Identification, synthesis and regulatory function of the N-acylated homoserine lactone signals produced by *Pseudomonas chlororaphis* HT66

Huasong Peng*, Yi Ouyang, Muhammad Bilal, Wei Wang, Hongbo Hu and Xuehong Zhang

**Abstract**

**Background:** *Pseudomonas chlororaphis* HT66 isolated from the rice rhizosphere is an important plant growth-promoting rhizobacteria that produce phenazine-1-carboxamide (PCN) in high yield. Phenazine production is regulated by a quorum sensing (QS) system that involves the N-acylated homoserine lactones (AHLs)—a prevalent type of QS molecule.

**Results:** Three QS signals were detected by thin layer chromatography (TLC) and high-performance liquid chromatography–mass spectrometry (HPLC–MS/MS), which identified to be N-(3-hydroxy hexanoyl)-l-homoserine lactone (3-OH-C6-HSL), N-(3-hydroxy octanoyl)-l-homoserine lactone (3-OH-C8-HSL) and N-(3-hydroxy decanoyl)-l-homoserine lactone (3-OH-C10-HSL). The signal types and methods of synthesis were different from that in other phenazine-producing *Pseudomonas* strains. By non-scar deletion and heterologous expression techniques, the biosynthesis of the AHL-signals was confirmed to be only catalyzed by PhzI, while other AHLs synthases i.e., CsaI and HdtS were not involved in strain HT66. In comparison to wild-type HT66, PCN production was 2.3-folds improved by over-expression of *phzI*, however, *phzI* or *phzR* mutant did not produce PCN. The cell growth of HT66ΔphzI mutant was significantly decreased, and the biofilm formation in *phzI* or *phzR* inactivated strains of HT66 decreased to various extents.

**Conclusion:** In conclusion, the results demonstrate that PhzI–PhzR system plays a critical role in numerous biological processes including phenazine production.

**Keywords:** *Pseudomonas chlororaphis*, Phenazine-1-carboxamide, Quorum sensing, N-Acylated homoserine lactones, *phzI*, Biofilm formation

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**Background**

Quorum sensing (QS) is a well-studied form of communication process used by a large variety of bacteria to regulate diverse cellular functions such as antibiotic production, biofilm development, gene expression, surface attachment and virulence in a cell-population density-dependent manner [1, 2]. The Gram-negative bacteria use N-acylated homoserine lactones (AHLs) to sense cell density, which is composed of a homoserine lactone ring (HSL) with varying acyl chain [3]. These diffusible small signaling molecules are synthesized by a member of the LuxI protein family. Bacteria can monitor cell-population density by measuring the concentration of small secreted signaling molecules, so-called AHLs. When AHL concentration reaches a certain threshold value, the cells can switch on the expression of a set of genes responsible for the production of bioluminescence [4], antibiotics [5], plasmid transfer [6], and symbiosis [7] etc. Therefore, the AHL-mediated QS play an essential role in numerous biological processes.

Phenazines are a class of pigmented heterocyclic metabolites, which are produced by the genera...
Pseudomonas, Burkholderia, and Streptomyces [8]. Phenazine and its derivatives, such as a phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), and 2-hydroxy-phenazine (2-OH-PHZ), exhibit potent antifungal activities against a wide range of eukaryotic microbes and therefore could be employed as a fungicide in agriculture production [9]. For example, PCA greatly reduces the risk of a severe wheat root disease caused by Gaeumannomyces graminis var. tritici [10, 11]. PCN possesses notable antifungal activity against Fusarium oxysporum f. sp. radices-lycopersici [12, 13]. Intriguingly, almost all of the phenazine compounds secreted by bacteria display antimicrobial, anti-tumor, antimalarial, and antiparasitic effects compared with some chemically synthesized phenazine derivatives [14–19]. In addition, natural phenazine products show great promise for use as electron acceptors and donors, components of microbial fuel cells (MFC), and environmental sensors and biosensors [20–23]. Most phenazine-producing microorganisms have been isolated from diverse terrestrial, freshwater, and marine environments [24, 25]. Fluorescent pseudomonads, which are members of the gamma subclass of the proteobacteria, are the best-studied phenazine producers, with strains of Pseudomonas fluorescens, P. chlororaphis, and P. aeruginosa known to produce these compounds [26]. PhzI, a LuxI homolog, is an AHL synthase mainly responsible for the synthesis of AHL signals in phenazine-producing Pseudomonas. Subsequently, the AHL-receptor protein, PhzR, binds to AHL-signals and activates the expression of downstream phenazine biosynthetic gene cluster [27].

In this study, an isolated microbe from rice rhizosphere namely Pseudomonas chlororaphis HT66 has been selected. Based on whole genome sequencing, three possible AHL synthesis genes phzI, csaI and hdtS were investigated. Among them, the phzI gene is located upstream of the phz cluster like other Pseudomonas. However, the types and biosynthesis of AHL signals are unclear and the regulation of phenazine biosynthesis needs to be studied. The AHLs were identified by Thin Layer Chromatography (TLC) and high-performance liquid chromatography-mass spectrometry (HPLC–MS/MS) analyses. Additionally, we elucidated the relationship between phenazine production, biofilm formation and AHLs biosynthesis, including which gene is responsible for the AHLs synthesis. The biocontrol activity of P. chlororaphis HT66 and its derivatives were also evaluated.

**Methods**

**Bacterial strains and growth conditions**

Selected bacterial strains and plasmids used in this study are summarized in Table 1. Bacterial strains were grown in Luria–Bertani (LB) medium at 37 °C (for Escherichia coli) or King’s B (KB) medium at 28 °C (for P. chlororaphis HT66). The bioreporter strains, Agrobacterium tumefaciens NTL4 (pZLR4) [28] and Chromobacterium violaceum CV026 biosensor strain for AHLs McClean et al. [29] and Pythium ultimum The pathogen causes ripe fruit rot of tomato Pearson et al. [33].

**Table 1 Bacterial strains, and plasmids used in this study**

| Strain, plasmid          | Characteristics                                      | Reference or source |
|-------------------------|------------------------------------------------------|---------------------|
| Escherichia coli strains |                                                      |                     |
| E. coli DH5α             | supE44ΔlacU169(φ80lacZM15)recAhsdR17 recA1 endA1 gyrA96 thi-1 relA-1 | Hanahan [45]        |
| E. coli S17              | res* pro mod* integrated copy of RP4, mob*, used for incorporating constructs in P. chlororaphis | Hoffmann et al. [46]|
| A. tumefaciens NTL4      | Biosensor strain for AHLs                            | Cha et al. [28]     |
| (pZLR4)                  |                                                      |                     |
| C. violaceum CV026       | Biosensor strain for AHLs                            | McClean et al. [29] |
| Pythium ultimum          | The pathogen causes ripe fruit rot of tomato         | Pearson et al. [33] |
| Pseudomonas chlororaphis |                                                      |                     |
| HT66                     | PCN, wild-type, Amp<sup>Sp</sup>                      | This study          |
| ΔphzI                    | HT66 derivative, phzI deleted                        | This study          |
| ΔphzR                    | HT66 derivative, phzR deleted                        | This study          |
| ΔcsaI                    | HT66 derivative, csal deleted                        | This study          |
| ΔhdtS                    | HT66 derivative, hdtS deleted                        | This study          |
| Plasmids                 |                                                      |                     |
| pK18mobsacB              | Broad-host-range gene replacement vector; sacB, Km<sup>+</sup> | Schafer et al. [47] |
| pME6032                  | IPTG-inducible expression vector, Tc<sup>+</sup>     | Heeb et al. [48]    |
| pME-phzI                 | phzI from HT66 in pME6032,Tc<sup>+</sup>             | This study          |
| pME-csaI                 | csal from HT66 in pME6032,Tc<sup>+</sup>             | This study          |
| pME-hdtS                 | hdtS from HT66 in pME6032,Tc<sup>+</sup>             | This study          |

<sup>*</sup> resistance
violaecum CV026 [29], were grown at 28 °C in AB minimal medium (ABM) [30] and LB medium, respectively. When required, antibiotics were used at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml) or tetracycline (20 μg/ml) for *E. coli* and ampicillin (100 μg/ml), kanamycin (50 μg/ml) and tetracycline (200 μg/ml) for *P. chlororaphis*; whereas the gentamicin (30 μg/ml) and kanamycin (20 μg/ml) were used for NTL4 (pZLR4) and CV026, respectively.

**DNA manipulation and mutant construction**

The non-scar gene deletion was carried out as previously described [31]. First, two pairs of primers designed to clone 100–600 bp fragments located upstream or downstream of the target gene. These two 100–600 bp fragments were amplified by polymerase chain reaction (PCR) from the genomic DNA of HT66. Next, an overlap PCR was used to combine the two fragments, which created a new sequence excluding the target gene. After digesting the overlap PCR product by restriction enzymes *EcoRI* and *HindIII*, the new sequence was ligated into pK18mobsacB by T4 DNA ligase. The resulting plasmid was first transferred into *E. coli* S17 and then mobilized into HT66 by conjugation. Afterwards, the colony carrying pK18mobsacB was inoculated into 15% sucrose containing LB medium for about 24 h and observed the blue spots indicating the location of AHLs. TLC analysis was repeated at least three times.

**Analysis of AHLs by HPLC–MS/MS**

AHL extracts from 100 ml cultures were partially purified by preparative TLC. For this, preparative TLC plate was cut into small strips and the compounds were carefully removed by scraping off the silica gel at the appropriate Rf and extracted three times with ethyl acetate. The dissolved compounds were centrifuged (at 12,000g for 10 min), and clear supernatant thus obtained was filtered by syringe filters (0.22 μm). The supernatant was concentrated and the residue was stored at −20 °C or dissolved in acetonitrile for bioassay or HPLC–MS/MS analysis. The residue contained 0.1% formic acid (analytical grade) and acetonitrile (AcN) (HPLC-grade). A flow rate of 0.5 mL/min was used, with increasing concentrations of 5 to 95% AcN in 15 min. Then, the flow was held for 3 min, and equilibration was performed for 7 min. All the mass spectra were recorded in the positive-ion mode. MS parameters were a spray voltage of 5 kV, a capillary temperature of 230 °C, and a sheath gas rate of 12 units N2.

**Determination of the PCN production**

HT66 and its mutants were grown overnight and inoculated into the fresh KB medium (1% inoculum ratio) followed by incubation for 24 h at an agitation speed of 180 rpm. Fermentation broth (300 μl) was acidified to pH
showed blue pigment, while CV026 did not produce purple pigment, suggesting that the AHLs produced by HT66 might be 3-OH and 3-oxo-acyl-HSLs.

Further, ethyl acetate extracts from HT66 culture supernatants were separated by C18-reverse TLC and then overlaid with the biosensor NTL4 strain. Notably, three blue spots were detected from the extracts of HT66 (Fig. 1). The detected compounds were compared with reported known \( R_f \) values [34] and speculated that AHLs might be 3-oxo or 3-OH-acyl-HSLs with side chains ranging from 6 to 10 carbons. To identify the signals more precisely, extracts were separated by preparative TLC, and then the corresponding parts were scrapped and dissolved in the sample for HPLC–MS/MS analysis. It is known that every member of the AHL family gives a particular lactone ring at a nominal mass of \( m/z \) 102.05 [35], which results from the cleavage of homoserine lactone ring at the N-acyclic side chain. This characteristic ion was the key to analyze AHLs by HPLC–MS/MS. After MS/MS analysis, we found that three ions generated a peak for the characteristic ion at \( m/z \) 102.05 and some other peaks of fragmentations (Fig. 2a–c). For example, the 3-OH-C8-HSL has a molecular weight of 243, and the detected mass fragmentation \([M + H]^{+}\) was 244.1546, almost same as the calculated mass 244.1543. Moreover, the ion \([M + H - H_2O]^{+}\) (226.1456) and \([M + Na]^{+}\) (266.1363) were also observed and confirmed that the AHL was 3-OH-C8-HSL. Combined with the results of TLC and bio-reporter experiments, we concluded that the AHLs produced by HT66 were 3-OH-C6-HSL, 3-OH-C8-HSL, and 3-OH-C10-HSL.

\textbf{Biofilm assay} 

The ability to form biofilms was analyzed by a highly reproducible 96-well plate assay as described earlier [32]. Overnight cultures of \textit{P. chlororaphis} HT66 and its mutants were diluted with 0.01 M phosphate-buffered saline (PBS) to \( 10^6 \) CFU/ml, and 100 \( \mu l \) diluted cultures were seeded into a 24-well plate followed by incubation at 28 °C for 24 h under static conditions. After the designated time, the cultures were gently removed by pipetting and each well was washed twice with 150 \( \mu l \) sterile PBS. Afterwards, 200 \( \mu l \) of 1% (w/v) crystal violet (CV) was added to each well to stain bacterial biofilm, and the plate was incubated at 28 °C for 20 min. The CV was then rinsed with 500 \( \mu l \) 95% ethyl alcohol and the amount of biofilm was quantified by measuring the optical density at 595 nm using Microtitre plate reader (Victor™ X series multi-label plate reader).

\textbf{In-vitro antifungal activity} 

\textit{Pythium ultimum} [33] was used to detect the antifungal activity of HT66 and its mutant derivatives. A mycelium disc of fungus was scrapped from the colonies grown on an agar plate, and spotted at the left of the agar plate, whereas the HT66 and its mutants were inoculated at the right of the agar plate. Fungal growth was monitored following incubation in darkness for 2 weeks at 28 °C. Three replicate plates were tested for each treatment.

\textbf{Results} 

Detection and identification of AHLs produced by HT66 

\textit{Chromobacterium violaeum} CV026 and \textit{A. tumefaciens} NTL4 plate assay were used to analyze the AHL-containing extracts of strain HT66. The CV026 indicator strain responds only to medium-chain alkanoyl-homoserine lactones (alkanoyl-HSLs) by producing purple spots, while NTL4 (pZLR4) responds strongly to 3-OH and 3-oxo-substituted HSLs, which react with X-gal resulting in blue pigment production. After the addition of AHL extracts to CV026 and NTL4 plates, the NTL4 assay
Fig. 2 Identification of AHLs produced by *Pseudomonas chlororaphis* HT66 using LC–MS/MS analysis, a 3-OH-C6-HSL b 3-OH-C8-HSL and c 3-OH-C10-HSL
luxI and luxR homologous genes were searched in the genome of HT66. According to the comparison results, one gene displayed extremely high identity (99%) to the AHL synthesis gene of PCL1391. This gene encodes 196 amino acids, of which only one amino acid is different from phzl of PCL1391. At the same time, an HdtS homolog was discovered, which has a similarity of 83% with the HdtS in P. fluorescens F113. The hdtS encodes 257 amino acids that direct the synthesis of C6-HSL, C10-HSL, and 3OH-C14:1-HSL in F113. Additionally, we also found another possible AHL synthesis gene in HT66 genome, CsaI, which had been reported to produce C4-, C5- and C6-HSL in P. chlororaphis 30-84 [36]. Although CsaI gene only contains 204 bp but shows high identity (over 93%) to the csaI (1416 bp) in strain 30-84. The CsaI–CsaR is a second quorum sensing system in 30-84, csaI quantity (over 93%) to the genome, also found another possible AHL synthesis gene in HT66 C10-HSL, and 3OH-C14:1-HSL in F113. Additionally, we knocked out phzI from the HT66 genome to construct HT66∆phzI mutant through a non-scar deletion method. Results showed that deletion of phzI caused the complete loss of PCN biosynthesis as well as the AHLs synthesis (Fig. 4); whereas the constitutive expression of phzI restored the production of PCN and AHLs in ∆phzI mutant by transforming into the pME6032-phzI plasmid, which could produce up to 83% of the PCN concentration in wild-type. These facts suggested that PCN production of HT66 was regulated by phzI and is also responsible for the synthesis of AHLs. Similar to HT66∆phzI strain, the HT66∆phzR derivative was also unable to produce PCN, demonstrating that the phenazine biosynthetic cluster was regulated by PhzR receptor protein.

To investigate the effects of exogenous AHLs on PCN production in HT66, we transformed the phzI over-expressed plasmid into the wild-type strain, and the empty plasmid was also transformed as a control. Results evidenced that the HT66/pME-phzI had a higher level of PCN up to 1045 mg/l, which was almost 2.3- and 4.9-folds higher than wild-type and control strain (HT66 carrying the empty pME6032 plasmid). Thus, the results implied that exogenous AHLs production had improved the PCN production. The absence of PCN production in ∆phzI or ∆phzR mutants indicated that a functional expressed

**Fig. 3** Detection of AHL extracts using *Agrobacterium tumefaciens* NTL4 plate bioassay. Samples contain extracts from cultures as follows (1) DH5α (pME-phzI) (2) DH5α (pME-csaI) and (3) DH5α (pME-hdtS)

**Fig. 4** PCN production of HT66 and its mutants; (1) wild-type HT66, (2) HT66 ∆luxI, (3) HT66 ∆luxR, (4) HT66 (pME-phzI), (5) HT66 (pME6032), (6) HT66∆phzI (pME-phzI), (7) HT66 ∆hdtS and (8) HT66 ∆csaI
quorum-sensing system was essential for the activation of the phz operon. The continuous over-expression of phzI resulted in an elevated level of PCN in wild-type HT66 strain. In addition, the PCN titers of ΔcsaI and ΔhdtS mutants were detected to be 425 and 469 mg/l, respectively, and had statistically no difference in contrast with the wild-type HT66 strain.

**Effect of phzI and phzR on growth and biofilm formation**

A comparative growth profile results portrayed that the HT66 strain reached stationary phase at almost 24 h, and started to decline after 48 h (Fig. 5a). No noticeable difference was observed between the growth of wild-type strain and its ΔphzR mutant. While ΔphzI mutant had a lower cell density in stationary phase compared to wild-type, which might be correlated with the absence of signals and negative impact on communication between microorganisms.

Biofilm structures, which protect bacteria from various physical and chemical stresses, are the major reason for bacterial persistence during chronic infections [37]. Quorum sensing regulation of swarming and DNA release has been reported, which play important roles in *P. aeruginosa* biofilm development. On the other hand, QS signaling and transcription of genes are connected with the biofilm matrix biosynthesis [38]. Reports have shown that in some bacteria phenazines were not limited to secondary metabolites but also could function as cell signals [39]. We used 24-well plate assay to assess the biofilm formation ability of HT66 strains and its mutant derivatives. Compared with the wild-type, a significant decrease in biofilm production was observed in ΔphzI and ΔphzR mutants (Fig. 5b). The biofilm formation of ΔphzI was markedly damaged and recorded only 34% amount of the wild-type. It also appeared delicate and loose in a structure following stained with crystal violet. The findings indicated that PhzI–PhzR system plays a significant role in biofilm formation in strain HT66.

**Effect on colonial morphology and antifungal activity**

On specialized colonial morphology medium (KB medium), the mutants had almost the identical growth rate as the wild-type to form colonies (Additional file 1: Figure S1). The colonies of HT66 were observed to be protruded, neat edge, and round with a semi-diameter of about 0.6 cm after 36 h growth. The surface was smooth with little viscosity. After inoculated on a plate for 48 h or a longer period, the wild-type colonies turned yellow and produced green pigment on the surface. The ΔphzI and ΔphzR mutants had no discernible changes in the morphology of colonies; however, their colonies appeared milky white and had no green pigment due to the lack of PCN production.

*Pythium ultimum* was chosen for antifungal activity experiment. HT66 and its mutants were inoculated on PDA agar plate, simultaneously with the pathogenic fungi on the other side. After cultivation together for 2 weeks, the development of pathogenic fungi was considerably inhibited by the wild-type presumably due to PCN production (Additional file 1: Figure S1). In contrary, the PCN-deficient mutant’s ΔphzI and ΔphzR could not inhibit the growth of mycelia. It is illustrated that the absence of phzI–phzR system reduced the antifungal activity of strain HT66 indicating the significance of PCN production in the biological control of soil-borne crop diseases.

**Discussion**

In this study, thin-layer chromatography and liquid chromatography-mass/mass spectrometry techniques were applied to identify three AHLs (3-OH-C6, C8, C10-HSL) in *P. chlororaphis* HT66. Strain HT66 was
a newly discovered phenazine-producing *P. chlororaphis* from rice rhizosphere, and the characteristic AHLs synthesis potential renders it distinguishing from other *Pseudomonads*. At first, we found three possible genes from the genome sequence that might produce AHLs. By heterologous expression in *E. coli* and non-scar deletion, *phzl* was evidenced to be the only responsible gene for the synthesis of these signals in HT66, while negating the contribution of *csaI* or *hdtS*. The knocking out of *csaI* or *hdtS* in HT66 has no obvious effect on PCN production and growth profile of HT66. In *P. aureofaciens* 30-84, Csa–CsaR was not required for the expression of the gene involved in phenazine biosynthesis [36]. In *P. fluorescens* F113, *hdtS* was able to direct the synthesis of AHLs in strain F113. Nevertheless, the protein HdtS not belongs to either of the known AHL synthase families (LuxI or LuxM) and has a relationship with the lysophosphatidic acid acyltransferase family [40].

In Table 2, *phzl* in several *Pseudomonas* were compared with that in HT66, including their signals products. Phzl proteins of HT66 and PCL1391 were found to be similar as high as 99% (Fig. 6). It was interesting to note that C4 and C6-HSL were the products of the *phzl* gene in PCL1391, while HT66 produces 3-OH-C6-HSL, 3-OH-C8-HSL, and 3-OH-C10-HSL. The AHLs discrepancy was due to a different amino acid in Phzl proteins of HT66 and PCL1391 and noted to be threonine and alanine respectively, at position 125. Additionally, PhzI of strain 30-84 was also compared with that of HT66. The results showed they shared almost 94% identical sequences (Fig. 6). Among the 197 amino acids sequence, there are five differences between Phzl HT66 and Phzl 30-84, and these five changes were: Glycine to Glutamine at position 10, Proline to Alanine at position 42, Threonine to Alanine at position 125, Glycine to Serine at position 179 and Alanine to Valine at position 181. As a consequence, AHLs produced by Phzl 30-84 were not only 3-OH-C6, C7, C8, C10-HSL but also C6-HSL and small amounts of C5- and C8-HSL. Regarding strain StFRB 508, there were 7 amino acids changes between Phzl 508 and Phzl HT66. Phzl 508 catalyzes the biosynthesis of C6-HSL as well as 3-OH-C6-HSL; whereas the second AHL synthase, AurI produces C4 and C6-HSL. In addition, we compared the *phzl* similarity of several phenazine-producing strains with HT66. The results revealed that these *phzl* genes were functionally same and were strongly conserved with the high sequence identity (Table 3).

Apart from the identification and production of AHLs in HT66, this study found that the over-expression of *phzl* had improved the level of PCN by 2.3-folds compared with the wild-type strain. More importantly, PCN production was abolished following the inactivation of *phzl* or *phzR* genes in HT66 indicating their roles in regulating the expression of the phenazine biosynthesis gene cluster. Previously, Morohoshi et al. [41] reported that the mutation in phzl caused a considerable reduction in phenazine biosynthesis by *P. chlororaphis* subsp. aurantiaca. On the other hand, no phenazine production was recorded in the triple mutant of phzI, aurI, and csal (508ΔPACI). Noticeably, phenazine production was supposed to be strongly stimulated by Phzl-mediated AHLs than produced by AurI and Csai that only induced a slight stimulation. Though, *Pseudomonas* sp. CMR12a and *P. chlororaphis* subsp. *aureofaciens* 30-84 display a second QS system apart from phzl/phzR system, phenazine biosynthesis was merely regulated by the AHLs produced by Phzl [36, 42, 43].

In order to appraise the involvement of AHL production to the biocontrol capacity of StFRB508, *P. ultimum* was used in bioassay. While cultivation together for 2 weeks, the development of pathogenic fungi was considerably inhibited by the wild-type presumably due to PCN production confirming the strain as an effective biocontrol agent. In *P. chlororaphis* PA23, it was examined that the biofilm development was reduced by approximately fivefold in the QS-deficient mutants and the motility was also altered [44]. Similarly, the biofilm formation in *phzl* and *phzR* deletion strains of HT66 had a decrease in different extents. With the absence of

| Table 2 Primers used in this study | Primer name | Primer sequence (5′–3′) |
|-----------------------------------|-------------|------------------------|
| phzl-F1                           | TCGGAATTCATGCACATGGAAGAGCACA  |
| phzl-R1                           | TGGCTCGAGTTAGCTCTATCCTCCATCATGTAATGT |
| csal-F1                           | TCGGAATTCATGCGGCGGAGGGCCGCT |
| csal-R1                           | TCGTCTCGACGTCCTCCATGAGGCGCTGA  |
| hdtS-F1                           | TCGGAATTCATGCTGACGTCCTCCATGAGGCGCTGA  |
| hdtS-R1                           | TCGTCTCGACGTCCTCCATGAGGCGCTGA  |
| Δphzl-F1                          | TCGGAATTCATGCCAGATTTGCTGAGAG  |
| Δphzl-R1                          | TCGGAATTCATGCCAGATTTGCTGAGAG  |
| Δcsal-F2                          | TCGGAATTCATGCCAGATTTGCTGAGAG  |
| Δcsal-R2                          | TCGGAATTCATGCCAGATTTGCTGAGAG  |
| ΔhdtS-F2                          | TCGGAATTCATGCCAGATTTGCTGAGAG  |
| ΔhdtS-R2                          | TCGGAATTCATGCCAGATTTGCTGAGAG  |

(Peng et al. *Microb Cell Fact* (2018) 17:9)
In conclusion, we identified three kinds of AHLs (3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C10-HSL) in P. chlororaphis HT66 by TLC and HPLC–MS/MS analyses. The production of AHLs exhibiting the activating role in phenazine biosynthesis gene cluster was only catalyzed by PhzI. In comparison to wild-type HT66, PCN production was 2.3-folds improved by over-expression of phzI, however, phzI or phzR mutant did not produce PCN. In addition, the growth, morphology and biofilm formation were all under the control of the PhzI–PhzR regulatory system. These results revealed that PhzI–PhzR system plays a pivotal role in PCN production and the biocontrol activity of HT66.

### Additional files

**Additional file 1: Figure S1.** a Colonial morphology changes in wild-type HT66, HT66ΔphzI and HT66ΔphzR strains during 7 days and b Influence of antifungal activity of HT66 and its mutants on the growth of Zyxium ultimum. P. ultimum was spotted at the left of the PDA plate, whereas HT66 and its mutants were inoculated on the right side.

### Authors’ contributions

PZ carried out the experimental-based work and drafted the manuscript. HBH, WW, and XHZ analyzed and interpreted the data. M8 analyzed the data and assisted in drafting the manuscript. All the research work was carried out...
under the dynamic guidance and supervision of PZ who designed, conceived and coordinated the experiments. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of supporting data
The dataset(s) supporting the conclusions of this article is (are) included within the article (and its additional file(s)).

Consent for publication
All authors read and approved the final manuscript.

Ethics approval and consent to participate
We state this is not applicable. The manuscript does not report on or involve the use of any animal or human data or tissue.

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