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Comparative genomic analysis and phenazine production of *Pseudomonas chlororaphis*, a plant growth-promoting rhizobacterium

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ABSTRACT

Pseudomonas chlororaphis HT66, a plant growth-promoting rhizobacterium that produces phenazine-1-carboxamide with high yield, was compared with three genomic sequenced *P. chlororaphis* strains, GP72, 30–84 and O6. The genome sizes of four strains vary from 6.66 to 7.30 Mb. Comparisons of predicted coding sequences indicated 4833 conserved genes in 5869–6455 protein-encoding genes. Phylogenetic analysis showed that the four strains are closely related to each other. Its competitive colonization indicates that *P. chlororaphis* can adapt well to its environment. No virulence or virulence-related factor was found in *P. chlororaphis*. All of the four strains could synthesize antimicrobial metabolites including different phenazines and insecticidal protein FitD. Some genes related to the regulation of phenazine biosynthesis were detected among the four strains. It was shown that *P. chlororaphis* is a safe PGPR in agricultural application and could also be used to produce some phenazine antibiotics with high-yield.

Introduction

Pseudomonas is a diverse genus known for their ubiquity in the environment and production of secondary metabolites [1]. Some pseudomonad strains are well-suited to be biocontrol agents [2], producing a wide range of bioactive metabolites. The general antibiotics produced by *Pseudomonas* include phenazine derivatives, pyoluteorin (Plt), pyrrolnitrin (Prn), hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (DAPG) and insect toxin [1]. The ability to release products with antimicrobial activity is the major mechanism by which pseudomonads suppress pathogens. Since the persistent use of chemical pesticides jeopardizes the health of some species like amphibians [3], biocontrol agents, including plant growth-promoting rhizobacteria (PGPR) such as *Pseudomonas chlororaphis* strains, become the focus of study [4].

P. chlororaphis is an important non-pathogenic biocontrol agent and is studied widely. Many strains show antagonistic activity against a number of disease-causing pathogens, such as *Fusarium oxysporum* f. sp. *radicis-lycopersici* [5], *Colletotrichum lagenarium*, *Phytophthora capsici*, *Pythium aphanidermatum*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, *F. oxysporum* f. sp. *cucumerinum*, *Corticium sasakii*, *Rhizoctonia solani* [6] and *Gaeumannomyces graminis* var. *tritici* [5]. *P. chlororaphis* competitively colonizes the roots, and is distributed worldwide [6–8]. Additionally, *P. chlororaphis* is well known for its ability to adapt well to the environment using several mechanisms, including the degradation of aromatic compounds [7], complex regulatory systems [9,10] and metabolism of nitrile compounds [11]. However, no systematic study of *P. chlororaphis* has been performed. It is important to study the shared and different traits of *P. chlororaphis* at the genomic level to better apply *P. chlororaphis* in agriculture and to promote the production of certain antibiotics. Whole-genome sequencing makes a comparative analysis among strains of *P. chlororaphis* possible. Up until January 2014, four *P. chlororaphis* strains have been whole-genome sequenced.

P. chlororaphis HT66 was isolated from the rhizosphere of rice in Shanghai, China by our group. This strain shows broad-spectrum resistance to plant pathogens and produces phenazine-1-carboxamide (PCN). *P. chlororaphis* GP72 was isolated from the green pepper rhizosphere by our group, and its genomic information was reported in 2012 [12]. This strain shows broad-spectrum antimicrobial activity, and can synthesize two phenazine derivatives, phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PHZ). *P. chlororaphis* 30–84 was isolated from the rhizosphere of wheat. Strain 30–84 is regarded as a

Abbreviations: PGPR, plant growth-promoting rhizobacteria; COGs, Clusters of Orthologous Groups; AAI, amino acid identity; GI, genomic island; Prn, pyrrolnitrin; PCN, phenazine-1-carboxamide; PCA, phenazine-1-carboxylic acid; 2-OH-PHZ, 2-hydroxyphenazine; HPR, 2-hexyl-5-propyl-alkylresorcinol; HCN, hydrogen cyanide; Pvd, pyoverdin; Acr, achromobactin; Fit, *P. fluorescens* insecticidal toxin; Mcf, makes caterpillars floppy; IAA, indole-3-acetic acid; ACC, 1-aminocyclopropane-1-carboxylate; PAA, phenylacetic acid; PQQ, pyrroloquinoline quinine; mGS, mGenomeSubtractor; MCP, methyl-accepting chemotaxis protein; Tad pili, type IVb tight adherence pili; MLSA, multilocus sequence analysis

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biocontrol strain because of its ability to control take-all disease. It provides protection mainly by producing phenazines, such as 2-OH-PCA, 2-hydroxyphenazine and PCA [13]. *P. chlororaphis* O6 was isolated from the rhizosphere of field-grown wheat. It produces phenazines similar to strain 30–84 [14].

This study first reports the sequencing of the HT66 genome. To identify the shared and divergent genomic characteristics among *P. chlororaphis* strains, we performed a comparative genomic analysis of the four known *P. chlororaphis* strains, HT66, GP72, 30–84 and O6. There are 4833 conserved genes among the four strains and 733 strain-specific genes in the genome of HT66. We focus on their characteristics, such as biocontrol activities, rhizosphere colonization, biosafety, and production of phenazines. Our research provides a theoretical foundation to develop and improve the antagonistic activities of *P. chlororaphis* for agricultural applications, as well as to use *P. chlororaphis* to produce phenazines with high-yield.

Results and discussion

General genome features and comparative genomes

The general features of the four *P. chlororaphis* genomes are summarized in Table 1. The genome sizes of HT66, GP72, 30–84 and O6 range from 6.66 to 7.30 Mb, with the number of CDSs ranging from 5869 to 6455. Compared with other pseudomonads, such as *Pseudomonas fluorescens* and *Pseudomonas putida*, these four genomes have higher GC contents [15,16], suggesting that this is a general characteristic of *P. chlororaphis*.

The COG database was used to functionally categorize predicted proteins [17], and we made a comparison of COG categories among the four strains (HT66, GP72, 30–84 and O6). The results are shown in Fig. 1. The COGs for the four strains showed highly similar distributions, especially COGs F and J, suggesting that the four strains have comparable biological niches. The percentage of genes in COG H was slightly higher in GP72 (approximately 3.95% of the total genes with COG annotations) than in the other three strains (3.77% in HT66, 3.78% in 30–84 and 3.78% in O6), suggesting that genes associated with coenzymes take more proportion in the genome of GP72.

We established a phylogenetic tree for completely sequenced representative strains of pseudomonad based on multilocus sequence analysis (MLSA) (Fig. 2). The tree showed that these four strains of *P. chlororaphis* fall into the same clade. The most closely related pseudomonad species to this clade was *P. fluorescens*, followed by *Pseudomonas syringae*. *P. chlororaphis* may share a more recent common ancestor with *P. fluorescens* than with *P. syringae*.

Conserved and specific regions in the genome can be identified through global alignments. BLASTatlas gives an overview of the whole genome homology, and the reference genome can be compared at the gene and/or protein level against many genomes [18]. In this study, the reference genome of HT66 was compared to the other three query genomes (Fig. 3).

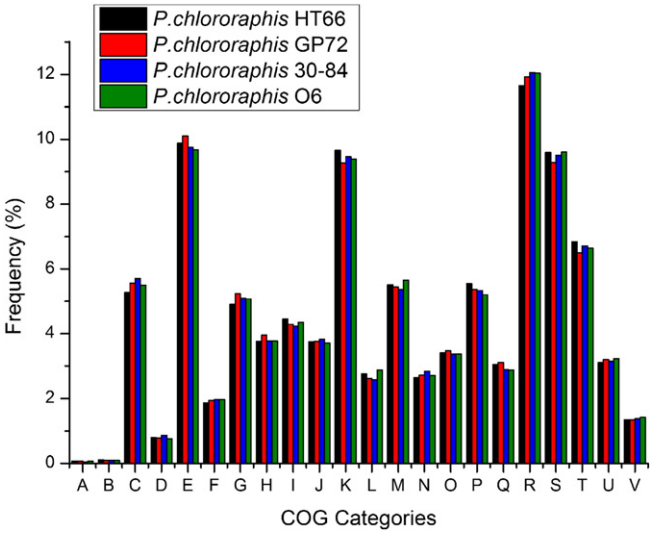

Fig. 1. Comparison of Clusters of Orthologous Group (COG) categories among the four *P. chlororaphis* strains. The comparison was based on 22 COG categories: RNA processing and modification (A), chromatin structure and dynamics (B), energy production and conversion (C), cell cycle control, cell division, and chromosome partitioning (D), amino acid transport and metabolism (E), nucleotide transport and metabolism (F), carbohydrate transport and metabolism (G), coenzyme transport and metabolism (H), lipid transport and metabolism (I), translation, ribosomal structure and biogenesis (J), transcription (K), replication, recombination and repair (L), cell wall, membrane, and envelope biogenesis (M), cell motility (N), posttranslational modification, protein turnover, and chaperones (O), inorganic transport and metabolism (P), secondary metabolite biosynthesis, transport and catabolism (Q), general function prediction only (R), function unknown (S), signal transduction mechanisms (T), intracellular trafficking, secretion and vesicular transport (U), and defense mechanisms (V).

mGenomeSubtractor performs a mpiBLAST based on in silico subtractive hybridization to identify conserved and strain-specific proteins. In this analysis, proteins with homology (H) values of less than 0.42 or more than 0.81 are defined arbitrarily as strain-specific or conserved, respectively [19]. The degree of protein conservation among HT66 and the other three genomes were determined by blastp based on homology value. The distribution of homology values for the 6455 predicted CDSs from *P. chlororaphis* HT66 compared with the other three genomes is shown in Fig. 4A. Genes conserved among all of four *P. chlororaphis* isolates comprised 4833 CDSs, representing 74.9%, 79.3%, 82.3% and 77.5% of the coding capacity in HT66, GP72, 30–84 and O6, respectively. Comparisons between HT66 and the other three strains (GP72, 30–84, and O6) revealed that 730 CDSs, 11.3% of the total coding capacity,

Table 1
General features of the four *P. chlororaphis* strains.

	HT66	GP72	30–84	O6
Number of bases	7,298,823 bp	6,663,241 bp	6,665,021 bp	6,977,251 bp
G + C (%)	62.60	62.89	62.9	62.8
Protein-coding genes	6455	6091	5869	6236
No. of protein-coding genes with function prediction	5423	5062	5023	5278
No. of protein-coding genes without function prediction	1032	1029	846	949
No. of protein-coding genes connected to KEGG Orthology	3299	3152	3118	3169
No. of protein-coding genes with COGs	4679	4454	4297	4489
No. of protein-coding genes coding signal peptides	719	662	652	664
No. of protein-coding genes coding transmembrane proteins	1509	1444	1400	1453
Coding percentage	88.33%	87.96%	88.12%	88.00%
RNA genes	147	88	156	142
rRNA genes (5S rRNA, 16S rRNA, 23S rRNA)	12 (6, 1, 5)	7 (2, 1, 4)	19 (7, 6, 6)	10 (5, 1, 4)
tRNA genes	57	61	69	60
Other RNA genes	78	20	68	72

Gene annotations and comparisons were obtained from the IMG database [51].

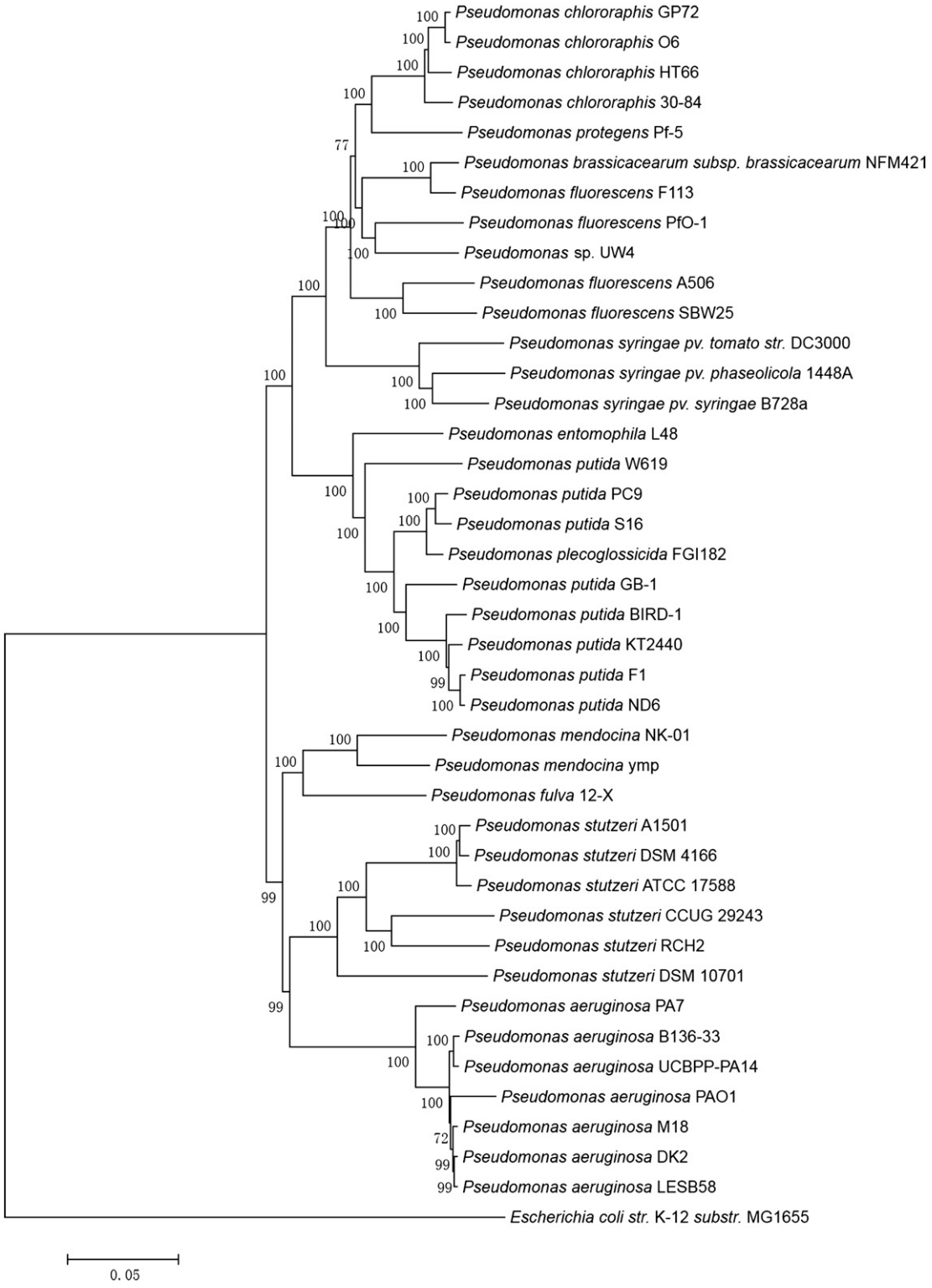

Fig. 2. Phylogenetic relationships among completely sequenced *Pseudomonas* species. A phylogenetic tree was constructed based on the sequences of 16s rRNA, *aroE*, *dnaA*, *guaA*, *gyrB*, *mutL*, *ppsA*, *pyrC*, *recA* and *rpoB* from each of the *Pseudomonas* genomes using the neighbor-joining method with 1000 bootstrap replicates. Numbers on nodes represent the percentages of individual trees containing that relationship.

were HT66 specific (E-value $< 10^{-5}$) (Fig. 4B). Among the 730 genes, we found that 192 genes formed 18 gene clusters. The 192 genes were individually blasted on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Four clusters showed high homology with *Pseudomonas protegens* Pf-5. One may be related to the metabolism of polysaccharides, one is similar with hemophore and may be responsible for the acquisition of heme, one may represent an *ofa* cluster responsible for the production of orfamide A which was first found in *P. chlororaphis*, and one's function remained undetermined. Two additional clusters showed high homology, with one being similar to *P. putida* HB3267, which may be related to phage, and the other having homology with *Pseudomonas* sp. UW4, which has unknown functions.

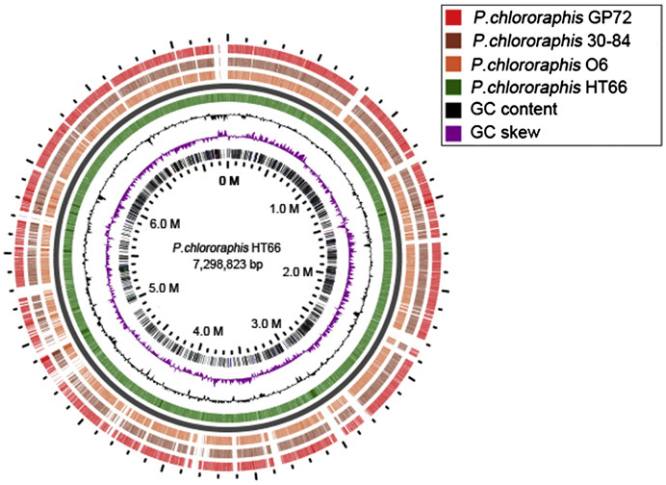

Fig. 3. Genome atlas diagram for the chromosome of *Pseudomonas chlororaphis* HT66. A GenomeAtlas diagram was drawn using comparisons between *P. chlororaphis* HT66 and three other strains. The HT66 genome was used as the reference sequence and is shown in green (line 4). Circles from outside to inside with different colors represent the strains as follows: red, *P. chlororaphis* GP72 (line 1); brown, *P. chlororaphis* 30–84 (line 2); and orange, *P. chlororaphis* O6 (line 3). A lack of color is used when HT66 genes do not exist in the genome of corresponding strain at that position. The GC content and GC skew are shown in line 5 and line 6, respectively. The scale is shown in line 7.

Genomic islands (GIs)

GIs were identified by IslandViewer [20]. Since the genome of GP72 contains many contigs, the GI predictions were not reliable (data not shown). The genome of HT66 contains 23 putative GIs (Fig. 5A), which is more than the other two genomes. The islands range in size from 4008 bp (HGI-5) to 16,094 bp (HGI-18). The largest, HGI-18, contains 10 genes and the smallest, HGI-5, is composed of four genes. Unlike the genome of 30–84 and O6, the GC contents of the putative islands, ranging from 42.65% (HGI-22) to 58.61% (HGI-1), are lower than the average GC content, 62.60%, of the HT66 genome. Compared with the genomes of 30–84 and O6, HT66 has more mobile genetic elements (MGEs). There are five islands (HGI-3, HGI-16, HGI-20, HGI-21 and HGI-23) that contain genes related to putative integrases and two islands (HGI-9 and HGI-14) contain genes encoding putative transposases. Of the 10 genes in HGI-18, five of them (M217_2064, M217_2065, M217_2066, M217_2067 and M217_2068) showed high similarities (84%, 85%, 87%, 89% and 84% at the amino acid level, respectively) with *batOPQRS* genes found in *P. fluorescens* strain BCCM_ID9359. The *bat* gene cluster is responsible for the biosynthesis of kalimantacin/batumin, which has a strong antibacterial activity [21]. However, the other essential genes in the *bat* cluster are not found in the HT66 genome. Thirteen putative GIs, ranging from 4158 bp (GI-11) to 43,856 bp (GI-6), were identified in 30–84 (Fig. 5B). The largest, GI 6, is composed of 19 genes, whereas the smallest, GI-11, contains six genes. In the 30–84 genome, nine GIs have lower GC contents, ranging from 43.96% (GI-3) to 59.74% (GI-4), two GIs have higher GC contents, 65.40% for GI-8 and 66.28 for GI-13, and two GIs have analogous GC contents, 61.52% for GI-2 and 60.17% for GI-7, when compared with the average GC content of 62.90%. GI-7 contains a gene (PCHL3084_3079) that encodes a putative transposase and both GI-11 and GI-6 contain genes (PCHL3084_4852 and PCHL3084_3061, respectively) that encode putative integrases. In the genome of O6, 11 putative GIs were identified (Fig. 5C), with sizes ranging from 4141 bp (OGI-9) to 22,717 bp (OGI-3). The largest GI, OGI-3, contained 13 genes, while only three genes were predicted in the smallest OGI-9. The GC contents of 10 of the islands are lower than the 62.80% average, ranging from 42.92% (OGI-5) to 59.46% (OGI-7). The remaining GI, OGI-11, has a similar GC content, 60.25%, to the average of the O6 genome. OGI-3 contains an integrase gene (PCHLO6_5969) and a transposase gene (PCHLO6_5968), while OGI-6 has two genes (PCHLO6_3566 and PCHLO6_3567) related to transposases. The GIs with MGEs may be able to self-mobilize. The comparative analysis revealed that none of the GIs found in HT66, 30–84 and O6 were in the GP72 genome.

Rhizosphere colonization

In contrast to the lack of nutrients in soil, the root of plant can secrete a series of compounds to provide rich nutrients for the growth of PGPR. Rhizosphere colonization is important for bacteria to adapt to the nutrient-lack environment. Also rhizosphere colonization is the first step in nearly all interactions between soilborne microorganisms and plants. *Pseudomonas* PGPR strains are regarded as good root colonizers, including the biocontrol bacterium *P. fluorescens* WCS365 and the model bacterium *P. fluorescens* F113 for rhizosphere colonization [22,23]. The major genes and traits involved in colonization competence are identified.

Martínez-Granero [24] reported that variants of *P. fluorescens* F113 with high motility were more competitive in rhizosphere colonization. Flagella play important roles in competitive tomato root tip colonization by *P. fluorescens*. Genes involved in chemotaxis and motility are found in the four genomes. For example, HT66 contains 45 genes related to chemotaxis, including 28 genes encoding methyl-accepting chemotaxis proteins (MCPs). The genome of HT66 contains 42 genes associated with flagellar biosynthesis, including the *fli* (M217_2401–2406), *flh* (M217_2407–2409) and *flg* (M217_5381–5385) operons. There are 40, 42 and 43 genes involved in flagellar biosynthesis in the genomes of GP72, 30–84 and O6, respectively. The *fli*, *flh* and *flg* operons are similarly organized in all four *P. chlororaphis* strains, and show 85% identity at nucleotide level to those located in F113. However, some genes were missing in *P. chlororaphis* strains, such as *flhC*, *flhD* and *fliT*. Whether these differences may affect the ability of motility for *P. chlororaphis* is unclear and remains to be clarified.

Root adhesion is just as important as motility for competitive colonization. Pili are appendages on the cell surface that are mainly involved in adhesion. Type IV pili are related to twitching motility and play very important roles in the colonization of plant hosts. Among the *P. chlororaphis* genomes, we identified three putative clusters of type IV pilus biosynthesis genes (Additional file 1), *pilACD*, *pilEYXWV*/*fimUT*, and *pilMNOPQ*, as well as five *pilZ* genes. One cluster, *pilGHIJ*/*chpAC*, was identified in the four genomes and involved in the complex regulatory system for pili biosynthesis. In addition to the biosynthesis of pili, we also found several genes involved in root adhesion in these four *P. chlororaphis* strains, such as genes related to the biosynthesis of filamentous hemagglutinin, hemolysin, lipopolysaccharide O-antigen and alginate (Additional file 1). The functions of these genes have been verified in other species.

The genes related to rhizosphere colonization in the four genomes showed a high similarity with each other. This suggests that *P. chlororaphis* may share a similar mechanism in root colonization.

Direct plant-growth promotion

Rhizobacteria can directly affect plant growth and development by producing or degrading phytohormones [25]. Indole-3-acetic acid (IAA) is a well-known plant growth regulator that controls many important plant physiological processes. GP72 and O6 can synthesize IAA via the indole-3-acetamide (IAM) pathway [26,27]. Genes homologous with those encoding tryptophan-2-monooxygenase (*iaaM*, M217_00681/02326) and indoleacetamide hydrolase (*iaaH*, M217_05950/02325/02091/03298) are defective in the HT66 genome, suggesting that HT66 may produce IAA only under certain conditions. 1-Aminocyclopropane-1-carboxylate (ACC) is the precursor of ethylene. The accumulation of ethylene results in the inhibition of root elongation and the acceleration of abscission and senescence [28]. Putative genes encoding ACC

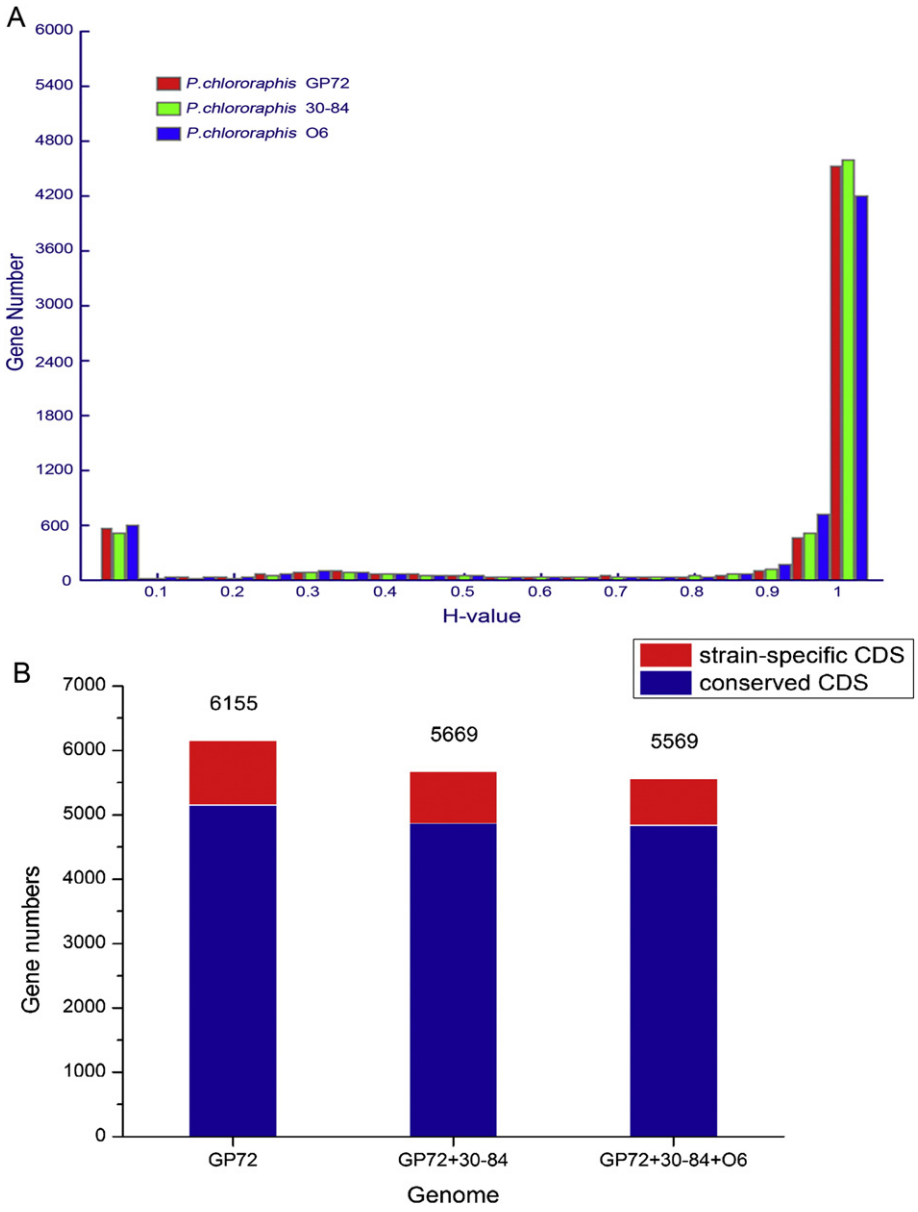

Fig. 4. Homology analysis between the *P. chlororaphis* HT66 genome and three other *P. chlororaphis* genomes. The mGenomeSubtractor defines coding sequences (CDSs) with a homology (H) value of less than 0.42 as strain-specific, and those with an H value of more than 0.81 as conserved [19]. (A) The H-value distribution of 6455 predicted CDSs from HT66 compared with the other three genomes: GP72 (red), 30–84 (green) and O6 (blue). (B) Numbers of conserved and strain-specific genes in the genome of HT66 compared with the other three genomes. The total numbers of conserved and strain-specific genes are marked above the bars.

deaminase (M217_04875, MOK_02139, PCHL3084_03963 and PCHLO6_04108) were identified in the four *P. chlororaphis* genomes. ACC deaminase counteracts the plants' ethylene response by degrading ACC into ammonia and α-ketobutyrate to enhance root growth [29]. We also found genes related to the catabolism of phenylacetic acid (PAA) (M217_03637–03649, MOK_00348–00336, PCHL3084_02993–02981 and PCHLO6_03084–03072), a plant auxin with antimicrobial activity, in the four genomes. The cluster found in *P. chlororaphis* is similar to the *paa* operon in *P. putida U*, and is involved in the degradation of phenylacetic acid under aerobic conditions.

Biocontrol activities

According to a previous study, the biocontrol abilities of *Pseudomonas* spp. strains play important roles in their capacity to inhibit pathogens. Like other pseudomonad species, *P. chlororaphis* secretes a series of broad spectrum of antibiotics to suppress pathogens [30]. According to our study, HT66 could suppress *R. solani*, *P. ultimum*, *F. oxysporum* f. sp. *niveum* and the pathogen of Stevia leaf spot disease (Additional file 2).

The putative secondary metabolites were predicted using antiSMASH [31]. According to our genomic analysis and research in the literature, *P. chlororaphis* produces phenazines, hydrogen cyanide, 2-hexyl-5-propyl-alkylresorcinol (HPR), two siderophores, pyoverdine (Pvd), achromobactin (Acr), *P. fluorescens* insecticidal toxin (Fit) and other antibiotics (Table 2). 2-Hexyl-5-propyl-alkylresorcinol (HPR) was reported to show moderate antifungal and antibacterial activities. In the genomes of GP72, 30–84 and O6, a locus similar to the HPR biosynthetic gene cluster (*darABCRS*) described in some other *Pseudomonas* strains [32–35] was identified. In the HT66 genome, we only found genes homologous with *darS* and *darR*, which are required to increase the production of HPR [32].

FitD is related to the potent insecticidal activity of *P. protegens* Pf-5 and CHA0 [36]. The complete *fit* locus detected in all of four *P. chlororaphis*

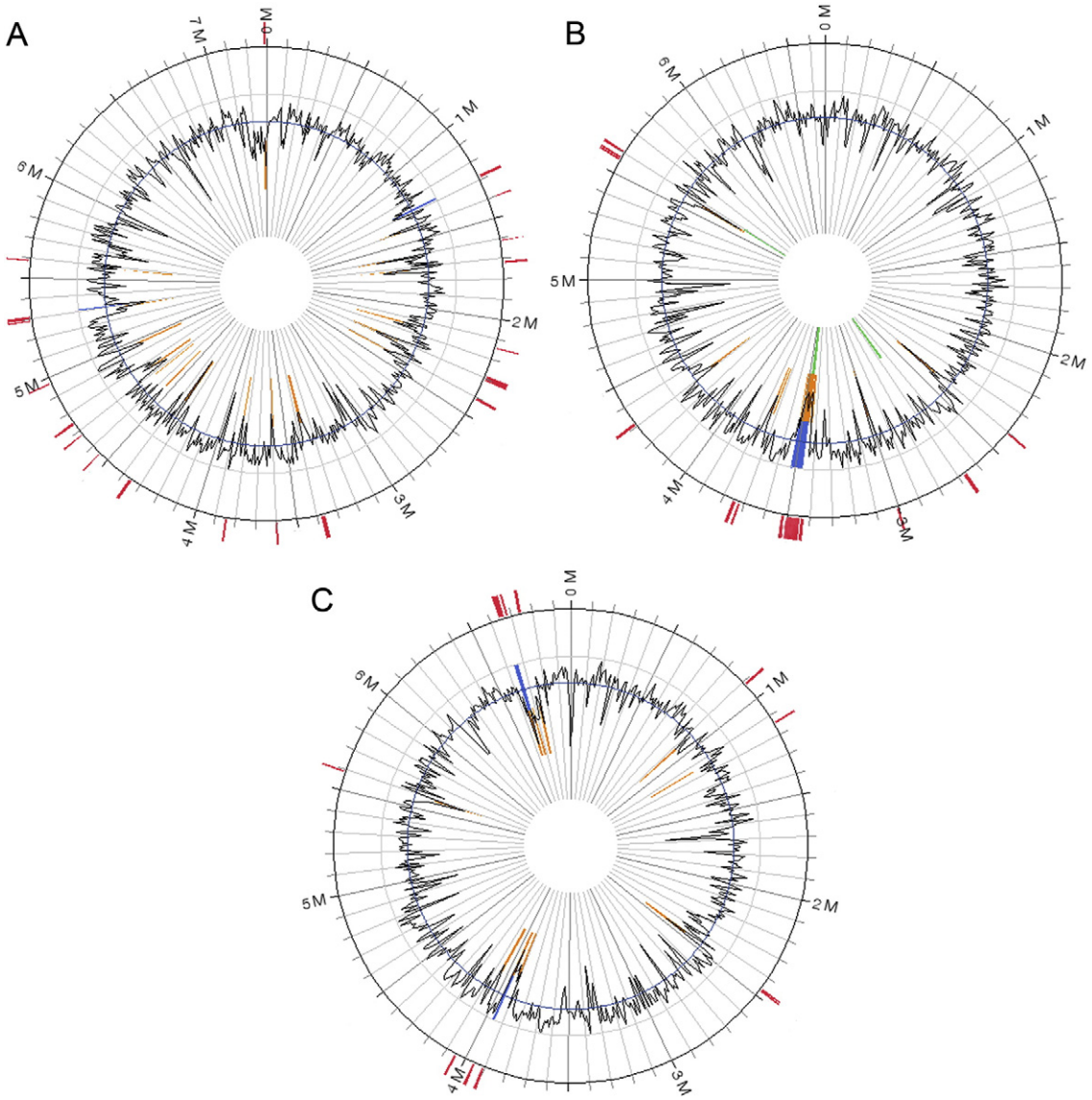

Fig. 5. Putative genomic islands of *P. chlororaphis* strains HT66, 30–84 and O6 as predicted by Island Viewer. The outer black circle represents the scale line in Mbps. Color indicates the putative genomic islands based on the following methods: Islandpick, green; SIGI-HMM, orange; IslandPath-DIMOB, blue; and integrated detection, red. (A) putative genomic islands of HT66; (B) putative genomic islands of 30–84; and (C) putative genomic islands of O6.

strains showed a high similarity with that in Pf-5, suggesting that *P. chlororaphis* strains also possess potent insecticidal activity.

Orfamide A was found by the genomisotopic approach in Pf-5. It exhibits a role in motility as well as biocontrol activity [37]. Orfamide A is encoded by an orphan gene cluster composed of *ofaA*, *ofaB* and *ofaC*. This *ofa* cluster was only found in the HT66 genome (M217_04407–04409) among four strains. Interestingly, no mobile elements have been detected near the cluster, thus the mechanism to obtain this cluster remains unknown. The biosynthesis of orfamide A may be conductive to the motility and antimicrobial activity of HT66.

The synthesis of siderophores also contributes to the biocontrol activity of *Pseudomonas* and promotes host plant growth. The complete gene cluster for the biosynthesis of Pvd was detected in the four strains. In addition, the four strains also contain putative genes to encode a

Table 2
Secondary metabolites produced by *Pseudomonas chlororaphis*.

	HT66	GP72	30–84	O6
Hydrogen cyanide (HCN)	HCN	HCN	HCN	HCN
Phenazine	PCN	PCA, 2-OH-PCA	PCA, 2-OH-PCA	PCA, 2-OH-PCA
Pyrrolnitrin (Prn)	–[a]	Prn	Prn	Prn
2-Hexyl-5-propyl-alkylresorcinol	–	HPR	HPR	HPR
Orfamide	Orfamide A	–	–	–
Pyoverdine (Pvd)	Pvd	Pvd	Pvd	Pvd
Achromobactin (Acr)	Acr	Acr	Acr	Acr
P. fluorescens insecticidal toxin (Fit)	FitD	FitD	FitD	FitD

[a] "–" indicates that the secondary metabolite is not present in the corresponding strain based on previous studies and genomic sequence data.

second siderophore, achromobactin (Acr). HCN is a secondary metabolite produced by some *Pseudomonas* spp., also has biocontrol activity, and the biosynthetic gene cluster (*hcnCBA*) was found in the four *P. chlororaphis* genomes. Prn is an important antibiotic and putative genes involved in the biosynthesis of Prn were detected in the genomes of GP72, 30–84 and O6.

P. chlororaphis can secrete a variety of secondary metabolites with biocontrol activities. Compared with *P. protegens* Pf-5, *P. chlororaphis* has one advantage to synthesize phenazine derivatives, which have shown impressive antibiosis activities. The presence of a gene cluster for HPR biosynthesis may allow *P. chlororaphis* to exhibit better inhibition to pathogens or competitors. The different antibiotics produced by *P. chlororaphis* enhance the diversity of antibiosis activities.

The production of phenazines

Phenazines play important roles in suppressing root diseases caused by pathogens [38]. There are a variety of phenazine derivatives found in different *Pseudomonas* spp., such as *P. fluorescens* [39], *Pseudomonas aeruginosa* [40], and *P. chlororaphis* [41]. Each genome of the four *P. chlororaphis* strains contains one phenazine biosynthetic gene cluster as shown in Fig. 6A, but the modifying genes differed among the four genomes. The genome of HT66 contains *phzH*, which encodes an asparagine synthetase that converts PCA to PCN by catalyzing the transaminase reaction [42], while GP72, 30–84 and O6 contain *phzO*, which encodes an aromatic monooxygenase involved in the hydroxylation of PCA to 2-OH-PCA [13]. It has been reported that the antifungal activity of PCN was more than 10 times higher than PCA at neutral pH [5]. Our studies also showed that after 24 h of incubation, only PCN was found in the fermentation broth with HT66, while PCA and 2-OH-PCA or 2-OH-PHZ existed in the fermentation broth with GP72, 30–84 and O6. This suggested that *phzH* was more active than *phzO*. Besides, our studies also showed that the yield of PCN in strain HT66 is obviously higher than the yield of phenazines detected in wild-type of GP72, O6 and 30–84 (Fig. 6B) [43–45].

The *P. chlororaphis* strains have high proportions of regulatory genes (Additional file 3), and HT66 contains the greatest number. Our analysis shows that there are 543 putative regulatory genes in the HT66 genome and the proportion of regulatory genes is 8.4%. There were 499 (8.2%), 501 (8.5%) and 526 (8.4%) regulatory genes in the genomes of GP72, 30–84, and O6, respectively. Also, genes reported to relate to the regulation of phenazine production were detected among the four strains, such as *psrA*, *rpoS*, *rpeA* and *lon* proteases [9,46,47]. The mutation of *psrA* could threefold increase the phenazine production in PCL1391, and the loss of *rpeA* significantly increased the phenazine production in 30–84 [46]. It is suggested that we can produce phenazines with high-yield by constructing gene engineering strain of HT66 or other three strains.

In addition to *phzH* and *phzO*, there are other modifying genes, such as *phzS* and *phzM*. *phzS*, which encodes a flavin-containing monooxygenase, and *phzM*, which encodes a putative phenazine-specific methyltransferase, are responsible for the conversion of PCA into PYO at certain

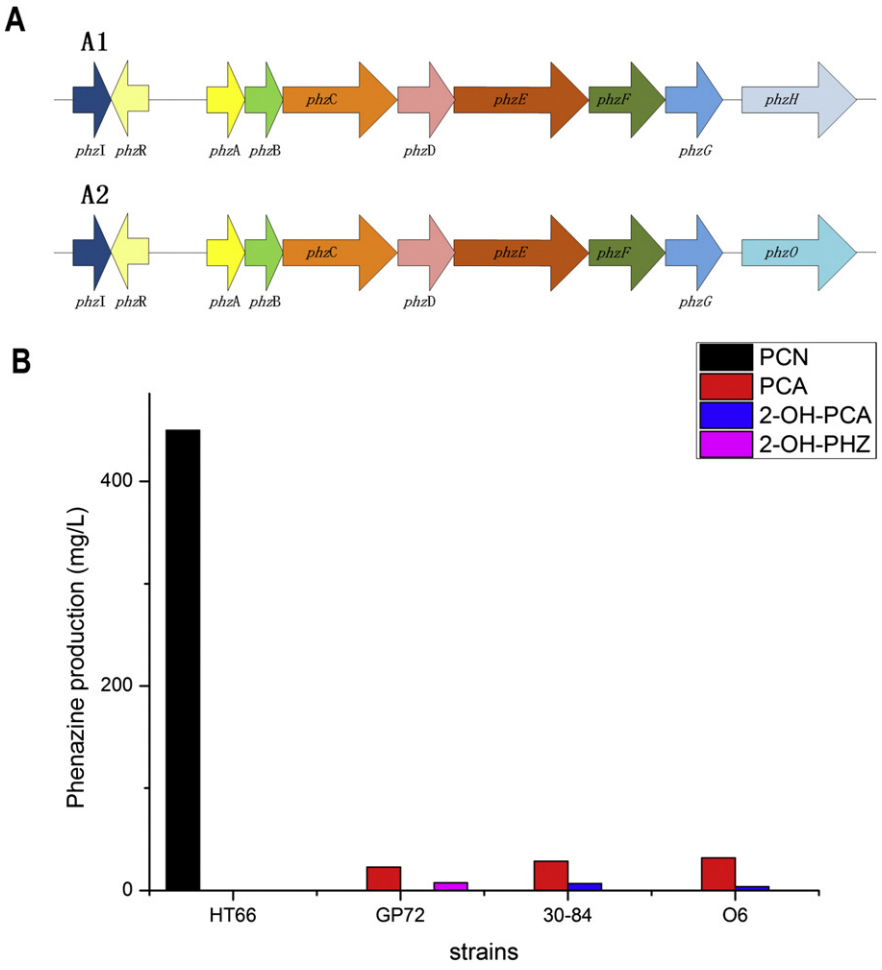

Fig. 6. Phenazine biosynthesis gene clusters and the production of phenazines in *P. chlororaphis*. Phenazine biosynthesis gene clusters are detected in the genomes of *P. chlororaphis* strains. Those genes are shown in different colors. The same color between two strains indicates that the genes are homologous. (A1) phenazine biosynthesis gene cluster in strain HT66; (A2) phenazine biosynthesis gene clusters in strains GP72, 30–84 and O6; (B) the production of phenazines in four *P. chlororaphis* strains [43–45].

conditions [40]. However, pyocyanin (PYO) is involved in pulmonary tissue damage [48], *P. chlororaphis* is unable to synthesize PYO and makes it more suitable for agricultural applications.

Virulence factors

Like other biosafety strains of *Pseudomonas*, *P. chlororaphis* lacks the key virulence or virulence-related factors. *P. chlororaphis* lacks genomic islands that are homologous with pathogenic islands such as PAPI-1 and PAPI-2 [49]. Also, genes required for the biosynthesis of phytotoxins (syringomycin, syringopeptin and coronatine) and exoenzymes (cellulases, pectinases and pectin lyases) involved in the degradation of plant cell walls are absent from the genomes of the *P. chlororaphis* strains. No evidence for a type III secretion pathway was found in the genomes of the four *P. chlororaphis* strains. This suggests that *P. chlororaphis* strains are safe for biocontrol applications.

Conclusion

A comparative genomic analysis of the genomes of HT66, GP72, 30–84 and O6 showed similarities and differences among traits of *P. chlororaphis* strains. It provided new insights into traits involved in the adaption of *Pseudomonas* to environmental niches and in the promotion of plant growth.

Our analysis showed that *P. chlororaphis* strains are highly similar in genomic level. Additionally, we analyzed genes related to plant growth promotion. The genomic information indicated that the production of antifungal metabolites differed but all of four strains have one phenazine biosynthesis gene cluster. But the phenazine derivative found in HT66 is PCN whereas the other three strains produce 2-OH-PHZ and PCA. However, only HT66 contains putative genes encoding orfamide A. Also, all of four *P. chlororaphis* strains contain the complete *fit* locus, suggesting that *P. chlororaphis* strains possess potent insecticidal activity. The diversity of antibiotics may allow *P. chlororaphis* to inhibit various pathogens, such as fungi, bacteria and some kinds of insects. Besides, the production of phenazines in HT66 is obviously higher than other strains, and some genes related to the regulation of phenazine biosynthesis have been detected in the four genomes. The analysis of genes contributing to the regulation and biosynthesis of antibiotics may lay the foundation for transforming *P. chlororaphis* to produce high levels of antibiotics. Finally, key virulence or virulence-related factors were absent from the *P. chlororaphis* strains, indicating that *P. chlororaphis* is safe and suitable to be applied in agriculture.

Materials and methods

Medium for HT66 and genomic DNA extraction

P. chlororaphis HT66 was isolated from a rice rhizosphere in Shanghai, China and showed antimicrobial activity to plant pathogenic bacteria. A single colony of HT66 grown on King's medium B plate (KB) was inoculated into 5 mL of KB broth and incubated overnight with shaking at 28 °C. Bacterial cells were collected by centrifugation and the genomic DNA was extracted with an Easy Pure Genomic DNA kit (TransGen Biotech) according to the manufacturer's instructions.

Genome sequencing and annotation

The genome of *P. chlororaphis* HT66 was sequenced using the Illumina Miseq platform (to 40-fold of the sequencing coverage) with paired-end reads. First, a paired-end library was prepared from 4 μg of DNA and subsequently sequenced, generating 590,886 reads in 296,624,772 bp of sequencing data. The data was initially assembled using a Celera Assembler 7.0 and 87 contigs ranging from 112 to 529,941 bp were obtained. 40 scaffolds with genome size of 7.30 Mb ranging from 1906 to 1,134,406 were obtained. The genomes of HT66, 30–84 and O6 were first automatically annotated using the RAST server [50] and IMG/ER system (https://img.jgi.doe.gov/cgi-bin/er/main.cgi) [51]. The annotations from these two programs were manual curated and combined.

Nucleotide sequence accession number

This Whole Genome Shotgun project of HT66 has been deposited in DDBJ/EMBL/GenBank under the accession number ATBG00000000.

Bioinformatics analysis

The genome sequence of HT66 was aligned against other *Pseudomonas* sequences from NCBI's database. BLASTatlases were generated using an online tool, GView Server (https://server.gview.ca/). Conserved and strain-specific genes were identified based on the homology (H) value (proteins with H values of less than 0.42 or more than 0.81 at E-value $< 10^{-5}$ are defined arbitrarily as strain-specific or conserved, respectively) using the mGenomeSubtractor web server (http://bioinfo-mml.sjtu.edu.cn/mGS/) [19]. A comparative genomic analysis of HT66, GP72, 30–84 and O6 was conducted using the IMG website's tool, which defined genes with a 60% identity at an E-value $< 10^{-2}$ as homologous to those in HT66. The genomic islands were identified using IslandViewer (http://www.pathogenomics.sfu.ca/islandviewer/query.php) [20]. Secondary metabolite production clusters were examined using the antiSMASH program (http://antismash.secondarymetabolites.org/) [31]. The phylogenetic relationships among completely sequenced *Pseudomonas* were determined using the sequences of 1) 16S rRNA and 2) concatenated alignments of 9 highly conserved housekeeping genes: *aroE*, *dnaA*, *guaA*, *gyrB*, *mutL*, *ppsA*, *pyrC*, *recA* and *rpoB*. The multiple-sequence alignments were carried out with ClustalW [52]. A neighbor-joining tree with 1000 bootstrap replicates was generated using MEGA 6.0 software [53].

Resistance to plant pathogens

Four normal plant pathogens: *R. solani*, *P. ultimum*, *F. oxysporum* f. sp. *niveum* and the pathogen of Stevia leaf spot disease, were chosen to test the biocontrol activities of HT66. The plant pathogenic bacteria were fully activated on PDA plate. After 3 days of incubation, hyphae block with 8 mm of diameter was added on one side of new PDA plate. The strain HT66 was activated on KB plate and then transferred into new KB broth and incubated to log phase. 10 μL cell suspension was added on filter paper whose diameter also remains to be 8 mm. The distance of the center of the filter paper and hyphae block was 25 mm. The plate was incubated under 28 °C for 5 days.

Quantification of phenazine production in HT66

400 μL supernatants of 24 h cultures were extracted with 9-times volumes of ethyl acetate and were acidified with adding 20 μL concentrated HCl. Following evaporation of the ethyl acetate under air, phenazines were resuspended in 100% acetonitrile and quantified with HPLC. HPLC was performed with a WondaSil C18-WR column (5μm; 4.6 × 250mm, Shimadzu, Japan) and a linear 8 to 60% (vol/vol) gradient of acetonitrile in water with a flow rate of 1 mL/min. UV detection was performed with wavelength scanning at 254 nm.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gdata.2015.01.006.

Conflict of interests

The authors declare that they have no competing interests.

Authors' contributions

XHZ, YWC, HBH, HSP and XMS conceived, coordinated and designed the research. WW, XHZ and YWC were responsible for sequencing, finishing and annotating data. YWC, XHZ, XMS and HSP performed experiments and data analyses. XHZ, YWC, HSP and XMS contributed to materials and analysis tools. XHZ, YWC, XMS and HBH drafted the manuscript. All authors read and approved the final manuscript.

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