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-diacytethylphloroglucinol (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; Trippe 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

Table 1. Bacterial strains and plasmids used in this study

| Strains and plasmid | Relevant characteristics | Source/reference |
|---------------------|-------------------------|------------------|
| **Strains**<br> E. coli<br> DH5α | Φ80 lacZΔM15 Δ(lacZYA-argF) U169 hsdR17 recA1 endA1 thi-1 | Lab collection |
| SM10 (λxfr) | F’ thi-1 thr-1 leuB6 recA1 tonA21 lacY1 supE44(Mu-6) λpir KanR | Lab collection |
| P. chlororaphis<br> G05 | The wild-type strain, phenazine-1-carboxylic acid and pyrrolnitrin producer, PCA’, PRN’, SpeR | Lab collection |
| G05ΔphzΔprn::lacZ | The phzABCDEFG and prnABCD operons deleted and the prnA’ fused with the truncated lacZ gene in frame in the wild-type strain G05, SpeRGenR | Luo et al., 2018 |
| G05Δvfr | The vfr deleted and inserted with gentamicin resistance cassette in the wild-type strain G05, SpeRGenR | This study |
| G05W02 | A white conjugant isolated on LB plates by transposon random insertion on the chromosome of the fusion mutant G05ΔphzΔprn::lacZ, SpeRGenR | This study |
| G05ΔphzΔprn::lacZΔvfr | The vfr deleted in the fusion mutant G05ΔphzΔprn::lacZ, SpeRGenR | This study |
| **Plasmids**<br> pUCm-T | T-vector, CoIE, 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 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 fragment containing the promoter region and the first 8 codon of phzA1 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 |
| pME22N | pME6522 carrying a 0.8 kb upstream region of prnA (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 (*prnABCD*, 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, Table 2. Oligonucleotide primers used in this study

| Primers | Sequences (5′-3′, artificial restriction enzyme site was underlined) |
|---------|---------------------------------------------------------------|
| V-1F    | CAGCGCACAAGGTCAGCCTGGTTTC                                        |
| V-1R<sub>phz</sub> | CATGATTCTGATAGCCGCGCGGCTGGCAGTGCACT (XbaI)          |
| V-2F<sub>prn</sub> | GATCATTTGACCTCAGAGAAGACGCAAACCTGACC (XbaI)       |
| V-2R    | CGGTGCTGTTGATTGTGGCGGCGGCTG                                    |
| V-3F<sub>Acc</sub> | CAAGTTGTTACCCGGGCGGATTCCTCGAGCAGATGCG (Acc65I) |
| V-3R<sub>HindIII</sub> | GATCATTGCTGATAGGCGGCGCAGTTGGAAGTGCATG (HindIII) |
| G-F     | GCAGCAACGTGTTACGCAG                                               |
| G-R     | TGTAGGTTGCGCGGTACTTTG                                             |
| G-LF    | GTCCAAGCAGCGGGGCGCCAAATTC                                         |
| G-LR    | CAGGCTTATGTCAATTTCGAGCTC                                          |
| V-WF<sub>EcoRI</sub> | GAACTTAGTCAATTTCGAGGATGCTGACCAGTCCAGAAG (EcoRI) |
| V-WR<sub>I</sub> | CAAAGTTGCTGACGGGAGAACCATGTTGCGGCGGCG (XhoI)   |
| TN5-inF | CGCTCCCAGTCCAGCAGCGCATCGC                                      |
| TN5-inR | CCAAGCGGCGGGAGAACCTGCGTGC                                       |
| M13-F   | TTGTTAAACGACGCACAG                                              |
| M13-R   | CAGGAACAGCCTATGAC                                               |
| RT-rpoDF | GTGTCTGATGACGGGCGGTTACGTGAC                                      |
| RT-rpoDR | GATGCAGTGCTTCCACGTCCCGG                                      |
| RT-prnAF | CAGCAGCAAGCAGCACATTACGCTC                                      |
| RT-prnAR | CGGTATCCCGAGAGAAGTCCAGAAGAC                                     |


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 Labo-
ratory, Hercules, CA, USA). The cycling program started
with a 2-min pre-denaturation at 94°C, followed by 33 cy-
cles (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 transpos-
on localization. Random mutagenesis was performed using
transposon mini-Tn5Kan which contains a kanamycin
resistance marker (de Lorenzo et al., 1990). Bacterial con-
jugations 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(Qpir)/pUT/
mini-Tn5 Kan and P. chlororaphis G05ΔphzΔprn::lacZ,
was harvested, washed twice with LB medium, mixed to-
gether 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 de-
veloped around many blue colonies after 3 days of growth,
suggesting that a double-crossover event had occurred
(Ge et al., 2007; Hoang et al., 1998). In addition, bipa-
rental mating was also performed between E. coli SM10/
pEXV and G05ΔphzΔprn::lacZ, generating the mutant
G05ΔphzΔprn::lacZ/vfr. All mutants were verified by
PCR using the primers G-F/G-R and G-LF/G-LR that an-
naled in the gentamicin resistance cassette specifically
data now shown).

Construction of the vfr expression vector for comple-
mentation 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_eco and V-WR_xho 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, Dalian, China) according to manufacturer’s in-
structions. The trace of genomic DNA in total RNA sam-
plings 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
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resulting cDNA was amplified and quantified by RT-qPCR with a ChamQ™ SYBR qPCR Master Mix (Vazyme) on ABI Q_ 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 gene in the conjugant.

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/pME10V 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.

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/pME10V 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, and 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 G05Δ*vfr*. For quantifying their pyrrolnitrin production, the wild-type strain G05, the mutant G05Δ*vfr* 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 G05Δ*vfr* was remarkably decreased. When the mutant G05Δ*vfr* was introduced with pME10V, pyrrolnitrin produced in the transformant G05Δ*vfr*/pME10V was almost same to that in the wild-type strain G05. The transformant G05Δ*vfr*/pME6010, however, looked like its parental strain G05Δ*vfr* 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 G05Δ*vfr* was same to that in the wild-type strain G05. The tranformant G05Δ*vfr*/pME6010, however, looked like its parental strain G05Δ*vfr* and produced a tiny amount of pyrrolnitrin. These results indicated that deletion of the *vfr* caused much less pyrrolnitrin production in *P. chlororaphis* G05.
gesting that Vfr did not exert any effects on the biosynthesis of phenazine-1-carboxylic acid.

**Down-regulation of the prn expression mediated by Vfr** occurred at the posttranscriptional level, but the phz expression was not regulated 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.
**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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