vfr, A Global Regulatory Gene, is Required for Pyrrolnitrin but not for Phenazine-1-carboxylic Acid Biosynthesis in Pseudomonas chlororaphis G05

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In our previous study, pyrrolnitrin produced in Pseudomonas chlororaphis G05 plays more critical role in suppression of mycelial growth of some fungal pathogens that cause plant diseases in agriculture. Although some regulators for pyrrolnitrin biosynthesis were identified, the pyrrolnitrin regulation pathway was not fully constructed. During our screening novel regulator candidates, we obtained a white conjugant G05W02 while transposon mutagenesis was carried out between a fusion mutant G05ΔphzΔprn::lacZ and E. coli S17-1 (pUT/mini-Tn5Kan). By cloning and sequencing of the transposon-flanking DNA fragment, we found that a vfr gene in the conjugant G05W02 was disrupted with mini-Tn5Kan. In one other previous study on P. fluorescens, however, it was reported that the deletion of the vfr caused increased production of pyrrolnitrin and other antifungal metabolites. To confirm its regulatory function, we constructed the vfr-knockout mutant G05Δvfr and G05ΔphzΔprn::lacZΔvfr. By quantifying β-galactosidase activities, we found that deletion of the vfr decreased the prn operon expression dramatically. Meanwhile, by quantifying pyrrolnitrin production in the mutant G05Δvfr, we found that deficiency of the Vfr caused decreased pyrrolnitrin production. However, production of phenazine-1-carboxylic acid was same to that in the wild-type strain G05. Taken together, Vfr is required for pyrrolnitrin but not for phenazine-1-carboxylic acid biosynthesis in P. chlororaphis G05.

Keywords : P. chlororaphis, phenazine-1-carboxylic acid, pyrrolnitrin, regulation, Vfr

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Now, some soil-borne fungal pathogens often cause diseases that lead to heavy yield losses in agriculture (Haas and Keel, 2003). Although some fungicides are effectively employed in protecting crops, their intensive applications are not permitted due to concern for the environment and public health (Chen et al., 2018; D’Mello et al., 1998). Therefore, more and more fluorescent Pseudomonas sp. are paid great attention because they can alleviate plant diseases and increase crop productivity (Baehler et al., 2005; Haas and Defago, 2005; Haas and Keel, 2003). Pseudomonas chlororaphis G05 is a root-colonizing biocontrol agent that bioprotects some plants from the diseases caused by fungal phytopathogens, such as Fusarium oxysporum, Rhizoctonia solani, and F. graminearum (Chi et al., 2017; Ge et al., 2008; Huang et al., 2018). It has been demonstrated that antifungal compounds, phenazine-1-carboxylic acid and pyrrolnitrin that are produced in this bacterium, mainly contribute to suppression of mycelial growth of these phytopathogenic fungi (Chi et al., 2017; Huang et al., 2018).
Up to date, besides phenazines and pyrrolnitrin, more and more antifungal compounds, including pyroleuteorin (PLT), hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (DAPG), lipopeptides, furanomycin and so on, have been identified in Pseudomonads’ strains and exhibited a remarkable biocontrol ability (Fenton et al., 1992; Ge et al., 2004; Laville et al., 1992; Mavrodi et al., 1998; Thomashow and Weller, 1988; Tripppe et al., 2013; Voisard et al., 1989).

In our previous study, we found that pyrrolnitrin played a more essential role than phenazines in growth suppression of *F. graminearum* and bioprotection of wheat crops against Fusarium head blight (FHB) disease (Huang et al., 2018). The production of pyrrolnitrin, however, is not high in the wild-type strain G05. Therefore, to increase pyrrolnitrin production and expand its application in agriculture, we should screen and identify more novel regulators and create regulatory pathway of pyrrolnitrin in detail. In *P. chlororaphis* PA23, ANR and PtrA were identified to mediate pyrrolnitrin production (Nandi et al., 2016; Shah et al., 2016). In *P. fluorescens* FD6, RetS and Vfr were reported to regulate pyrrolnitrin biosynthesis (Zhang et al., 2015, 2016). In *P. chlororaphis* O6, RpoS and GacS deficiency could change the production of pyrrolnitrin (Oh et al., 2013; Park et al., 2018). Although pyrrolnitrin can be biosynthesized in many different genera of bacteria and some regulators

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<th>Table 1. Bacterial strains and plasmids used in this study</th>
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<td><strong>Strains</strong></td>
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<td>E. coli</td>
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<td>SM10(λvfr)</td>
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<td><em>P. chlororaphis</em> G05</td>
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<tr>
<td>G05ΔphzΔprn::lacZ</td>
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<td>G05Δvfr</td>
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<td><em>P. chlororaphis</em> G05ΔphzΔprn::lacZvfr</td>
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**Plasmids**

| Plasmids | Relevant characteristics | Source/reference |
| T-vector, ColE, AmpR | | Sangon |
| pUCTW02 | Transposon-flanking DNA fragment amplified by inverse PCR cloned into pUCm-T, AmpR | This study |
| pEX18Tc | Gene replacement vector with MCS from pUC18, oriT sacB’, TetR | Hoang et al., 1998 |
| pEXV | pEX18Tc containing a 2.0 kb vfr-flanking PCR fragment, TetR | This study |
| pEXVG | A 0.8 kb XbaI-digested *aacC1* fragment (gentamicin resistance cassette) inserted in XbaI site in pEXV, TetRGenR | This study |
| pME6010 | Low-copy shuttle vector between *E. coli* and *Pseudomonas* spp., TetR | Heeb et al., 2000 |
| pME10V | A 1.2 kb vfr amplified by PCR cloned in pME6010, TetR | This study |
| pME6015 | Pvs1-p15A shuttle vector for translational *lacZ* fusion, TetR | Heeb et al., 2000 |
| pME15N | A 0.9 kb DNA DNA fragment containing the promoter region and the first 10 codon of *prnA* cloned in pME6015, TetR | Zhang et al., 2018 |
| pME15Z | A 0.9 kb DNA DNA fragment containing the promoter region and the first 8 codon of *phzA* cloned in pME6015, TetR | Zhang et al., 2018 |
| pME6522 | pVS1-p15A shuttle vector for transcriptional *lacZ* fusion and promoter probing, TetR | Blumer et al., 1999 |
| pME6522 | pME6522 carrying a 0.8 kb upstream region of *prn* (promoter region) and transcriptional fusion *prnA-lacZ*, TetR | Zhang et al., 2018 |
| pME22Z | pME6522 carrying a 0.8 kb upstream region of *phz* (promoter region) and transcriptional fusion *phzA1-lacZ*, TetR | Zhang et al., 2018 |
| pUCGm | Gentamicin resistance gene cassette (*aacC1*) resource, cloning vector, AmpRGenR | Schweizer, 1993 |
that mediate its biosynthesis have been identified, its regulatory pathway in detail is not fully made clear. To identify more novel regulatory candidate genes involving in pyrrolnitrin biosynthesis, in our study with *P. chlororaphis* G05, we first constructed the fusion mutant G05ΔphzΔprn::lacZ (Luo et al., 2018). In this mutant, the *phz* operon (*phz*-ABCDEFG, phenazine biosynthetic loci) was knocked out and the *prn* operon (*prn*ABCD, pyrrolnitrin biosynthetic loci) was deleted and its promoter zone was in-frame fused with the truncated *lacZ* reporter gene (Minton, 1984). With the fusion mutant G05ΔphzΔprn::lacZ as recipient cell, conjugation mating was then carried out with random insertion of transposonMini-Tn5Kan (de Lorenzo et al., 1990). One white colony was fortunately found and isolated in an LB agar plate supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). By inverse PCR, we cloned and identified the site of transposon insertion. vfr, a novel candidate gene mediating the pyrrolnitrin biosynthesis was then identified. In this study, we confirmed that *vfr* was indeed required for pyrrolnitrin, but not for phenazine-1-carboxylic acid biosynthesis in *P. chlororaphis* G05.

### Materials and Methods

**Bacterial strains, plasmids, primers and culture conditions.** All strains and plasmids employed in this work are listed in Table 1. All oligonucleotide primers used for regular PCRs or RT-qPCRs in this study are showed in Table 2. *Escherichia coli* strains were routinely cultivated in Luria-Bertani (LB) medium at 37°C (Sambrook and Russell, 2001). *P. chlororaphis* strains were regularly grown in LB medium at 30°C (Ge et al., 2008), or in glycerol-alanine medium (GA) at 30°C for phenazine assays (Chieda et al., 2005). If required, ampicillin (Amp, 100 μg/ml), chloramphenicol (Chl, 30 μg/ml), spectinomycin (Spe, 100 μg/ml), kanamycin (Kan, 50 μg/ml), and gentamicin (Gen, 20 μg/ml) were supplemented in medium for *E. coli* growth. For *P. chlororaphis* growth, tetracycline (Tet, 125 μg/ml), gentamicin (40 μg/ml) were used in its medium.

**Recombinant DNA techniques.** Standard techniques were employed for gel electrophoresis, restriction endonuclease digestion, and ligation (Sambrook and Russell, 2001). Plasmid DNA isolation from *E. coli* and *P. chlororaphis* strains was carried out with alkaline lysis method or with the recommended protocols provided by Plasmid DNA Extraction Kit (Sangon, Shanghai, China). Chromosomal DNA was isolated from *P. chlororaphis* using the Genomic DNA Extraction Kit (Solarbio, Beijing, China) or the regular method as described by Chen and Kuo (1993). Regular PCR amplifications were carried out with a 25 μl reaction mixture containing 1 × LA with GC buffer, 2 mM MgSO₄, 200 μM (each) dATP, dGTP, dCTP, and dTTP, 10 pmol of each primer, 0.2 μl LA DNA polymerase (Takara Bio,
Dalian, China), and 10 ng of purified genomic DNA of the strain G05 or its derivative mutants. All the amplifications were performed in T100™ thermal cycler (Bio-Rad Laboratory, Hercules, CA, USA). The cycling program started with a 2-min pre-denaturation at 94°C, followed by 33 cycles (30-sec denaturation at 94°C, 30 s anneal at 60-66°C, 2-min extension at 72°C), and ended with 7-min final extension at 72°C. PCR amplicons were routinely purified using PCR Purification Kit (Sangon, Shanghai, China). To do transformation, P. chlororaphis competent cells were first prepared and electroporation was then performed as described by Smith and Iglewski (1989).

Transposon mutagenesis and identification of transposon localization. Random mutagenesis was performed using transposon mini-Tn5Kan which contains a kanamycin resistance marker (de Lorenzo et al., 1990). Bacterial conjugations were carried out to introduce mini-Tn5Kan into the P. chlororaphis chromosome. Briefly, a 500 μl sample of each of two overnight cultures, E. coli S17-1(λpir)/pUT/ mini-Tn5 Kan and P. chlororaphis G05ΔphzΔprn::lacZ, was harvested, washed twice with LB medium, mixed together into a 100 μl aliquot, then transferred onto a 25-mm-diameter filter (0.22 μm pore size) that was placed on the surface of an LB agar plate, and grown for at least 12 h at 30°C. The cells grown on the filter surface were then suspended in 1 ml of LB broth, diluted and spread on LB agar plates that contained Kanamycin, chloramphenicol, and X-gal. Plates were kept in an incubator at 30°C till blue colonies developed. A white colony named G05W02 developed around many blue colonies after 3 days of growth, and then isolated.

To identify the localization of transposon insertion, chromosomal DNA from the white conjugant G05W02 was isolated, digested with SalI, purified, and self-ligated. With the purified ligation as a template, inverse PCR amplification was then carried out using a pair of primers TN5-inF and TN5-inR. The PCR amplicons were finally cloned into pUCm-T (T-vector), and sequenced using primers M13-F and M13-R.

Construction of the vfr knockout mutant with homologous recombination. To confirm the vfr function in P. chlororaphis G05, we constructed a vfr-defective mutant G05Δvfr using a homologous recombination strategy (Hoang et al., 1998), in which the vfr DNA region was deleted and replaced with the gentamicin resistance genes (aacC1) in chromosome. Firstly, two PCR amplifications were performed with two pairs of primers (V-1F/V-1R XbaI, and V-2F XhoI/V-2R), obtaining two 1.0 kb amplicons: one is a 1007 bp portion of the G05 genome upstream of the vfr; another is a 1100 bp region downstream of the vfr. Two amplicons were pooled, purified, digested with XbaI, re-purified, and finally ligated. The purified ligation was used as the template and the nested PCR was performed with a pair of primers (V-3FAcI/V-3Rluu) to obtain 2.0 kb PCR products. After simultaneous digestion with Acc65I and HindIII, the digested PCR products were cloned into the suicide plasmid pEX18Tc, resulting in pEXV (Hoang et al., 1998). Secondly, an 878 bp gentamicin resistance gene (aacC1) was purified with XbaI-digestion of pUCGm (Schweizer, 1993), then inserted into the XbaI site in pEXV to yield pEXVG.

After sequence confirmation, biparental mating was carried out and the derivative pEXVG was mobilized to P. chlororaphis G05 from E. coli SM10. The potential mutant G05Δvfr was screened and isolated on LB medium plates supplemented with 10% sucrose and gentamicin, suggesting that a double-crossover event had occurred (Ge et al., 2007; Hoang et al., 1998). In addition, biparental mating was also performed between E. coli SM10/pEXV and G05ΔphzΔprn::lacZ, generating the mutant G05ΔphzΔprn::lacZΔvfr. All mutants were verified by PCR using the primers G-F/G-R and G-LF/G-LR that annealed in the gentamicin resistance cassette specifically (data now shown).

Construction of the vfr expression vector for complementation assay. To complement the mutant G05Δvfr, pME10V was constructed as follows. The 1.0 kb DNA amplicons containing the whole vfr amplified by PCR with primers V-WF EcoRI and V-WR XhoI were cleaved with EcoRI and XhoI, and then cloned into the same sites of a low-copy shuttle vector pME6010, creating pME10V (Heeb et al., 2000). After sequence confirmation, pME10V and pME6010 were respectively transformed into G05Δvfr and other derivatives for complementation assay.

RNA extraction and real-time quantitative PCR (RT-qPCR). Pseudomonas strains were cultivated in GA broth similarly to genomic DNA preparation. The prnA was selected for qRT-PCR analysis. Cells grown for 24 h, 48 h, and 72 h were harvested. The total RNAs was isolated from cells of the strain G05 and G05Δvfr using a TRIZol reagent (Takara, Dailian, China) according to manufacturer’s instructions. The trace of genomic DNA in total RNA samples was removed with digestion using RNase-free DNase I. Reverse transcription to cDNA was performed at 42°C for 60 min using random hexamer primer with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The
resulting cDNA was amplified and quantified by RT-qPCR with a ChamQ™ SYBR qPCR Master Mix (Vazyme) on ABI Q5 Flex PCR system. The rpoD gene was used as a reference (Liu et al., 2018; Mulet et al., 2009). The primers RT-prnAF/RT-prnAR were designed to amplify 125-bp DNA fragment in prnA. The qPCR amplifications were carried out at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, and 58°C for 30 s, and a final dissociation curve analysis step from 58 to 95°C. The transcriptional level of prnA between G05 and G05Δvfr was compared by the 2^ΔΔCt method (Livak and Schmittgen, 2001).

Phenazine-1-carboxylic acid assay. To quantify phenazine-1-carboxylic acid, the wild-type strain G05 and its derivatives were respectively inoculated in 150 ml GA broth at 30°C for 72 h. Samples of each cultures were collected and quantified once every 12 h. Samples were prepared with previously established methods and PCA was quantified spectrophotometrically at 252 nm (Cui et al., 2016; Kim, 2000).

Pyrrolnitrin assay. To quantify pyrrolnitrin, bacterial strains were cultivated with same methods above. Samples were prepared with previously created methods (Huang et al., 2018). Pyrrolnitrin quantified by high performance liquid chromatography (HPLC) with reverse phase C18 column (Ovadis et al., 2004). Standard sample of pyrrolnitrin was purchased from Sigma-Aldrich (St. Louis, MO, the U.S.A.).

β-Galactosidase activity assay. For β-galactosidase enzyme assay, the wild-type strain G05 and its derivative were grown in 150 ml of GA or LB medium at 30°C. Samples were harvested after a specified period of growth. After treated with SDS and chloroform in appropriate amounts, β-galactosidase activities were released and quantified with standard methods (Miller, 1972).

Statistical analysis. All statistical data in this work were analyzed and processed with an analysis of variance test (ANOVA) or a two-tailed paired Student t-test using the statistical software package SPSS (Chicago, IL, USA), and Duncan’s multiple range test was employed for means separation of antifungal compound production and β-galactosidase activities. Values of P < 0.05 were considered statistically significant, and values of P < 0.01 were extremely significant.

Nucleic sequence accession number. The vfr gene sequence was deposited in GenBank and accession number was assigned with MK288018.

Results

Isolation and characterization of the blue-changed mutant G05W02. To identify more novel regulators that modulate the prn expression, mini-Tn5-mediated mutagenesis was carried out between E. coli and P. chlororaphis G05ΔphzΔprn::lacZ. In an LB medium plate containing X-gal and kanamycin, a white colony, called G05W02, was screened and picked up. To confirm its color change and mutagenesis, we streaked it in another X-gal-supplemented LB medium plate again, using its parental strain G05ΔphzΔprn::lacZ as a control. As shown in Fig. 1A,
the exconjugant G05W02 totally differed from its parental strain G05ΔphzΔprn::lacZ with white color. Meanwhile, we quantified its β-galactosidase activities while it grown in GA medium for 72 h. As shown in Fig. 1B, in comparison with the fusion mutant G05ΔphzΔprn::lacZ, β-galactosidase activities produced by the transposon-mediated mutant G05W02 were extremely low, suggesting that the expression of the prn operon was suppressed in this white exconjugant.

Localization of transposon insertion and identification of the vfr. To clone the flanking DNA fragment of transposon insertion, we employed inverse-PCR to amplify and identify the transposon-disrupted gene. Before PCR, the template of the genomic DNA of the conjugant G05W02 was prepared as described in Material and methods. After inverse PCR, 3.0 kb amplicon was cloned into the pUCm-T (T-vector) and created pUCTW02 for sequencing. Sequencing results verified that the transposon mini-Tn5Kan was actually inserted the vfr gene in the conjugant G05W02. According to the sequence of the vfr gene in the conjugant G05W02, the predicted Vfr in the strain P. chlororaphis G05 contains 214 amino acid residues with a molecular mass of 24 KDa, showing closest similarity to that in the strain P. chlororaphis (99%), P. fluorescens (98%), P. aeruginosa PAO1 (83%), and E. coli K12 (63%).

Deletion of the vfr caused decreased expression of the prn operon. To examine regulatory effects of Vfr on the expression of the prn operon, we first created the vfr-knockout mutant G05ΔphzΔprn::lacZΔvfr. As shown in Fig. 2A, the mutant G05ΔphzΔprn::lacZΔvfr turned out to be white on a LB medium plate supplemented with X-gal. As it was complemented with bearing the shuttle plasmid pME10V, the transformant could turn blue again. The transformant harboring the original plasmid pME6010, however, did not turn blue. Meanwhile, we inoculated the fusion mutant G05ΔphzΔprn::lacZ and its derivatives in GA medium, and then quantified their β-galactosidase activities. As shown in Fig. 2B, β-galactosidase activities produced in the vfr-knockout mutant G05ΔphzΔprn::lacZΔvfr were much lower than those in the parental strain G05ΔphzΔprn::lacZ. When the mutated vfr gene was complemented with introduction of pME10V, however, the transformant G05ΔphzΔprn::lacZΔvfr/pME10V produced almost same β-galactosidase activities as the parental strain G05ΔphzΔprn::lacZ did. In addition, we also found that the transformant G05ΔphzΔprn::lacZΔvfr/pME6010 expressed same β-galactosidase activities as the vfr-knockout mutant G05ΔphzΔprn::lacZΔvfr did.

These results indicated that the expression of the prn operon was indeed decreased in the absence of the vfr gene, suggesting that the expression of the prn operon requires the presence of Vfr in the wild-type strain G05.

Fig. 2. Characterizations of the site-directed knockout mutant G05ΔphzΔprn::lacZΔvfr and its derivatives. (A) Color of colonies shown in the LB medium plate supplemented with X-gal. Arabic numbers from 2 to 7 stand for the fusion mutant G05ΔphzΔprn::lacZ, the vfr-knockout mutant G05ΔphzΔprn::lacZΔvfr, the transformant G05ΔphzΔprn::lacZΔvfr/pME6010, respectively. (B) β-Galactosidase activities were quantified when they grown in GA medium at 30°C for 72 h. The values from three independent experiments were presented as the average ± standard deviation. Different superscript lowercase letters followed strains indicate significant difference (P < 0.05) according to duncan’s multiple range test, and different superscript uppercase letters indicate extremely significant difference (P < 0.01).
Deletion of the vfr brought much less pyrrolnitrin production, but no change of phenazine-1-carboxylic acid.

To assess regulatory effects of Vfr on pyrrolnitrin production, we also created the mutant G05Δvfr. For quantifying their pyrrolnitrin production, the wild-type strain G05, the mutant G05Δvfr and its derivative transformants were respectively grown in GA medium. As shown in Fig. 3A, in comparison with the wild-type strain G05, the production of pyrrolnitrin in the mutant G05Δvfr was remarkably decreased. When the mutant G05Δvfr was introduced with pME10V, pyrrolnitrin produced in the transformant G05Δvfr/pME10V was almost same to that in the wild-type strain G05. The transformant G05Δvfr/pME6010, however, looked like its parental strain G05Δvfr and produced a tiny amount of pyrrolnitrin. These results indicated that deletion of the vfr caused much less pyrrolnitrin production in P. chlororaphis G05.

In addition, we also determined phenazine-1-carboxylic acid production while they were inoculated and grown in GA medium. According to the Fig. 3B, it was shown that phenazine-1-carboxylic acid produced in the mutant G05Δvfr was same to that in the wild-type strain G05. The strain G05Δvfr/pME6010, however, looked like its parental strain G05Δvfr and produced a tiny amount of pyrrolnitrin. These results indicated that deletion of the vfr caused much less pyrrolnitrin production in P. chlororaphis G05.

Fig. 3. Regulatory effects of deletion of the vfr on fungal metabolites production in P. chlororaphis G05. All experiments were performed in triplicate, and each value was presented as the means ± standard deviation. (A) Pyrrolnitrin produced by the wild-type strain G05 and its derivatives in GA broth. According to duncan’s multiple range test, different superscript lowercase letters followed the strains indicated significant difference (P < 0.05), and different superscript uppercase letters followed the strains indicated extremely significant difference (P < 0.01). (B) Phenazine-1-carboxylic acid produced by the wild-type strain G05 and its derivatives in GA broth. Asterisks at top of columns mean no significant difference (P > 0.05).

Fig. 4. Translational lacZ fusion vectors pME15Z and pME15N were employed to examine Vfr regulation in P. chlororaphis G05. (A) β-Galactosidase activities produced by pME15N in the wild-type strain G05 and the mutant G05Δvfr were quantified. The transformants G05/pME6015 and G05Δvfr/pME6015 were used as negative controls. (B) β-Galactosidase activities produced by pME15Z in the wild-type strain G05 and the mutant G05Δvfr were quantified. The transformants G05/pME6015 and G05Δvfr/pME6015 were used as negative controls. All experiments were performed in triplicate, and each value was presented as the means ± standard deviation. Asterisks at top of columns mean no significant difference (P > 0.05).
suggesting that Vfr did not exert any effects on the biosynthesis of phenazine-1-carboxylic acid.

**Down-regulation of the prn expression mediated by Vfr.** To further confirm the results above, we also employed the translational fusions (phzA'-lacZ and prnA'-lacZ) (Heeb et al., 2000; Zhang et al., 2018) and transcriptional fusions (phzA-lacZ and prnA-lacZ) (Blumer et al., 1999; Zhang et al., 2018), did transformation and quantified their β-galactosidase activities in the wild-type strain G05 and its derivative mutants. As shown in Fig. 4, β-galactosidase activities expressed by pME15N (prnA-lacZ) in the mutant G05Δvfr were much less than those in the wild-type strain G05. However, β-galactosidase activities expressed by pME15Z (phzA-lacZ) in the mutant G05Δvfr were almost same to those in the wild-type strain G05. As shown in Fig. 5, β-galactosidase activities expressed by pME22N (prnA-lacZ) in the mutant G05Δvfr were almost same to those in the wild-type strain G05. Similarly, β-galactosidase activities expressed by pME22Z (phzA-lacZ) in the mutant G05Δvfr were also same to those in the wild-type strain G05. To verify these results with direct evidences, we also carried out RT-qPCRs to check the transcription of the prnA. As shown in Fig. 6, the copies of mRNA transcribed from the prnA in the mutant G05Δvfr were almost same to those in the wild-type strain G05, confirming that there were no remarkable differences in transcriptional levels of the prn operon in the vfr-deletion mutant G05Δvfr and its parental strain G05. Taken together, no matter whether the vfr gene was mutated with the random transposon insertion or the site-directed deletion in *P. chlororaphis* G05, deficiency of Vfr dramatically down-regulated the prn operon expression at the posttranscriptional level, but not at the transcriptional level. Meanwhile, Vfr did not exert any regulatory effects on the phz expression.

**Fig. 5.** Translational lacZ fusion vectors pME22Z and pME22N were employed to examine Vfr regulation in *P. chlororaphis* G05. (A) β-Galactosidase activities produced by pME22Z in the wild-type strain G05 and the mutant G05Δvfr were quantified. The transformant G05/pME6522 and G05Δvfr/pME6522 were used as negative controls. (B) β-Galactosidase activities produced by pME22N in the wild-type strain G05 and the mutant G05Δvfr were quantified. The transformant G05/pME6522 and G05Δvfr/pME6522 were used as negative controls. All experiments were performed in triplicate, and each value was presented as the means ± standard deviation. Asterisks at top of columns mean no significant difference ($P > 0.05$).

**Fig. 6.** Gene expression of prnA by RT-qPCR assay in *P. chlororaphis* G05 and its derivative mutant G05Δvfr. Expression level of the tested prnA in the wild-type strain G05 was considered 1. Relative expressions of prnA in the mutant G05Δvfr compared to the wild-type strain G05 grown in GA medium for 24 h, 48 h, and 72 h were determined by the $2^{-\Delta\Delta CT}$ method. Asterisks at top of columns mean no significant difference ($P > 0.05$).
**Vfr is Required for Pyrrolnitrin Biosynthesis**

**Discussion**

As an important global regulator, Vfr first was identified and designated in *P. aeruginosa* due to its regulatory effects on the biosynthesis of virulence factors (West et al., 1994). In fact, it is a homologue of a transcriptional regulator cyclic AMP receptor protein (Crp) in *E. coli*, which mediates the expression of more than 100 genes, as well as the biosynthesis of at least 60 proteins (Suh et al., 2002; Wolfgang et al., 2003). Today, a few of homologues of the Crp regulator have been identified in different bacterial genera and their many regulatory effects on virulence-associated phenotypes have been elucidated, such as iron uptake ability and virulence-host relationships (Taguchi and Ichinose, 2013). In general, Vfr is not only related tightly to the pathogenicity of some bacteria, but also plays a critical role in their infection. In one other previous study, it was reported Vfr in *P. fluorescens* had a negative regulation on the biosynthesis of secondary antifungal metabolites, such as pyrrolnitrin, PLT, and DAPG (Zhang et al., 2016). Knockout of the vfr gene brought increased production of antifungal compounds. Surprisingly, we happened to find that transposon insertion mutagenesis in the vfr gene in the fusion mutant G05ΔphzΔprn::lacZ led to much less β-galactosidase activities, suggesting that mutation of the vfr could inhibit the biosynthesis of pyrrolnitrin in *P. chlororaphis* G05. To confirm this hypothesis, we made a site-directed knockout of the vfr gene in the wild-type strain G05 and the fusion mutant G05ΔphzΔprn::lacZ. Their pyrrolnitrin production and β-galactosidase activities verified that deletion of the vfr actually suppressed the expression of the prn operon and biosynthesis of pyrrolnitrin in *P. chlororaphis* G05. Meanwhile, we also found that Vfr did not exert any regulatory effects on the expression of the phz operon and phenazine-1-carboxylic acid biosynthesis. This is the first report about Vfr-mediated regulation on phenazine production although phenazine biosynthesis is regulated by many well-known regulators (Bilal et al., 2017; Mavrodi et al., 2006). The fact that Vfr differentially regulates two antifungal compounds production in a strain suggests each of two secondary metabolites, pyrrolnitrin and phenazine-1-carboxylic acid, has respectively been synthesized under the control of their own specific regulatory cascade. Obviously, this differential regulation mechanism helps to keep stability of total production of antifungal compounds in the strain G05 and also is helpful in maintaining its biological control function.

Although it has been reported that Vfr could regulate a quite few of metabolites production, the regulation mechanism of Vfr has not been elucidated in detail. Using the translational and transcriptional fusions and RT-qPCR, in this study, we tried to understand whether the Vfr-mediated regulation of the prn operon occurs at the transcriptional level or the posttranscriptional level. β-Galactosidase activities and qPCR indicated that the expression of the prn operon is regulated by Vfr at the posttranscriptional level, not the transcriptional level. Based on these data, we deduced that there might be an intermediate(s) at the downstream of the Vfr-mediated regulatory cascade. This intermediate should be controlled by the Vfr, and in turn, it might directly or indirectly regulate the prn operon expression in *P. chlororaphis* G05. For the detailed Vfr regulation pathway, therefore, further study should be conducted later.

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