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Construction of a β-galactosidase-gene-based fusion is convenient for screening candidate genes involved in regulation of pyrrolnitrin biosynthesis in *Pseudomonas chlororaphis* G05

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In our recent work, we found that pyrrolnitrin, and not phenazines, contributed to the suppression of the mycelia growth of *Fusarium graminearum* that causes heavy Fusarium head blight (FHB) disease in cereal crops. However, pyrrolnitrin production of *Pseudomonas chlororaphis* G05 in King's B medium was very low. Although a few regulatory genes mediating the *prnABCD* (the *prn* operon, pyrrolnitrin biosynthetic locus) expression have been identified, it is not enough for us to enhance pyrrolnitrin production by systematically constructing a genetically-engineered strain. To obtain new candidate genes involved in the regulation of the *prn* operon expression, we successfully constructed a fusion mutant G05Δ*phz*Δ*prn*::*lacZ*, in which most of the coding regions of the *prn* operon and the *phzABCDEFG* (the *phz* operon, phenazine biosynthetic locus) were deleted, and the promoter region plus the first thirty condons of the *prnA* was in-frame fused with the truncated *lacZ* gene on its chromosome. The expression of the fused *lacZ* reporter gene driven by the promoter of the *prn* operon made it easy for us to detect the level of the *prn* expression in terms of the color variation of colonies on LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). With this fusion mutant as a recipient strain, mini-Tn5-based random insertional mutagenesis was then conducted. By picking up colonies with color change, it is possible for us to screen and identify new candidate genes involved in the regulation of the *prn* expression. Identification of additional regulatory genes in further work could reasonably be expected to increase pyrrolnitrin production in G05 and to improve its biological control function.

Key Words: *Pseudomonas chlororaphis*; pUT/mini-Tn5Kan; regulation; the *prn* operon; the truncated *lacZ*

Introduction

As is well known, there are quite a lot of bacteria in soil, but most of them actually inhabit the rhizospheric zone, including plant roots and seed surfaces. Thus, they are also known as rhizobacteria. However, only a very small portion of them (2–5%) are referred as to plant growth promoting rhizobacteria (PGPR) because their presence around roots is beneficial to plant growth (Antoun and Kloepper, 2001; Lugtenberg and Kamilova, 2009). In all of the PGPR, *Pseudomonas* spp. has been one genus of the most widely studied bacteria for several decades, such as *P. fluorescens* 2-79, CHA0, F113, Pf-5, and *P. chlororaphis* 30–84 (Carroll et al., 1995; Kraus and Loper, 1995; Laville et al., 1992; Thomashow et al., 1990; Weller, 1984). While they colonize and grow in the rhizosphere, an array of antifungal metabolites are biosynthesized and contribute to the suppression of mycelial growth of many fungal phytopathogens (Haas and Défago, 2005; Raaijmakers and Mazzola, 2012; Singh et al., 2012; Weller et al., 2002). Up to now, many of these antifungal com-

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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 (*λpir*)	F- *thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44*(Mu_C^+) *λpir* Kan^R	Lab collection
S17-1 (*λpir*)	(F-) RP4-2-Tc::Mu *aphA*::Tn7 *recA λpir* lysogen Tp^R Sm^R	Lab collection
P. chlororaphis		
G05	Wild type, phenazine-1-carboxylic acid and pyrrolnitrin producer, PCA^+, PRN^+, Amp^RChl^R	Lab collection
G05Δ*prn*::*lacZ*	The *prnABCD* deleted and the *prnA* fused with the truncated *lacZ* gene in frame in the wild type strain G05, Amp^RChl^R	This study
G05Δ*phz*Δ*prn*::*lacZ*	*phzABCDEFG* deleted and inserted with gentamicin resistance cassette (*aacC1*) in fusion mutant G05Δ*prn*::*lacZ*, $Amp^RChl^RGen^R$	This study
Plasmids		
pUCm-T	T-vector, ColE, Amp^R	Sangon (Shanghai, China)
pUT/mini-Tn5Kan	Mini-Tn5 suicide donor plasmid, Amp^RKan^R	de Lorenzo et al. (1990)
pEX18Tc	Gene replacement vector with MCS from pUC18, *oriT*+ *sacB*+, Tet^R	Hoang et al. (1998)
pEXZ	pEX18Tc containing a 2.0-kb *phz*-flanking PCR fragment, Tet^R	This study
pEXZG	0.8-kb *Sma*I-digested *aacC1* fragment (gentamicin resistance cassette) inserted in *Sca*I site in pEXZ, Tet^R Gen^R	This study
pEXN	pEX18Tc containing a 1.8-kb *prn*-flanking PCR fragment, Tet^R	This study
pEXNZ	The *prnA* fused in frame with the truncated *lacZ* gene in *Sca*I site in pEXN, Tet^R	This study
pME10N	A 6.1-kb *prnABCD* operon by PCR amplification cloned in pME6010, Tet^R	This study
pME10Z	A 6.8-kb *phzABCDEFG* operon by PCR amplification cloned in pME6010, Tet^R	Chi et al. (2017)
pME6010	Low copy vector in *Pseudomonas* sp., Tet^R	Heeb et al. (2000)
pNM480	*lacZ* fusion vector, Amp^R	Minton (1984)
pUCGm	Gentamicin resistance gene cassette (*aacC1*) resource, cloning vector, Amp^RGen^R	Schweizer (1993)

pounds have been purified and identified, such as phenazine, hydrogen cyanide, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, and their derivatives (Fenton et al., 1992; Ge et al., 2004; Laville et al., 1992; Mavrodi et al., 1998, 2013; Thomashow and Weller, 1988; Voisard et al., 1989). Due to their biocontrol properties, these bacterial strains exhibit great application potential in bio-protecting crops from different diseases caused by fungal phytopathogens in modern agriculture (Haas and Défago, 2005; Mauchline and Malone, 2017; Mavrodi et al., 2013).

P. chlororaphis G05 is a novel root-colonizing biological control agent that was isolated from greenhouse soil in the Jiangsu province of China. In our previous reports, we found that it could suppress the growth of *Fusarium oxysporum* and *Rhizoctonia solani* around the roots of pepper, cucumber, and other vegetables in a greenhouse (Ge et al., 2008). It was proved later that biosynthesis and secretion of phenazine-1-carboxylic acid in our isolate contributed to its biocontrol property against the diseases caused by the above-mentioned fungal phytopathogens. Recently, we found that besides phenazine-1-carboxylic acid, another antifungal compound, pyrrolnitrin, is also biosynthesized and secreted in *P. chlororaphis* G05. Moreover, we confirmed that pyrrolnitrin, and not phenazine, produced in our isolate contributed to the growth suppression of *Fusarium graminearum*, a fungal phytopathogen that often causes FHB disease on wheat and leads to a heavy loss in agriculture (Huang, 2017). In fact, although *P. chlororaphis* G05 exhibited its biocontrol function against fungal diseases, the production yield of pyrrolnitrin was extremely low. In addition, the *phz* and *prn* operons that are respectively responsible for the biosynthesis of phenazine-1-carboxylic acid and pyrrolnitrin were cloned and identified in wild-type strain G05. According to the results of BLAST, the wild-type strain G05 is like other phenazine-producing and pyrrolnitrin-producing strains and has the same number and arrangement of genes in the *phz* and *prn* operon on its chromosome. Although some regulators, such as the GacS/GacA system, the Quorum Sensing system, ANR, Vfr, and RpoS, were identified in certain *Pseudomonas* spp. to mediate pyrrolnitrin biosynthesis positively or negatively (Selin et al., 2010, 2012, 2014; Haas and Keel, 2003; Nandi et al., 2016; Oh et al., 2013; Zhang et al., 2016), we still do not know much about the regulatory pathway of pyrrolnitrin biosynthesis in detail.

In order to promote pyrrolnitrin biosynthesis in *P. chlororaphis* G05 and improve its bio-protection of vegetables and crops against fungal phytopathogens, we sought, in this study, to screen and identify novel candidate genes that regulate the *prn* operon expression. To achieve this purpose, we first constructed and confirmed a chromosomal fusion mutant G05Δ*phz*Δ*prn*::*lacZ*, in which most coding regions of the *phz* and *prn* operon were deleted and the DNA region upstream of the *prnA* was in-frame fused with the truncated *lacZ* reporter gene on its chromosome. With this mutant as the recipient strain, we then conducted transposon mutagenesis using a pUT/mini-Tn5Kan delivery system. Based on the variation in the colony color (deep blue, light blue or white), the target exconjugants on LB agar plates supplemented with X-Gal and kanamycin were screened and picked up, in which the potential regulatory genes for the *prn* operon expression would be disrupted by a random insertion of a

Table 2. Primers used in this study.

Primers	Sequences (5′-3′, artificial restriction enzyme site underlined and in italics)
N-1F	CTGGAGGATGTTGGGACGGCGTTGC
N-1R	GAACTT*AGTACT*CTGCTGGAGCGCCCGGACGAGGTAC (*Sca*I)
N-2F	GTTCAA*AGTACT*ACCGGGGCTGGGTCGACCGCGTC (*Sca*I)
N-2R	CCAGATAGCTGCCGCCGAACTTGCC
N-3F	CAAGTT*GAATTC*TTCCAGGTGCGCGGCCTGAAGCTG (*Eco*RI)
N-3R	CAAGTT*GGATCC*GCCCGCGAGGAATACACCG (*Bam*HI)
Z-1F	GTGATGGTAATTCACGGTATCGGTG
Z-1R	CAAGTA*AGTACT*AGGGGAAAGCGAAGCAGGCATGG (*Sca*I)
Z-2F	GAACTT*AGTACT*TTGAAGTGCCTGGCTGCTCCAAC (*Sca*I)
Z-2R	GCGTTGGCCAGTTCCGGCACGTTCG
Z-3F	CAAGTT*GGTACC*CCCATGATCACGCCGATTTCC (*Kpn*I)
Z-3R	CTTGAA*CTCGAG*CGGGTGGTCATAGGCGCTGGCG *(Xho*I)
G-F	GCAGCAACGATGTTACGCAG
G-R	TGTTAGGTGGCGGTACTTGG
L-F	GAGCGCCGGTCGCTACCATTACCAG
L-R	CTGGTGCCGGAAACCAGGCAAAGCG
N-WF	CTTGAA*TCTAGA*ATGGGAAATGGTGTTCTCCCTGGG (*Xba*I)
N-WR	CTTGAA*GGATCC*AGCACGAGTTGCAACAGCCAGATAG (*Bam*HI)
AmpF	CATAGTTGCCTGACTCCCCGTCGTG
AmpR	GTTGGGTGACG AGTGGGTTACATC
Tn5-F	GCACGCAGGTTCTCCGGCCGCTTGG
Tn5-R	GGCGATGCGCTGCGAATCGGGAGCG

transposon. With cloning and identification of the regulatory genes, the regulation mechanism involving the pyrrolnitrin biosynthesis could be updated later.

Materials and Methods

Bacterial strains, plasmids, primers and culture conditions. All bacterial strains and plasmids involved in this study are shown in Table 1. All designed PCR primers employed in this work are listed in Table 2. *Escherichia coli* DH5α and other strains were routinely cultivated in Luria-Bertani (LB) medium at 37°C (Sambrook and Russell, 2001). *P. chlororaphis* G05 and its derivatives were regularly grown in LB medium at 30°C (Ge et al., 2008), or in glycerol-alanine supplemented (GA) medium at 30°C for phenazine assays (Chieda et al., 2005). Antibiotic compounds, when required, were applied at the following final concentrations in the medium (μg/mL): Ampicillin (Amp), 100; chloramphenicol (Chl), 30; spectinomycin (Spe), 100; kanamycin (Kan), 50; gentamicin (Gen), 20; and tetracycline (Tet), 25 for *E. coli* and 100 for *P. chlororaphis* strains.

In addition, two fungal phytopathogens, *F. oxysporum* HB-1 and *F. graminearum* PH-1, were prepared on potato dextrose agar (PDA) medium at 25–28°C.

Recombinant DNA techniques. Small-scale plasmid DNA isolation from *E. coli* and *P. chlororaphis* strains were prepared with alkaline lysis or standard methods provided by Plasmid DNA Extraction Kit (Sangon, Shanghai, China). Chromosomal DNA as a PCR template was isolated from *P. chlororaphis* using the Genomic DNA Extraction Kit (Sangon, Shanghai, China) or the method as described by Chen and Kuo (1993). For DNA fragment sequencing, PCR products were regularly purified using the PCR Purification Kit (Sangon, Shanghai, China) and cloned into pUCm-T vector. Standard techniques were employed for restriction endonuclease digestion, ligation, agarose gel electrophoresis, and isolation of DNA fragments from low melting point agarose gels. Transformation of competent bacterial cells with plasmid DNA was performed as described by Smith and Iglewski (1989).

Construction of the fusion mutant G05Δprn::lacZ. To easily detect the level of the *prn* operon expression in *P. chlororaphis* G05, we constructed a fusion mutant, in which the most DNA region of the *prnABCD* operon on chromosome was deleted and the promoter DNA region and upstream of the *prnA* were fused in-frame with the truncated *lacZ*. Firstly, a 1094 bp fragment covering the DNA region upstream of the *prnA* gene was amplified with primers N-1F and N-1R. A second fragment of 1191 bp, which was located at the downstream regions of the *prnD*, was amplified using primers N-2F and N-2R. Two PCR amplicons were pooled, purified, digested with *Sca*I, then re-purified, and finally ligated. The ligation was purified and served as the template, and the nested PCR was performed with a pair of primers N-3F/N-3R to obtain a 2.0 kb PCR product. After double digestion with *Eco*RI and *Bam*HI, the PCR product was cloned into the suicide plasmid pEX18Tc (Hoang et al., 1998), resulting in pEXN. Secondly, a 3.1 kb DNA fragment of the truncated *lacZ* gene was obtained with the *Sma*I-*Dra*I double digestion of pNM480 (Minton, 1984), was then in-frame inserted in the *Sca*I site in pEXN to yield pEXNZ.

Biparental mating was performed by mobilizing the suicide vector pEXNZ from *E. coli* SM10 λ*pir* (the donor strain) to *P. chlororaphis* G05 (the recipient strain). The potential mutant G05Δ*prn*::*lacZ* that lacked tetracycline resistance was isolated on LB agar plates supplemented with 10% (w/v) sucrose, indicating that a double-crossover event had occurred (Ge et al., 2007).

Deletion of the phz operon in the fusion mutant G05Δprn::lacZ. To knock out the *phzABCDEFG* operon, a disruption plasmid pEXZ was first created with the same

method as above, using three pair of primers Z-1F/Z-1R, Z-2F/Z-2R, and Z-3F/Z-3R. A gentamicin resistance cassette (*aacC1*) was obtained with the *Sma*I-digestion of the cloning vector pUCGm (Schweizer, 1993), and cloned into the unique *Sca*I site in the center of the 2.0 kb *phz*-flanking PCR fragment in pEXZ to generate pEXZG.

After confirmation, the suicide plasmid pEXZG was mobilized from *E. coli* SM10 *λpir* to the fusion mutant G05Δ*prn*::*lacZ* by biparental mating. The *phz-prn*-deleted fusion mutant (designated as G05Δ*phz*Δ*prn*::*lacZ*) was selected on plates containing 10% sucrose and gentamicin due to its gentamicin resistance and tetracycline sensitivity.

The phz and prn operon complementation assay. To check the biocontrol ability of the mutant G05Δ*phz*Δ*prn*::*lacZ* against fungal phytopathogens, we constructed shuttle vectors pME10N. Firstly, 6.1 kb DNA fragments containing the whole *prn* operon were amplified by PCR with a pair of primers (N-WF/N-WR). Digested with *Xba*I and *Bam*HI, they were then cloned into the *Xba*I-*Bgl*II site of the shuttle vector pME6010 (Heeb et al., 2000), to generate pME10N. After sequencing, pME10N and the previously-constructed pME10Z (Chi et al., 2017) were respectively transformed into the competent G05Δ*phz*Δ*prn*::*lacZ* cells. The positive transformants on LB agar plates supplemented with tetracycline were confirmed by plasmid isolation and restriction enzyme digestion.

Randomly-insertional mutagenesis by pUT/mini-Tn5Kan. A typical randomly-insertional mutagenesis mediated by transposon mini-Tn5Kan was carried out using biparental mating (de Lorenzo et al., 1990). Briefly, the donor strain *E. coli* S17-1 *λpir* bearing pUT/mini-Tn5Kan and the recipient strain G05Δ*phz*Δ*prn*::*lacZ* were separately cultivated in LB medium with the required antibiotics at an appropriate concentration overnight at 30°C. 100 μL of each culture was harvested with centrifugation at 8,000 × *g* for 1 min. Each pellet was resuspended and washed at least two times in 1.0 mL of sterile 10 mM $MgSO_4$. Two samples were then mixed and resuspended in 100 μL $MgSO_4$ solution, and finally transferred onto a sterile filter membrane (Pore Diameter = 0.22 μM) that was placed on an LB agar plate. After 24 h of incubation at 37°C, The cells grown on the surface of the filter membrane were washed with 200 μL of sterile 10 mM $MgSO_4$, and 0.1–100 μL aliquots of the suspension were spread onto an LB agar plate supplemented with gentamicin, kanamycin and X-Gal. The blue colonies of exconjugants appeared after 2 days of incubation at 30°C and were randomly chosen for further identification.

Phenazine-1-carboxylic acid, pyrrolnitrin and β-galactosidase activity assay. For phenazine-1-carboxylic acid, the cell cultures were grown in 500 mL shaking flasks with 150 mL GA broth at 30°C for 72 h. Samples of bacterial cultures were collected and quantified once every 12 h. Briefly, 180 μL of each culture was acidified to pH 4.0 with HCl before the addition of 540 μL of chloroform. After being mixed thoroughly for 2 min on a vortex mixer, chloroform extracts were clarified by centrifugation at 10,000 × *g* for 5 min. Phenazine samples were diluted with chloroform appropriately, and phenazine-1-carboxylic acid was quantified spectrophotometrically at 252 nm (Cui et al., 2016; Kim, 2000). The equation of linear regression [concentration (μg/mL) = 2.9667 × OD_{252} – 0.0979, R^2 = 0.9998] was created with a purified sample of phenazine-1-carboxylic acid provided by Dr. Xu (Shanghai Jiaotong University, Shanghai, China).

To quantify pyrrolnitrin, cultures of the strains were inoculated into GA medium and cultivated under the same conditions as above. Once every 12 h, 6.0 mL of each culture sample was harvested and added with 3.0 mL of chloroform, mixed, and then vortexed for 5 min. Chloroform extracts were clarified by centrifugation at 10,000 × *g* for 5 min. After 1.0 mL of extract was dried in air, pyrrolnitrin was resolved in 1.0 mL of acetonitrile and quantified by HPLC (Agilent 1200 system with a diode array detector) with a reverse-phase C18 column (Agilent SB-C18, 250 by 4.6 mm) (Costa et al., 2009; Selin et al., 2010). Samples were eluted isocratically (45% water, 30% acetonitrile, and 25% MeOH) at a flow rate of 1.0 mL/min. Pyrrolnitrin samples were monitored with a diode-array detector (UV6000) at 250 nm of the revised wavelength. Purified pyrrolnitrin was purchased from Sigma-Aldrich (St. Louis, MO, USA) as a standard.

For the β-galactosidase enzyme assay, the wild-type strain G05 and its derivative were grown in 150 mL of GA or LB medium with shaking at 30°C after inoculation with 1.5 mL of overnight cultures. Samples were harvested after specified periods of growth. After treatment of cells with SDS and chloroform in appropriate amounts, β-galactosidase activities were quantified with the Miller method (Cui et al., 2016; Sambrook and Russell, 2001).

Pathogen inhibition in vitro assay. Phenazine-1-carboxylic acid and pyrrolnitrin could suppress the growth of pathogen *F. oxysporum* and *F. graminearum* (Huang, 2017; Upadhyay and Srivastava, 2011). We made assays to confirm the bioprotection of the wild-type strain G05 and its derivatives against them. All bacterial strains were grown in GA medium for 72 h at 30°C. 20 μL aliquots of cell suspensions were separately spotted onto triplicate PDA plates. A 6-mm-diameter plug of *F. oxysporum* HB-1 or *F. graminearum* PH-1 grown on a PDA plate was separately placed in the center of the plates. The zone of inhibition developed 4 days after growth in an incubator at 30°C and the distance between the edge of the bacterial colony and the fungal mycelium was measured (Ge et al., 2006). The assays were carried out three times to obtain real data on the inhibition of mycelial growth.

Southern blot. To confirm random insertion of mini-Tn5kan in the chromosome of exconjugants, we also carried out a Southern blot analysis. The chromosomal DNA of exconjugants was digested with *Sal*I and transferred to a nitrocellulose membrane. The probe was prepared with a Dig-labeled DNA fragment amplified with a kanamycin resistance gene as a template in mini-Tn5 transposon. All hybridization steps were carried out with standard methods provided by DIG High Prime DNA Labeling and Detection Starter Kit I (for color detection with NBT/BCIP) (Roche, Germany).

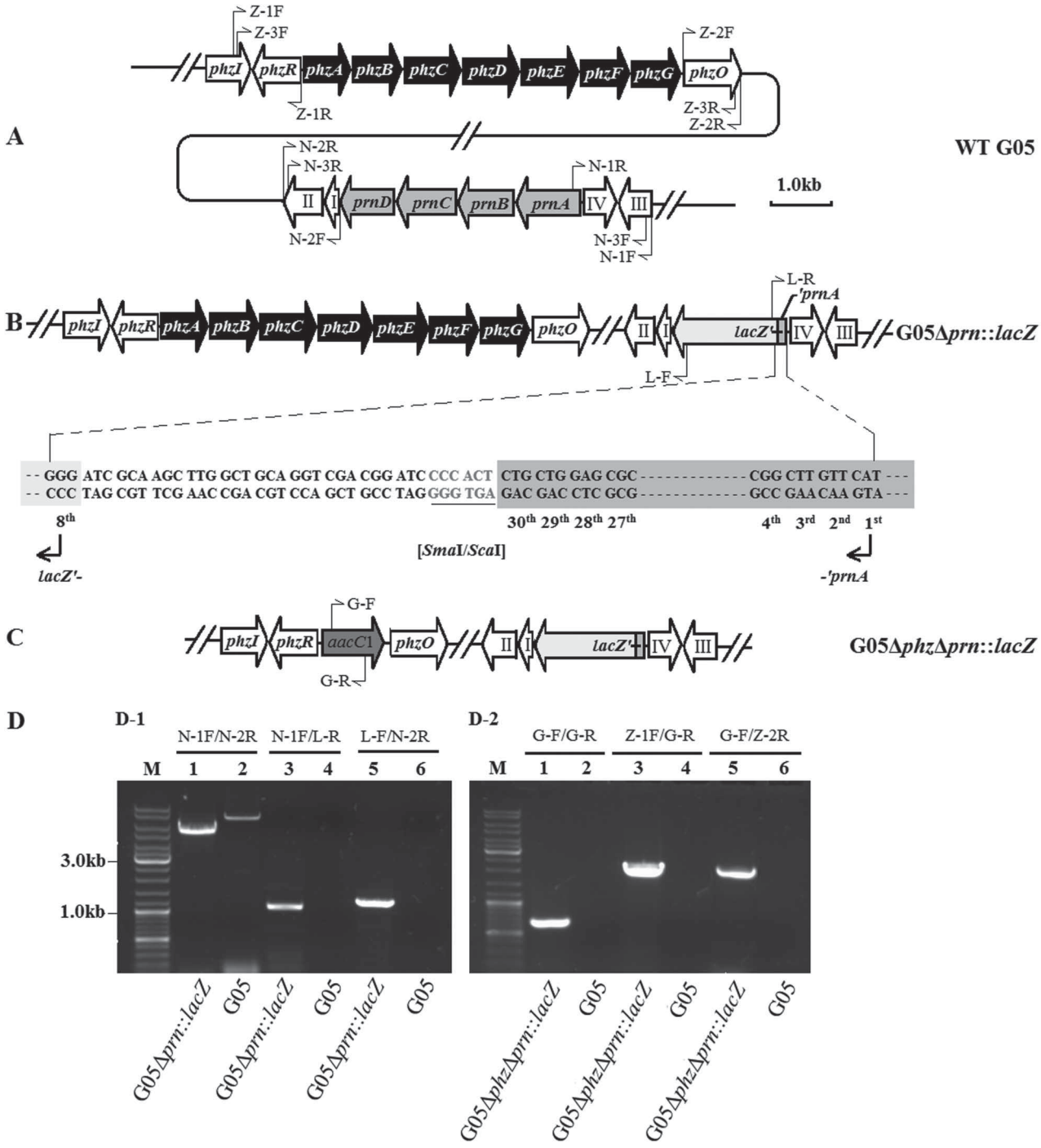

Fig. 1. Construction and identification of the fusion mutants, G05Δ*prn*::*lacZ* and G05Δ*phz*Δ*prn*::*lacZ*.

A. Diagram of the phenazine-producing gene cluster *phzABCDEFG* (black arrows) and the pyrrolnitrin-producing gene cluster *prnABCD* (light grey arrows) and their adjacent ORFs. The binding sites of six pairs of primers, i.e., Z-1F/Z-1R, Z-2F/Z-2R, Z-3F/Z-3R, N-1F/N-1R, N-2F/N-2R, and N-3F/N-3R, are also indicated. B. Diagram of the fusion mutant G05Δ*prn*::*laZ*, indicating the most DNA region of the *prn* operon (the *prnABCD*) was deleted and the upstream of the *prnA* (containing the promoter DNA region and the first thirty codons) was fused in-frame with the eighth codon of the truncated *lacZ* reporter gene (yellow arrow). The binding sites of the primers L-F and L-R are also indicated within the *lacZ* reporter gene. C. Diagram of the fusion mutant G05Δ*phz*Δ*prn*::*laZ*, indicating that the most partial *phzABCDEFG* region was deleted and inserted with a gentamicin resistance cassette (*aacC1*, dark grey arrow) on the chromosome. The binding sites of the primers G-F and G-R are also indicated within the gentamicin resistance gene. D. Identification of the fusion mutant G05Δ*prn*::*lacZ* and G05Δ*phz*Δ*prn*::*lacZ* by PCR amplification. The genomic DNA isolated from the wild-type strain G05, the fusion mutant G05Δ*prn*::*lacZ* (D-1) or the fusion mutant G05Δ*phz*Δ*prn*::*lacZ* (D-2) as template are shown at the bottom of the lanes. Several pairs of primers for PCR amplification are indicated at the top of the lanes. Letter M stands for standard DNA ladder (No. SM0331, MBI Fermentas, Canada).

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 inhibition of mycelial growth *in vitro*. Values of $p < 0.05$ were considered statistically significant, and values of $p < 0.01$ were extremely significant.

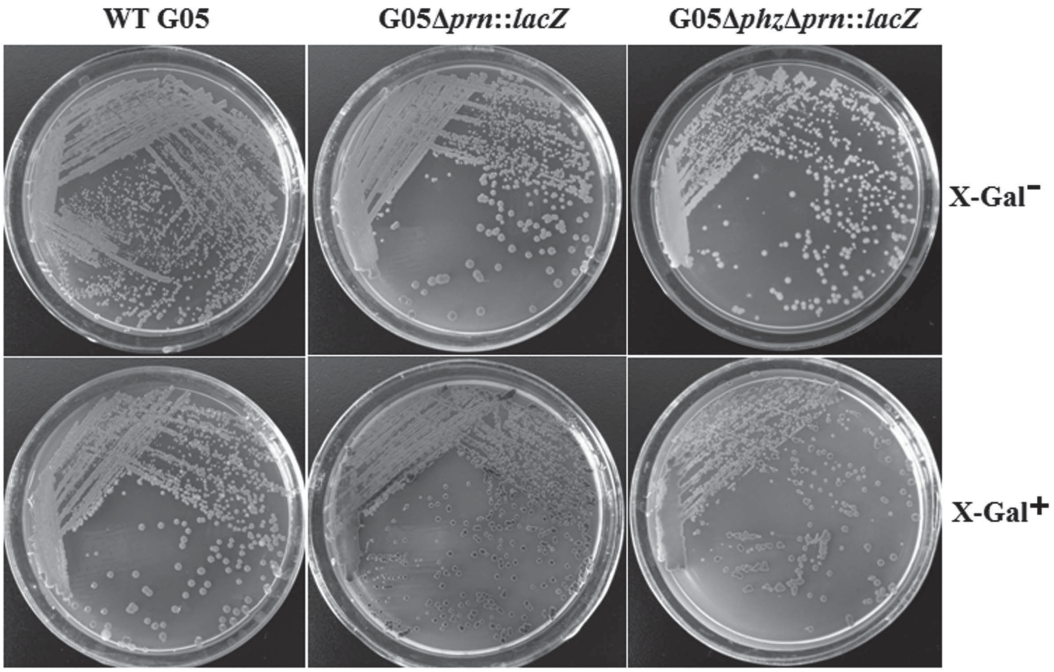

Fig. 2. The wild-type strain G05 and its derivative mutants grown on LB agar plates.

The colonies of the fusion mutant G05Δ*prn*::*lacZ* and G05Δ*phz*Δ*prn*::*lacZ* developed and turned blue after they were separately streaked on an LB agar plate with the addition of X-Gal, but did not turn blue on an LB agar plate with no addition of X-Gal. The wild-type strain G05 streaked on LB agar plates with X-Gal or without X-Gal was used as a negative control.

Results and Discussion

The construction and PCR identification of the fusion mutant G05Δprn::lacZ, G05ΔphzΔprn::lacZ

As the *prnABCD* operon is responsible for encoding a series of enzymes for pyrrolnitrin biosynthesis in *P. chlororaphis*, regulation of the *prn* operon expression definitely plays a key role in increasing production of pyrrolnitrin. In order to easily screen novel regulatory genes and to obtain more insight into the regulatory mechanism of pyrrolnitrin biosynthesis, we first constructed a fusion mutant G05Δ*prn*::*lacZ* with homologous recombination, in which most of the coding region of the *prnA* and the whole *prnBCD* were deleted, but the promoter DNA region and the first thirty codons of the *prnA* were in-frame fused with the truncated *lacZ* reporter gene. Furthermore, in order to erase the interference caused by the color of phenazines, we deleted the *phz* operon in the mutant G05Δ*prn*::*lacZ* and created a phenazine-deficient mutant G05Δ*phz*Δ*prn*::*lacZ*.

To confirm the deletion of the *phz* and *prn* operons and the insertion of the truncated *lacZ* gene on the chromosome of the mutants above, we designed two pairs of specific primers and amplifications were carried out by PCRs. As shown in Figs. 1B and 1C, the primers L-F and L-R were located at the two ends of the truncated *lacZ* reporter gene, and the primers G-F and G-R at two ends of the gentamicin resistance cassette (*aacC1*) region. As shown in Fig. 1D-1, with the genomic DNA isolated from the wild-type strain G05 (Lanes 2, 4, and 6) and the mutant G05Δ*prn*::*lacZ* (Lanes 1, 3, and 5) as templates, PCR amplifications were conducted with three pair of primers (N-1F/N-2R, N-1F/L-R, and L-F/N-2R), respectively. Positive amplification bands with expected sizes were observed in Lanes 1, 2, 3, and 5, and no bands in Lanes 4 and 6, indicating that the *prn* operon was deleted and that the truncated *lacZ* was successfully inserted in the chromosome of the mutant G05Δ*phz*Δ*prn*::*lacZ*. As shown in Fig. 1D-2, PCR amplifications were carried out with different genomic DNAs isolated from the wild-type strain G05 and the mutant G05Δ*phz*Δ*prn*::*lacZ* as templates and relevant primers (G-F/G-R, Z-1F/G-R, and G-F/Z-2R). Similarly, positive bands with expected sizes were amplified in the expected lanes, suggesting that the most of the DNA region of the *phz* operon was deleted and replaced with a gentamicin resistance cassette in the chromosome of the mutant G05Δ*phz*Δ*prn*::*lacZ*.

Characterization of the fusion mutant G05Δprn::lacZ and G05ΔphzΔprn::lacZ

Color of colonies. Although we did PCRs and confirmed that the truncated *lacZ* reporter gene was inserted into the deleted DNA region of the *prn* operon in the mutants, we did not know whether the reporter gene was in-frame fused with the coding region of the *prnA*. If it was, then the fused β-galactosidase would be expressed under the control of the promoter of the *prn* operon, and, thus, the colony color of the mutants would turn blue on an LB agar plate supplemented with X-Gal. To verify this hypothesis, the mutant G05Δ*prn*::*lacZ* and G05Δ*phz*Δ*prn*::*lacZ* were respectively streaked on an LB agar plate with X-Gal and without X-Gal. The plates were incubated at 30°C for two days. As shown in Fig. 2, colonies of the mutant G05Δ*prn*::*lacZ*

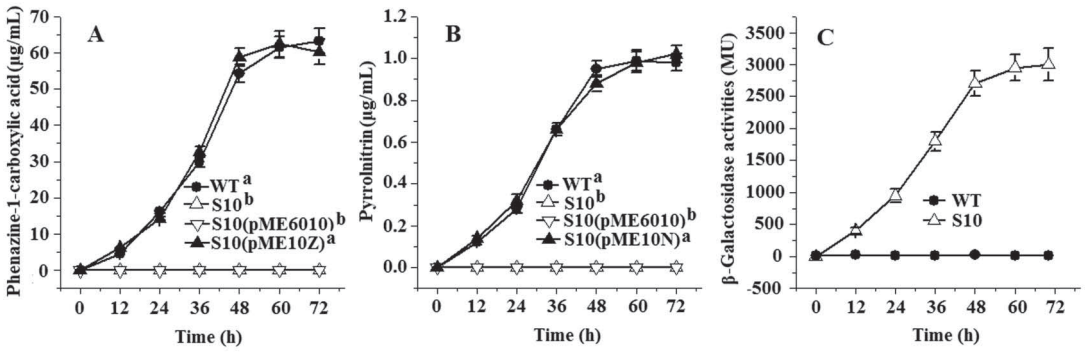

Fig. 3. Antifungal compounds production and β-galactosidase activities detected in the *P. chlororaphis* G05 and its derivatives.

Phenazine-1-carboxylic acid (A), pyrrolnitrin (B) and β-galactosidase activities (C) were respectively measured in the 72-hour cultivations of the wild-type strain G05 (black circles), the fusion mutant G05Δ*phz*Δ*prn*::*lacZ* (S10, white triangles), the transformant G05Δ*phz*Δ*prn*::*lacZ* (pME10Z) [S10 (pME10Z), black triangles in A], the transformant G05Δ*phz*Δ*prn*::*lacZ* (pME10N) [S10 (pME10N), black triangles in B], and the transformant G05Δ*phz*Δ*prn*::*lacZ* (pME6010) [S10 (pME6010), white inverted triangles]. All experiments were carried out in triplicate, and each value was presented as the average plus standard deviation. Different superscript lowercase letters following the wild-type strain and its derivatives indicate a significant difference ($p < 0.05$) according to Duncan's multiple range test.

and G05Δ*phz*Δ*prn*::*lacZ* actually turned blue on LB medium with X-Gal. These results confirmed that the truncated *lacZ* reported gene was not only inserted in the *prn*-deleted gap in the chromosome, but also fused in-frame with the *prnA*.

Due to phenazines produced with the presence of the *phz* operon in the mutant G05Δ*prn*::*lacZ*, they definitely make the medium and colonies on the plates turn yellowish-brown. So, the blue color of colonies developed in X-Gal-supplemented LB agar plates was not bright and clear (Fig. 2). However, the colony of the mutant G05Δ*phz*Δ*prn*::*lacZ* was truly pure and bright in the medium on the plates, because the interference of phenazines was eradicated with the deletion of the *phz* operon in the mutant G05Δ*phz*Δ*prn*::*lacZ*. In comparison with the mutant G05Δ*prn*::*lacZ*, the mutant G05Δ*phz*Δ*prn*::*lacZ* is therefore better and more suitable to next transposon mutagenesis.

Production of antifungal compound and β-galactosidase. If the *phz* and *prn* operons were deleted, the mutant G05Δ*phz*Δ*prn*::*lacZ* would lose its phenazine-carboxylic acid and pyrrolnitrin production. To check this point, we inoculated the mutant G05Δ*phz*Δ*prn*::*lacZ* and the wild-type strain G05 into GA medium and measured the production of antifungal compounds. As shown in Figs. 3A and 3B, phenazine-1-carboxylic acid and pyrrolnitrin were biosynthesized in the wild-type strain G05, but neither phenazine-1-carboxylic acid nor pyrrolnitrin was produced in the fusion mutant G05Δ*phz*Δ*prn*::*lacZ*. However, the biosynthesis of phenazine-1-carboxylic acid and pyrrolnitrin was recovered when the mutant G05Δ*phz*Δ*prn*::*lacZ* was complemented with pME10Z and pME10N, respectively. These results confirmed that the wild-type strain G05 just carries one copy of the *phz* and *prn* operon on its chromosome, and two operons were indeed knocked out in the mutant G05Δ*phz*Δ*prn*::*lacZ*. In addition, with the Miller method, we quantified β-galactosidase activities produced in the parental strain G05 and the mutant G05Δ*phz*Δ*prn*::*lacZ*. As shown in Fig. 3C, in GA medium, β-galactosidase activities in the mutant G05Δ*phz*Δ*prn*::*lacZ* were very high. On an average, after 72 h of inoculation, the β-galactosidase activity of the mutant G05Δ*phz*Δ*prn*::*lacZ* could reach 3000 Miller units. Meanwhile, no β-galactosidase activities were measured with the parental strain G05 ($P < 0.01$, two-tailed paired Student *t*-test), also confirming that the truncated *lacZ* reporter gene was indeed inserted and fused with the *prnA* in-frame.

Table 3. Pathogen inhibition assay.

Strains	Zone of inhibition in GA medium plates*	
	F. oxysporum HB-1	*F. graminearum* PH-1
G05	8.68 ± 0.50$^{\$}$	10.52 ± 0.50$^{\#}$
G05Δ*phz*Δ*prn*::*lacZ*	NA	NA
G05Δ*phz*Δ*prn*::*lacZ* (pME10Z)	9.25 ± 0.45$^{\$}$	NA
G05Δ*phz*Δ*prn*::*lacZ* (pME10N)	NA	10.65 ± 0.45$^{\#}$
G05Δ*phz*Δ*prn*::*lacZ* (pME6010)	NA	NA

*Inhibition zone expressed as distance ± standard error in millimeters from leading edge of mycelia of *F. oxysporum* HB-1and *F. graminearum* PH-1 to bacterial spot from triplicate plates. NA indicated no inhibition zones developed around the fusion mutant G05Δ*phz*Δ*prn*::*lacZ* or its transformants.

$^{\#,\$}$The data were processed with a two-tailed Student *t*-test ($P > 0.05$).

The fusion mutant G05ΔphzΔprn::lacZ lost its ability to suppress the mycelial growth of F. oxysporum and F. graminearum. To check the bio-control ability of the wild-type strain G05 and the mutant G05Δ*phz*Δ*prn*::*lacZ*, we employed two fungal phytopathogens, *F. oxysporum* and *F. graminearum*, and made a pathogen inhibition assay *in vitro*. As shown in Table 3, we found that the zones of inhibition against *F. graminearum* developed by the wild-type strain G05. However, no inhibition zones developed around the mutant G05Δ*phz*Δ*prn*::*lacZ*. When the *phz* or *prn* operon was introduced into the mutant G05Δ*phz*Δ*prn*::*lacZ*, the inhibition zones developed again around these transformants. These results confirmed that the phenazine-1-carboxylic acid and pyrrolnitrin produced

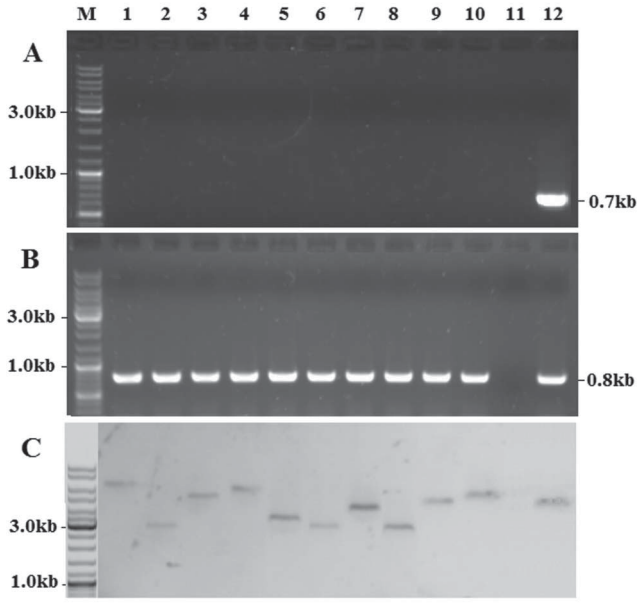

Fig. 4. Verification of transposon insertion in the chromosome of exconjugants.

A. PCR confirmation with amplification of an ampicillin resistance gene fragment in the pUT delivery system. B. PCR confirmation with amplification of a kanamycin resistance gene fragment in transposon mini-Tn5Kan. C. Southern blot analysis. Lanes 1–10 showed amplifications and hybridization with the different genomic DNA samples that were isolated from 10 randomly-picked-up exconjugants. Lane 11 showed amplification and hybridization with the chromosomal DNA of the parental strain G05Δ*phz*Δ*prn*::*lacZ* as negative controls. Lane12 showed amplification and hybridization with the pUT/mini-Tn5Kan as positive controls. The first lane M indicated standard DNA ladder (No. SM0331, MBI Fermentas, Canada).

in the wild-type strain G05 did contribute to its bio-control function against these fungal phytopathogens, and the knockout of the *phz* and *prn* operon was indeed made in the chromosome of the fusion mutant G05Δ*phz*Δ*prn*::*lacZ*.

Random mutagenesis and verification of the exconjugants G05ZNK$_{1\text{-}10}$. To screen novel regulatory genes that are responsible for pyrrolnitrin biosynthesis, random insertional mutagenesis mediated by mini-transposon was carried out. All exconjugants that had acquired the mini-transposon antibiotic resistance were spread on LB agar plates supplemented with Kan50, Gen40 and X-Gal that counter-selects the donor strain and selects recipient cells carrying the transposon marker. Blue colonies of exconjugants on each medium plate were developed after 24 h of incubation at 30°C.

To confirm that exconjugants were derived by authentic transposition of the mini-Tn5Kan rather than by co-integration of the whole pUT delivery system into the target chromosome, 10 blue colonies of the G05ZNKs were randomly picked up from the LB agar plates and their genomic DNAs were isolated from each of these exconjugants as PCR templates. We employed a pair of primers (Amp-F/Amp-R) to amplify the DNA fragments in an ampicillin resistance cassette on the pUT delivery system and another pair of primers (Tn5-F/Tn5-R) to amplify the DNA fragment in a kanamycin resistance cassette in transposon mini-Tn5Kan. As shown in Fig. 4A, no bands of the ampicillin resistance gene fragment (about 0.7 kb) were obtained in any of the lanes of the 10 samples, indicating that the pUT delivery system was not integrated into the target chromosome. These results were consistent with a previous report on the analysis of *P. putida* exconjugants (de Lorenzo et al., 1990). Meanwhile, the expected bands of the kanamycin resistance gene fragment (about 0.8kb) in transposon were positively observed and shown in all the lanes of the samples (Fig. 4B), suggesting that transposon mini-Tn5Kan were actually transferred and integrated into the chromosomes of exconjugants. Furthermore, we carried out Southern blotting with genomic DNA of exconjugants. As shown in Fig. 4C, the positive bands with different sizes (containing the partial fragment of the kanamycin resistance gene in transposon) appeared in different lanes, indicating that the mini-Tn5 transposon inserted in the chromosome of exconjugants randomly.

At present, diseases caused by infection with fungal phytopathogens often lead to a significant decrease of crop yield in modern agriculture. Many chemical fungicides are popularly employed to prevent and control these diseases that damage the field crops (D'Mello et al., 1998; Hu et al., 2014). Long-term application of these chemical fungicides, however, results in directly or indirectly deleterious effects on the soil, environment, and human and animal health (Hu et al., 2014). Due to their safe and more environmentally-friendly properties, some bio-control agents, such as *Pseudomonas* and *Bacillus* spp., are popular and are now being applied in the treatment of some diseases caused by infection of some fungal phytopathogens in certain areas. As a matter of fact, the amounts of antifungal compounds biosynthesized by these bacteria are too low to boost their widespread application in modern agriculture. Because of this problem, farmers prefer to re-apply chemical fungicides for protection of crops against soil-borne fungal diseases (Haas and Défago, 2005). In this study, we first constructed the fusion mutant *P. chlororaphis* G05Δ*phz*Δ*prn*::*lacZ*, in which the expression level of the *prn* operon was easily reflected by the expression of the fused *lacZ* reporter. We then employed this fusion mutant as a recipient strain and did transposon mutagenesis. If a colony (an exconjugant) turns deep blue on an LB medium plate spread with X-Gal, the promoter of the *prn* operon in this colony should be up-regulated and overexpressed. Otherwise, a light blue or white colony means that the *prn* operon promoter in it should be down-regulated and expressed weakly. Based on the color changes of colonies on LB medium with X-Gal, we could easily identify those color-changed colonies, pick them up and clone the transposon-interrupted genes or DNA region in these mutants with plasmid rescue or reverse PCR. Novel genes or DNA regions potentially involving the regulation of pyrrolnitrin biosynthesis could be found and identified later. In general, our work and the methods created in this study would finally be helpful in elucidating the regulatory mechanism of the *prn* expression in detail in *P. chlororaphis*, and would make it possible to increase pyrrolnitrin production and thereby improve its future application in agriculture.

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