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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 G05Δ*phz*Δ*prn*::*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 G05Δ*vfr* and G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*. 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 G05Δ*vfr*, 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 duo 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 pyoleuteorin (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; 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

Strain and plasmid	Relevant characteristics	Source/reference
Strains		
E. coli		
DH5α	Φ80 *lacZ*ΔM15 Δ (*lacZYA-argF*) U169 *hsdR17 recA1endA1 thi⁻1*	Lab collection
SM10 (λ*vfr*)	F⁻ *thi⁻1 thr⁻1 leuB6 recA tonA21 lacY1 supE44*(Mu_C^+) λ*pir* Kan^R	Lab collection
P. chlororaphis		
G05	The wild-type strain, phenazine-1-carboxylic acid and pyrrolnitrin producer, PCA^+, PRN^+, Spe^R	Lab collection
G05Δ*phz*Δ*prn*::*lacZ*	The *phzABCDEFG* and *prnABCD* operons deleted and the *prnA'* fused with the truncated *lacZ* gene in frame in the wild-type strain G05, Spe^RGen^R	Luo et al., 2018
G05Δ*vfr*	The *vfr* deleted and inserted with gentamicin resistance cassette in the wild-type strain G05, Spe^RGen^R	This study
G05W02	A white conjugant isolated on LB plates by transposon random insertion on the chromosome of the fusion mutant G05Δ*phz*Δ*prn*::*lacZ*, Spe^RKan^R	This study
G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*	The *vfr* deleted in the fusion mutant G05Δ*phz*Δ*prn*::*lacZ*, Spe^RGen^R	This study
Plasmids		
pUCm-T	T-vector, ColE, Amp^R	Sangon
pUCTW02	Transposon-flanking DNA fragment amplified by inverse PCR cloned into pUCm-T, Amp^R	This study
pEX18Tc	Gene replacement vector with MCS from pUC18, *oriT*$^+$ *sacB*$^+$, Tet^R	Hoang et al., 1998
pEXV	pEX18Tc containing a 2.0 kb *vfr*-flanking PCR fragment, Tet^R	This study
pEXVG	A 0.8 kb *Xba*I-digested *aacC1* fragment (gentamicin resistance cassette) inserted in *Xba*I site in pEXV, Tet^RGen^R	This study
pME6010	Low-copy shuttle vector between *E. coli* and *Pseudomonas* spp., Tet^R	Heeb et al., 2000
pME10V	A 1.2 kb *vfr* amplified by PCR cloned in pME6010, Tet^R	This study
pME6015	Pvs1-p15A shuttle vector for translational *lacZ* fusion, Tet^R	Heeb et al., 2000
pME15N	A 0.9 kb DNA DNA fragment containing the promoter region and the first 10 condon of *prnA* cloned in pME6015, Tet^R	Zhang et al., 2018
pME15Z	A 0.9 kb DNA DNA fragment containing the promoter region and the first 8 condon of *phzA* cloned in pME6015, Tet^R	Zhang et al., 2018
pME6522	pVS1-p15A shuttle vector for transcriptional *lacZ* fusion and promoter probing, Tet^R	Blumer et al., 1999
pME22N	pME6522 carrying a 0.8 kb upstream region of *prn* (promoter region) and transcriptional fusion *prnA-lacZ*, Tet^R	Zhang et al., 2018
pME22Z	pME6522 carrying a 0.8 kb upstream region of *phz* (promoter region) and transcriptional fusion *phzA1-lacZ*, Tet^R	Zhang et al., 2018
pUCGm	Gentamicin resistance gene cassette (*aacC1*) resource, cloning vector, Amp^RGen^R	Schweizer, 1993

Table 2. Oligonucleotide primers used in this study

Primers	Sequences (5′-3′, artificial restriction enzyme site was underlined)
V-1F	CAGCGACAAGGTCAGCCTGGGTTTC
V-$1R_{Xba}$	CATGATTCTAGATAGCGGCGGCGCTGGCAGTGCATC (*Xba*I)
V-$2F_{Xba}$	GATCATTCTAGACCTCGAAGAACGCAACCTGGTCC (*Xba*I)
V-2R	CGTGCTGTTGATTGTGGCGGCGCTG
V-$3F_{Acc}$	CAAGTTGGTACCCGGGCGATTCTCGAGCAGATGCG (*Acc*65I)
V-$3R_{Hin}$	GATACTAAGCTTGATGTGCTGGTTGAAGTGCATG (*Hin*dIII)
G-F	GCAGCAACGATGTTACGCAG
G-R	TGTTAGGTGGCGGTACTTGG
G-LF	GTCACAACGCCGCGGCCAATTC
G-LR	CAGGCTTATGTCAATTCGAGCTC
V-WF_{Eco}	GAACTTGAATTCAGGATGCTGACCACGTCGAAG (*Eco*RI)
V-WR_{Xho}	CAAGTTCTCGAGCGGGAAACCATGGTCGCGGCG (*Xho*I)
TN5-inF	CGCTCCCGATTCGCAGCGCATCGCC
TN5-inR	CCAAGCGGCCGGAGAACCTGCGTGC
M13-F	GTTGTAAAACGACGGCCAG
M13-R	CAGGAAACAGCTATGAC
RT-rpoDF	GTGGTCGTGAGCAGGGTTACCTGAC
RT-rpoDR	GGATGATGTCTTCCACCTGTTCCGG
RT-prnAF	CAGCAGCAAGCGAACATTACGCTC
RT-prnAR	CGGTATCCCGAGGAAGTCGAAGAAC

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 G05Δ*phz*Δ*prn*::*lacZ* (Luo et al., 2018). In this mutant, the *phz* operon (*phzABCDEFG*, 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 G05Δ*phz*Δ*prn*::*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 $MgSO_4$, 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-Tn*5*Kan into the *P. chlororaphis* chromosome. Briefly, a 500 µl sample of each of two overnight cultures, *E. coli* S17-1(λ*pir*) /pUT/ mini-Tn*5* Kan and *P. chlororaphis* G05Δ*phz*Δ*prn*::*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 was then isolated.

To identify the localization of transposon insertion, chromosomal DNA from the white conjugant G05W02 was isolated, digested with *Sal*I, 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 G05Δ*vfr* 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_{Xba} and V-2F_{Xba}/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 *Xba*I, 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-3F_{Acc}/V-3R_{Hin}) to obtain 2.0 kb PCR products. After simultaneous digestion with *Acc*65I and *Hin*dIII, 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 *Xba*I-digestion of pUCGm (Schweizer, 1993), then inserted into the *Xba*I 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 G05Δ*vfr* 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 G05Δ*phz*Δ*prn*::*lacZ*, generating the mutant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*. 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 G05Δ*vfr*, 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 *Eco*RI and *Xho*I, 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 G05Δ*vfr* 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 havested. The total RNAs was isolated from cells of the strain G05 and G05Δ*vfr* 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 Q_6 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 G05Δ*vfr* was compared by the $2^{-\Delta\Delta Ct}$ 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* G05Δ*phz*Δ*prn*::*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 G05Δ*phz*Δ*prn*::*lacZ* as a control. As shown in Fig. 1A,

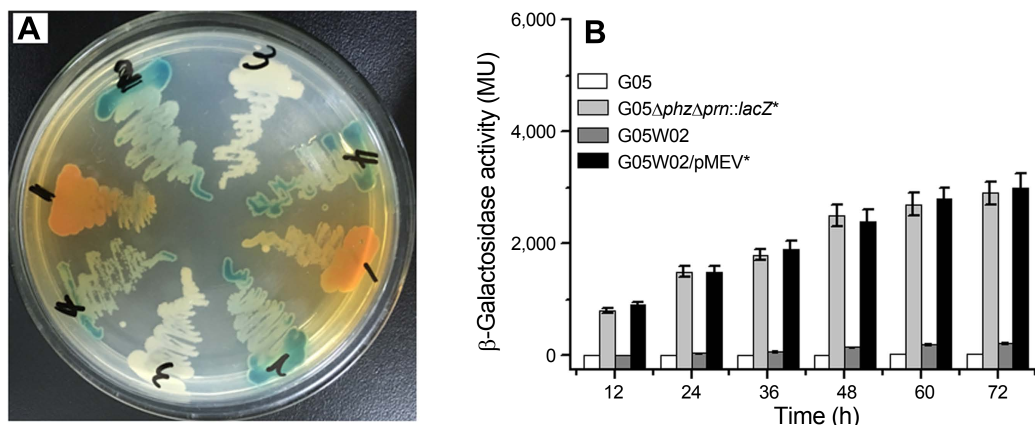

Fig. 1. Characterizations of the conjugant G05W02 and its derivatives. (A) Color of colonies shown in the LB medium supplemented with X-gal. Arabic numbers from 1 to 4 stand for the wild-type strain G05, the fusion mutant G05Δ*phz*Δ*prn*::*lacZ*, the transposon mutant G05W02, and the transformant G05W02/pME10V, respectively. (B) β-Galactosidase activities were quantified when they were grown in GA medium at 30°C for 72 h. The values from three independent experiments were presented as the average ± standard deviation. Superscript of asterisk followed the strains indicated no significant differences ($P > 0.05$).

the exconjugant G05W02 totally differed from its parental strain G05Δ*phz*Δ*prn*::*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 G05Δ*phz*Δ*prn*::*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*, 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 G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*. As shown in Fig. 2A, the mutant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr* 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 G05Δ*phz*Δ*prn*::*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 G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr* were much lower than those in the parental strain G05Δ*phz*Δ*prn*::*lacZ*. When the mutated *vfr* gene was complemented with introduction of pME10V, however, the transformant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*/pME10V produced almost same β-galactosidase activities as the parental strain G05Δ*phz*Δ*prn*::*lacZ* did. In addition, we also found that the transformant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*/pME6010 expressed same β-galactosidase activities as the *vfr*-knockout mutant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr* 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.

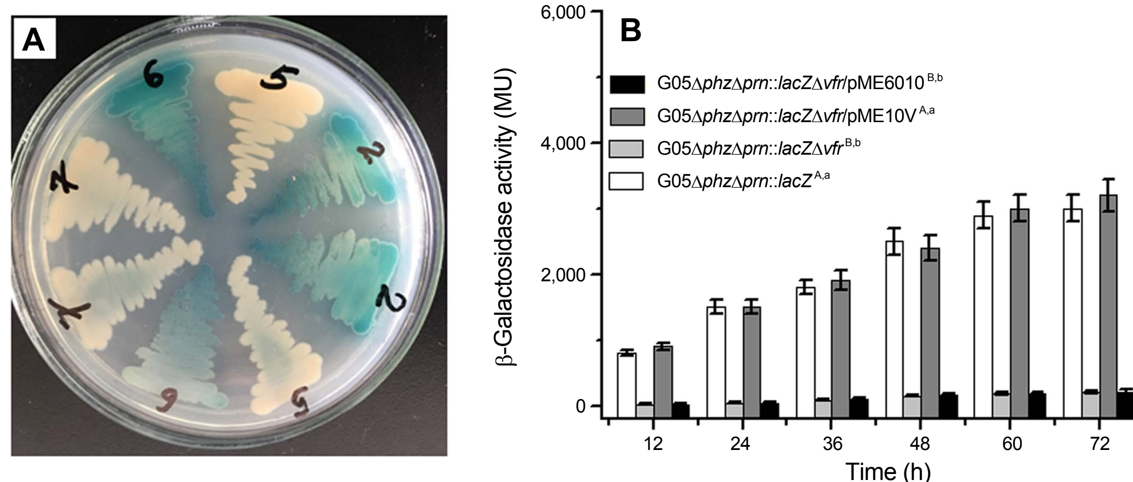

Fig. 2. Characterizations of the site-directed knockout mutant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr* and its derivatives. (A) Color of colonies shown in the LB medium plate supplemented with X-gal. Arabic numbers from 2 to 7 stand for the fusion mutant G05Δ*phz*Δ*prn*::*lacZ*, the *vfr*-knockout mutant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*, the transformant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*/pME10V, and the transformant G05Δ*phz*Δ*prn*::*lacZ*Δ*vfr*/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 ($P < 0.01$).

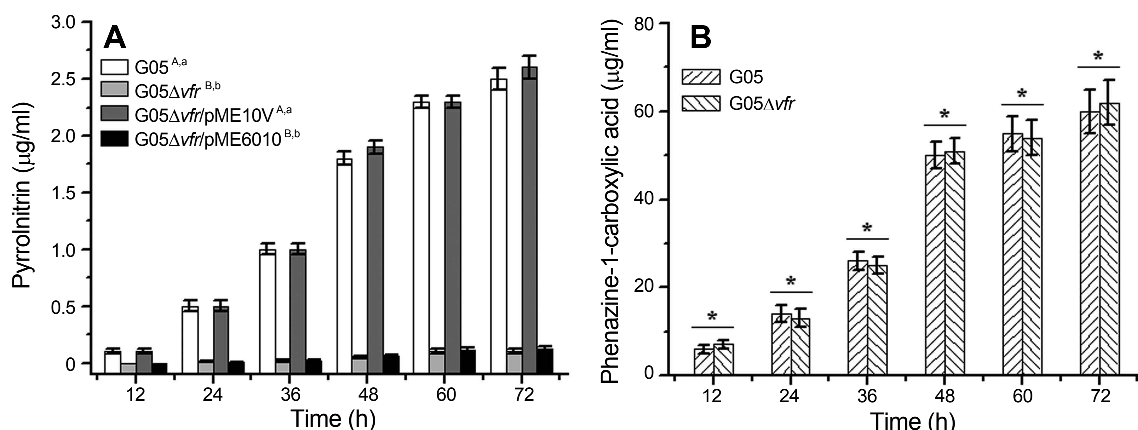

Fig. 3. Regulatory effects of deletion of the *vfr* on fungal metabolites production in *P. chlororaphis* G05. All experiments were performed in triplicate, and each value was presented as the means ± standard deviation. (A) Pyrrolnitrin produced by the wild-type strain G05 and its derivatives in GA 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 ($P > 0.05$).

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 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.

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, sug-

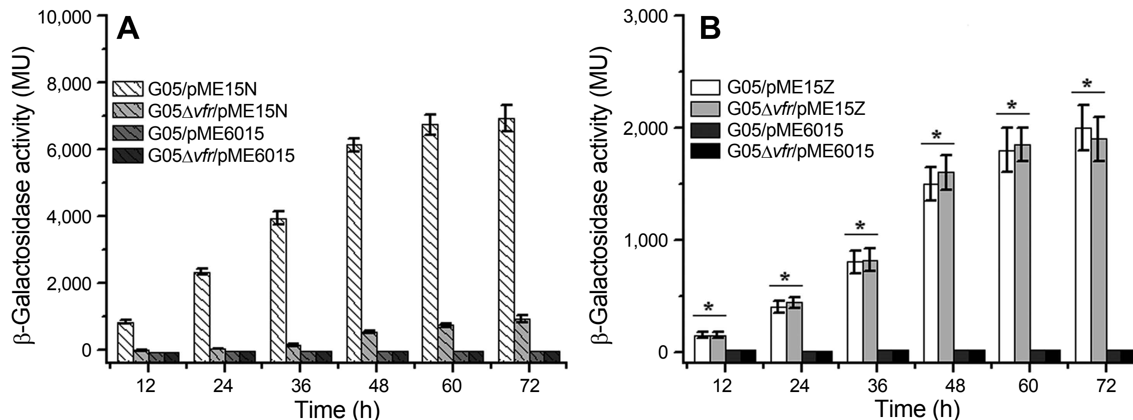

Fig. 4. Translational *lacZ* fusion vectors pME15Z and pME15N were employed to examine Vfr regulation in *P. chlororaphis* G05. (A) β-Galactosidase activities produced by pME15N in the wild-type strain G05 and the mutant G05Δ*vfr* were quantified. The transformants G05/pME6015 and G05Δ*vfr*/pME6015 were used as negative controls. (B) β-Galactosidase activities produced by pME15Z in the wild-type strain G05 and the mutant G05Δ*vfr* were quantified. The transformants G05/pME6015 and G05Δ*vfr*/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 ($P > 0.05$).

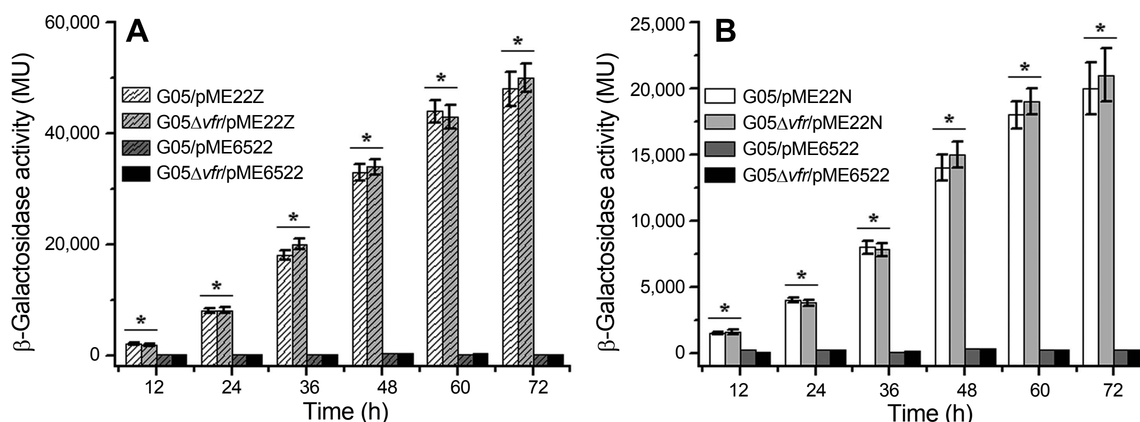

Fig. 5. Translational *lacZ* fusion vectors pME22Z and pME22N were employed to examine Vfr regulation in *P. chlororaphis* G05. (A) β-Galactosidase activities produced by pME22Z in the wild-type strain G05 and the mutant G05Δ*vfr* were quantified. The transformant G05/pME6522 and G05Δ*vfr*/pME6522 were used as negative controls. (B) β-Galactosidase activities produced by pME22N in the wild-type strain G05 and the mutant G05Δ*vfr* were quantified. The transformant G05/pME6522 and G05Δ*vfr*/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 ($P > 0.05$).

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 G05Δ*vfr* were much less than those in the wild-type strain G05. However, β-galactosidase activities expressed by pME15Z (*phzA'-'lacZ*) in the mutant G05Δ*vfr* 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 G05Δ*vfr* were almost same to those in the wild-type strain G05. Similarly, β-galactosidase activities expressed by pME22Z (*phzA-lacZ*) in the mutant G05Δ*vfr* 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 G05Δ*vfr* 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

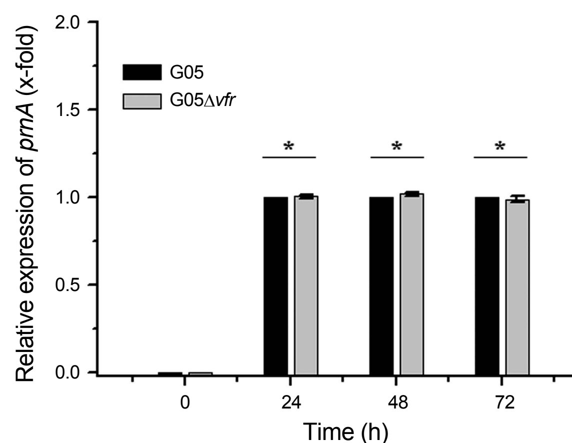

Fig. 6. Gene expression of *prnA* by RT-qPCR assay in *P. chlororaphis* G05 and its derivative mutant G05Δ*vfr*. Expression level of the tested *prnA* in the wild-type strain G05 was considered 1. Relative expressions of *prnA* in the mutant G05Δ*vfr* compared to the wild-type strain G05 grown in GA 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 ($P > 0.05$).

mutant G05Δ*vfr* 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.

Disscussion

As an important global regulator, Vfr first was identified and designated in *P. aeruginosa* duo 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 G05Δ*phz*Δ*prn*::*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 G05Δ*phz*Δ*prn*::*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, pyrrlnitrin 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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