxyR* overexpression, which was likely due to the disrupted PhrS/MvfR complex.

DISCUSSION

Phenazines are aromatic nitrogen-containing compounds that are produced by pseudomonads, among other microorganisms, as secondary metabolites. They exhibit strong redox activities and can react with oxygen to produce a variety of toxic ROS. Consequently, these compounds can affect a wide diversity of organisms, including cells that produce it, in effects ranging from deleterious oxidative stress to beneficial effects like survival (Fröhlich and Gottesman 2018; Imlay 2015). Phenazines play important roles in *P. aeruginosa* by serving as signaling molecules that facilitate biofilm formation (Wang et al. 2011) and by promoting redox homeostasis (Price-Whelan et al. 2007). Given the paradoxical role of phenazines in *Pseudomonas* spp., the biosynthesis of these compounds should be tightly regulated to prevent oxidative damage. Moreover, because phenazine is an important regulator of antioxidant defense systems, we hypothesized that OxyR might be involved in the regulatory network of *Pseudomonas* PCA biosynthesis. The results described here indicate a crucial role of OxyR in physiological responses to oxidative stress and that OxyR is an important regulator of PCA synthesis.

Fig. 7. OxyR directly binds to the *phrS* promoter to positively regulate *phrS* expression. **A**, Overexpression of *oxyR* significantly increased *phrS* promoter-dependent β -galactosidase activities. **B**, Identification of a putative OxyR binding site in the *phrS* promoter region. **C**, Electrophoretic mobility shift assay analysis showing that OxyR directly binds to the *phrS* promoter region.

Fig. 8. Point mutation of base-pairing residues in *phrS* significantly affects *oxyR*-dependent phenazine-1-carboxylic acid (PCA) production. **A**, The proposed base-pairing mechanism between *phrS* and *pqsR* (also known as *mvfR*) by Sonnleitner et al. (2011). **B**, Point mutation of CG...C to GT...T in *phrS*. **C**, Overexpression of *oxyR* in the strain with mutations in *phrS* was less influential on PCA production than in the strain with wild-type *phrS*. Data are averages for two technical repeats. Statistically significant differences are indicated by one ($P \leq 0.05$) or two asterisks ($P \leq 0.01$).

A previous investigation of *P. aeruginosa* PAO1 indicated that the null mutation of *oxyR* leads to a significant increase in the production of another phenazine compound (pyocyanin) and that overexpression of *oxyR* by pBBR1MCS leads to a further increase of pyocyanin (Vinckx et al. 2010). Increased PCA production was observed in the $\Delta oxyR$ or $\Delta oxyR(oxyR)$ strains in the present study, which coincides with previous studies (Fig. 2). The *oxyR* overexpression strain $\Delta oxyR(oxyR)$ produced considerably more PCA than the *oxyR* deletion strain, suggesting that OxyR protein levels are involved in MSH gene regulation. In the wild-type strain, normal expression levels of OxyR are responsible for scavenging ROS that are induced by the presence of PCA. When complemented with a high copy plasmid, higher OxyR levels can activate more genes to remove ROS. Thus, the reduction of ROS may ameliorate the oxidative stress exerted by PCA production, which in turn stimulates PCA production. However, this feedback mechanism requires further investigation to validate.

Both of the *phz1* and *phz2* gene clusters collectively contribute to PCA biosynthesis in MSH (Sun et al. 2016). In the present study, the first evidence is shown that OxyR influences PCA production by differentially regulating *phz1* and *phz2* gene clusters in strain MSH (Figs. 2 and 3). Overexpression of *oxyR* decreased *phz2*-dependent PCA production and increased *phz1*-dependent PCA production (Figs. 2 and 3). Moreover, the present study provides the first evidence that OxyR directly binds to the promoter region of *phz2* and thereby decreases transcriptional levels of *phz2*. When *oxyR* was inactivated, the repression was relieved and *phz2*-dependent PCA production increased (Fig. 3C). When *oxyR* is overexpressed, *phz2* exhibit similar transcriptional levels to $\Delta phz1$ (Fig. 3D) and the transcriptional levels of *phz1* were higher than $\Delta phz2$ (Fig. 3B). The *phz1*-dependent PCA production increased about 1.6-fold independent of *phz2* (Fig. 3A). A regulatory feedback loop involves the expression of *phz1* and *phz2* gene clusters; in the presence of *phz2*, the *phz1* cluster was induced to produce a large amount of PCA (Li et al. 2011). These results indicate that overall increases in PCA biosynthesis in strain $\Delta oxyR(oxyR)$ are a result of multiple interactions between the opposing regulatory effects elicited by OxyR on the two *phz* clusters.

Previous studies have shown that QS systems are one of the most important mechanisms for regulating PCA biosynthesis in strain MSH (Fang et al. 2018; Sun et al. 2016). In the present study, HHQ and PQS production were both positively regulated by *oxyR* overexpression. This result suggested the possibility that the *pqs* QS system is involved in *oxyR*-activated PCA production. Given that *mvfR* expression was not altered based on transcriptomic analysis, the regulation between OxyR and the *pqs* QS system likely does not involve a direct interaction at the transcriptional level. Based on our results, we concluded that the direct inhibitory effect on *phz2* was counteracted by the positive effect through the *pqs* system in the *oxyR* overexpression strain.

Several small regulatory RNAs mediate the fine-tuned regulation of the QS system in *P. aeruginosa* (Balasubramanian et al. 2013). The small RNA *phrS* has been reported as an activator of MvfR translation. Further, the sRNA *phrD* positively affects RhlR expression in *P. aeruginosa* without the involvement of any *Pseudomonas*-specific proteins (Malgaonkar and Nair 2019). Unlike other base-pairing sRNAs, *PhrS* is an activator of MvfR synthesis and uses a base-pairing mechanism to activate a short upstream open reading frame, to which the *mvfR* is translationally coupled (Sonnleitner et al. 2011). In addition, the expression of *phrS* requires the oxygen-responsive regulator ANR (Sonnleitner et al. 2011). In the present study, transcriptomic analysis and subsequent screening revealed that *phrS* plays a key role in OxyR-dependent PCA production (Fig.

6). OxyR activates the transcription of *phrS* by directly binding to its promoter region and the base-pairing mechanism is required for OxyR-dependent PCA production (Figs. 7 and 8). Thus, the results described here demonstrate an association between OxyR, oxygen availability, QS, and PCA biosynthesis in strain MSH.

MvfR is a key regulator of the biosynthesis of the QS signals HHQ and PQS. It directly binds to the promoter region of the *pqsABCD* gene cluster to initiate its expression (Xiao et al. 2006). In addition, MvfR is essential for *phz1* and *phz2* expression and PCA biosynthesis (Sun et al. 2016). Moreover, recent results indicated that MvfR can bind to the promoter region of *phz1* to regulate PCA biosynthesis (Fang et al. 2018). These results are consistent with a significant increase in *phz1*-dependent PCA production in $\Delta oxyR(oxyR)$ (Fig. 3). Taken together, these results point to a general working model for OxyR-dependent PCA production in MSH through *phrS*. In the model, the peroxide stress response regulator OxyR senses endogenous ROS levels and activates the transcription of *phrS*. *phrS* then interacts with *mvfR* through a base-pairing mechanism to activate its function. The activated MvfR then binds to the *phz1* promoter to activate PCA biosynthesis and, also, to the *pqsA* promoter to promote HHQ and PQS production. Although more detailed mechanisms remain to be elucidated, a brief working model for the regulation of PCA biosynthesis in $\Delta oxyR(oxyR)$ is proposed (Fig. 9).

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation.

The bacterial strains, plasmids, and amplification primers that were used in this study are listed in Supplementary Tables S1, S2, and S3, respectively. PA1201 and its derived strains were grown at 28°C in PPM (Shtark et al. 2003; Sun et al. 2016). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth. The media were supplemented with the following antibiotics when needed: spectinomycin (50 µg/ml), tetracycline (100 µg/ml for *P. aeruginosa* strains and 10 µg/ml for *E. coli* strains), kanamycin (50 µg/ml), and gentamycin (100 µg/ml for *P. aeruginosa* strains and 20 µg/ml for *E. coli* strains).

Generation of in-frame deletion and overexpression mutants.

In-frame deletion mutants were generated via homologous recombination in pEX18Gm cells, as previously described (He et al. 2006; Sun et al. 2016). The new markerless mutants were validated by colony PCR and subsequent DNA sequencing. Triparental mating between PA1201 and *E. coli* was carried out with the helper strain *E. coli* (pRK2013). To overexpress plasmids, the coding and promoter regions were cloned into the pBBR-1-MCS plasmid (Kovach et al. 1995). Plasmid DNA preparation, enzyme digestion, agarose gel electrophoresis, and ligation were performed using previously described standardized methods (Sambrook and Russell 2001).

PCA extraction and quantification by HPLC analysis.

To extract PCA from cultures, 180 µl of fermentation culture was mixed with 20 µl of 6 M HCl and were then extracted with 540 µl of chloroform, as previously described (Jin et al. 2015). A 3-µl aliquot of the extracted PCA sample was then subjected to HPLC analysis (Agilent Technologies 1260 Infinity), using a C18 reversed-phase column (5 µm, 4.6 × 150 mm) eluted with acetonitrile-5 mM ammonium acetate (60:40, vol/vol). PCA production was quantified by HPLC peak area (A) using the formula $PCA\ (mg/liter) = 0.0146A - 0.341$, which was derived from a dose-peak area plot with a correlation coefficient (R^2) of 0.999, using purified PCA.

Construction of promoter-lacZ fusion and β -galactosidase assays.

To generate promoter-lacZ fusions, promoter regions (approximately 500 bp) with coding sequences (approximately 30 bp) from the target genes, *phz1*, *phz2*, *phrS*, *pqsA*, and *mvfR* were individually PCR-amplified and were cloned into the mini-CTX-lacZ vector (Becher and Schweizer 2000). The resultant plasmids were integrated into the chromosome of PA1201-derived strains as previously described (Sun et al. 2016). GUS activity was evaluated in the resultant strains, as previously described (Sambrook and Russell 2001). The experiments were performed in triplicate at different points of the bacterial growth curves in PPM medium.

Bioassays of C4-HSL and 3-oxo-C12-HSL production.

The synthesis of C4-HSL and 3-oxo-C12-HSL in PA1201 and derived mutant strains were detected semiquantitatively by diffusion plate assays, using *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* CF11 biosensors, respectively, as previously described (McClellan et al. 1997; Zhang et al. 2007). Blue or purple spots on the plates indicated that the diffusible QS signals were detected by the reporter cells. The production of C4-HSL and 3-oxo-C12-HSL was measured in proportion to the diffusion distance of the molecules from the PA1201 strain colonies.

Extraction and quantification of QS signaling molecules by LC-MS.

QS signal molecule extraction and quantification was conducted as previously described (Sun et al. 2016). Briefly, 270 μ l of culture supernatant was adjusted to pH 4.0 with the addition of 6 M HCl and was then extracted with an equal volume of ethyl acetate. A total of 100 μ l of ethyl acetate extract was subsequently collected and evaporated at 40°C. The dried pellet was dissolved in 500 μ l of methanol, and a 10- μ l aliquot of this sample was then injected into an ultra-performance LC column that was coupled with a mass spectrometer (Agilent UPLC1290-TOF-MS6230) using an Agilent Zorbax XDB C18 reverse-phase (5 μ m, 4.6 $\times$ 150 mm) system separated by a

acetonitrile gradient with 0.5% acetic acid and H₂O with 0.5% (vol/vol) acetic acid at a rate of 0.4 ml/min. The MS analysis was performed under positive mode with a scanning range of $m/z = 100 - 1,700$. QS molecule concentrations were quantified from the peak area (A) of the specific extracted ion chromatogram (EIC) of the total ion chromatogram (TIC) according to the following formula: 3-oxo-C12-HSL (μ M) = $6.0 \times 10^{-7}A$. The formula was derived from a dose–peak area plot using standard 3-oxo-C12-HSL at different concentrations, which exhibited a correlation coefficient of $R^2 = 0.979$. The same procedure was used to determine the concentrations of C4-HSL, HHQ, and PQS, based on the following formulations, respectively: C4-HSL (μ M) = $2.0 \times 10^{-6}A$ ($R^2 = 0.995$), HHQ (μ M) = $2 \times 10^{-7}A$ ($R^2 = 0.9826$), and PQS (μ M) = $3 \times 10^{-6}A + 0.4728$ ($R^2 = 0.966$).

Transcriptomic analysis.

Transcriptomic analysis was conducted using RNA-Seq sequencing at the BGI sequencing center (Shenzhen, China). Briefly, total RNA was extracted from 1.5 ml of MSH and $\Delta oxyR(oxyR)$ cultures using the RNeasy miniprep kit. DNA contamination was removed with an RNase-Free DNase reagent kit (Qiagen). Total RNA (1 μ g) was then used to construct RNA-Seq libraries as previously described (Sun et al. 2016). Gene expression levels were calculated using the reads per kilobase transcriptome per million mapped reads (RPKM) method, with the following formula: $RPKM = 10^6 C / (NL/10^3)$. The raw sequence data are available in the National Center for Biotechnology Information Sequence Read Archive (KX173291-173307).

OxyR protein expression and purification.

The OxyR coding region was amplified by PCR, using the primers shown in Supplementary Table S3, and was then cloned into the expression vector pET28a (Merck KgaA). The resultant plasmid was introduced into *E. coli* BL21 (DE3, pLysS) cells for heterologous protein expression. OxyR expression was induced by incubation with isopropyl- β -D-thiogalactoside (0.2 mM) at 16°C for 16 h, and the recombinant protein was

Fig. 9. A brief working model for the regulation of phenazine-1-carboxylic acid (PCA) biosynthesis in $\Delta oxyR(oxyR)$. Solid arrows with plus symbols indicate positive regulation, T-lines with minus symbols indicate negative regulation.

purified by Ni²⁺-affinity chromatography from the soluble cellular fraction, using His-Pur Ni-NTA resin (Thermo Scientific, Pierce Biotechnology) according to manufacturer instructions. OxyR was eluted with a buffer containing 20 mM sodium phosphate, 300 mM sodium chloride, and 250 mM of imidazole (pH 7.4). The purified protein was then dialyzed against buffer containing 10 mM Tris-HCl, 150 mM NaCl, and 10% (vol/vol) glycerol at pH 7.5. Protein purity was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

EMSA.

EMSA was performed as previously described (Hoot et al. 2010) with minor modifications. Briefly, DNA fragments containing the promoter regions of interest were amplified at the 5'-end by Cy5-labeled primers. The labeled probes were then incubated with different concentrations of purified OxyR in binding buffer (4 mM Tris-HCl, 4 mM MgCl₂, 40 mM NaCl, 5% [vol/vol] glycerol, and 0.5 mg salmon sperm DNA at pH 8.0). The unlabeled oligonucleotides were also amplified for competitive binding. Subsequent reactions were carried out in a 20-μl volume. After 20 min of incubation at 25°C, the reaction mixtures were loaded and run on a 5% (wt/vol) polyacrylamide gel under nondenaturing conditions. The DNA-protein complexes were then visualized using a molecular imager (Typhoon Trio Plus, GE Health Sciences).

qRT-PCR analysis.

Total PA1201 RNA was extracted and purified using a RNeasy miniprep kit (Qiagen). Genomic DNA was removed by DNase I (Qiagen) and cDNA synthesis was then conducted using the PrimeScript RT reagent kit (Takara). Gene expression quantification and melting curve analyses were performed on a Mastercycler ep Realplex 4S (Eppendorf), using SYBR Premix EX Taq polymerase (Takara). Relative expression levels were calculated using the 2^{-ΔΔCT} method, and the assays were performed in triplicate.

Statistical analyses.

All the experiments were performed at different timepoints during growth curves and were conducted in triplicate. The statistical significance of differences in mean were determined by calculating *P* values using two-tailed Student's *t* tests for unpaired data sets.

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