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ORIGINAL PAPER

Antibiotics of *Pseudomonas protegens* FD6 are essential for biocontrol activity

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Abstract

Pseudomonas protegens (formerly *Pseudomonas fluorescens*) FD6, which was isolated from the canola rhizosphere, is a biocontrol agent that protects against the fungal pathogens *Botrytis cinerea* and *Monilinia fructicola*. The complete sequence of the 6.7-Mb *Pseudomonas protegens* FD6 genome was determined. Genomic analysis of strain FD6 led to the identification of twelve putative gene clusters of secondary metabolites, including the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT) and pyrrolnitrin (PRN). To assess the role of 2,4-DAPG and PLT in the biocontrol activity of *P. protegens* FD6, two mutants of *P. protegens* FD6, including strains ΔphlC and ΔpltD, were constructed using homologous recombination technology. The results showed that no significant differences were observed in the antagonistic action of the ΔphlC mutant compared with wild-type FD6. However, the ΔpltD mutant no longer exhibited antifungal activity. HPLC analysis indicated that ΔphlC mutant could not biosynthesize 2,4-DAPG, whereas PLT production in the ΔphlC mutant was significantly increased by 67-fold compared with wild-type FD6. Biosynthesis of PLT in the ΔpltD mutant was not detected, whereas the level of 2,4-DAPG was vastly decreased over sixteenfold compared with strain FD6. The major antagonistic activity produced by *P. protegens* FD6 corresponded to 2,4-diacetylphloroglucinol and pyoluteorin. There is an inverse interaction between 2,4-DAPG and PLT.

Keywords *Pseudomonas protegens* · 2,4-diacetylphloroglucinol · Pyoluteorin · Secondary metabolite · Biocontrol agent

Introduction

The genus *Pseudomonas* in the family *Pseudomonadaceae* is one of the most ubiquitous environmental bacterial genera due to its metabolic versatility. Some strains can produce water-soluble fluorescent pigments. In addition, the genus *Pseudomonas* has received much attention as biological control agents (Raaijmakers and Mazzola 2012). Several studies have suggested that antimicrobial compounds produced by *Pseudomonas* spp. are involved in the biological control of plant diseases. *P. corrugate* GFBP 5454 and *P. mediterranea* GFBP 5447 inhibited the growth of plant pathogenic fungi and bacteria due to the production of diffusible compounds and cyclic lipopeptides (Strano et al. 2017). *P. fluorescens* 2P24 produced a variety of antimicrobial compounds, and 2,4-diacetylphloroglucinol (2,4-DAPG) is the key determinant of its protection against various soilborne diseases (Wei et al. 2004). Some biocontrol agents may produce a wide range of antifungal compounds. For example, *P. chlororaphis* PA23 produces multiple secondary metabolites, including phenazine-1-carboxylic acid (PCA), PRN, HCN, proteases, lipases and siderophores, some of which have been shown to contribute to antagonism (Poritsanos et al. 2006). The antibiotics 2,4-DAPG and PLT are essential for the biocontrol of soil borne diseases of cotton caused by *Rhizoctonia solani* and *Pythium ultimum* by *P. fluorescens* Pf-5 (Nowak-Thompson et al. 1994; Howell and Stipanovic 1980). Hu et al. (2005) reported that PLT and PCA are responsible for the ability of *Pseudomonas* sp. M18 to suppress many soilborne phytopathogenic fungi. PCA derived from strain M18 has been registered as a new biogenic pesticide to prevent sheath blight of rice in 2011, illustrating the potential prospects of biocontrol pseudomonads in agricultural production.

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The antibiotic 2,4-DAPG, a phloroglucinol derivative, can suppress the activities of many bacteria, fungi, oomycetes, and nematodes (Haas and Defago 2005). The biosynthesis of 2,4-DAPG begins with the synthesis of phloroglucinol (PG) by the type III polyketide synthase PhlD (Achkar et al., 2005). PG is subsequently acetylated through the action of PhlABC to form DAPG. These four structural genes constitute a single operon phlACBD (Bangera and Thomashow, 1999). Pyoluteorin is a broad spectrum polyketide that is composed of a resorcinol and a pyridine containing two chlorine atoms. The structural genes *pltLABCDEFG* for biosynthesis constitute a single operon (Nowak-Thompson et al. 1999). The Pro-S-PltL intermediate is oxidized by PltE and chlorinated by the halogenase enzymes PltA to synthesize 4,5-dichloropyrrolyl-S-PltL (Dorrestein et al. 2005). PltA and PltD are $FADH_2$-dependent halogenases that chlorinate secondary metabolites. PltD lacks a putative conserved FAD-binding site and is likely not functional. Tn5 insertion in *pltD* disrupts pyolutrorin production, suggesting a role of *pltD* in synthesis of the antibiotic (Nowak-Thompson et al. 1999). The PltD is a rate-limiting enzyme in PLT biosynthesis. The pyoluteorin production of *pltD* overexpression mutant was increased by 77.5% (Li et al. 2012). The physiological role of *pltD* in pyoluteorin assembly is not clear. Mutation in genes for antibiotics biosynthesis has provided strong evidence of the role of these antibiotics in pathogen suppression.

Pseudomonas fluorescens FD6 has shown antagonistic activity against various plant pathogenic fungi. The mechanisms of action for the biocontrol activity of strain FD6, which include 2,4-DAPG, PLT, PRN, extracellular proteases, siderophores and HCN, have been identified (Chang et al. 2011). Certain isolates of the *Pseudomonas* genus produce 2,4-DAPG but not PLT (Wei et al. 2004; Weller 2007). Ramette et al. (2011) proposed the new species name *P. protegens* for these fluorescent *Pseudomonas* strains, which could produce both 2,4-DAPG and PLT. At present, only certain strains of *P. protegens* are known to produce these two antibiotics (Loper et al. 2012). Therefore, *P. fluorescens* FD6 was redesignated as *P. protegens* FD6 based on the types of antibiotics it produces. Only indirect evidence was analysed to determine the role of antibiotic production by FD6 in its biocontrol efficacy (Chang et al. 2014). To shed some light on the biocontrol traits of *P. protegens* FD6, its genome was sequenced and genomic analysis of secondary metabolites was performed. The aim of this study was to investigate the role of the *pltD* and *phlC* genes in biocontrol activity of *P. protegens* FD6 on the basis of the genomic analysis.

Materials and methods

Strains, plasmids, and growth conditions

The strains, plasmids, and primers used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) media with shaking overnight at 37 °C. *P. protegens* strains were cultured in LB liquid media at 28 °C. Antibiotics were used as necessary at the following

Table 1 Strains, plasmids and primers used in this study

Strains	Characteristics[a]	Reference or source
Pseudomonas protegens		
FD6	Wild type, 2,4-DAPG$^+$, PLT$^+$, PRN$^+$, Ap^r	(Chang et al. 2011)
ΔpltD	*pltD* gene in-frame deletion, Ap^r	This study
ΔphlC	*phlC* gene in-frame deletion, Ap^r	This study
Escherichia coli		
DH5α	*φ80 lacZ ΔM15Δ(lacZYA_argF)U169 hsdR17 recA1 endA1 thi_1*	Lab collection
S17–1	*recA pro hsdR- M$^+$ RP4 2-Tc::Mu-Km::Tn7* Sm^r Tp^r	Lab collection
Botrytis cinerea	The fungal pathogen causing tomato grey mould	Lab collection
Monilinia fructicola	The fungal pathogen causing peach brown rot	Lab collection
Plasmids		
pEX18Km	Suicide plasmids for *Pseudomonas* spp. used for homologous recombination, Km^r	(Hoang et al. 1998)
pEX18-ΔpltD	pEX18 carrying an 873 bp insert with a deletion in *pltD*, Km^r	This study
pEX18-ΔphlC	pEX18 carrying a 436 bp insert with a deletion in *phlC*, Km^r	This study
Primers	Sequence (5′-3′)	
phlA138-F1	ATGGATCCTGCAACCSGAYGAAGATGTCAT	This study
phlC1222-R1	AGGTGTTCGGCCACCAGGATCGCGCCCTTGTAGGCGGACTCGACGATCAT	This study
phlC1763-F2	ATGATCGTCGAGTCCGCCTACAAGGGCGCGATCCTGGTGGCCGAACACCT	This study
phlB2743-R2	ATCAAGCTTATARGCRTATTGCCAGGCC	This study
pltF1–240	ATGGATCCCACCTGGTTGTTGCACGAAC	This study
pltR1–1291	CACTCAAGCTGATGATGCTGTCCTGAAGACGGCATCACCTTCTGCTGGAT	This study
pltF2–2164	ATCCAGCAGAAGGTGATGCCGTCTTCAGGACAGCATCATCAGCTTGAGTG	This study
pltR2–2748	ATGAAGCTTAATTGCTCGGCACACACCTG	This study

[a] Ap^r and Km^r indicate resistance to ampicillin and kanamycin, respectively. 2,4-DAPG$^+$, 2,4-diacetylphloroglucinol; PLT$^+$, pyoluteorin; PRN$^+$, pyrrolnitrin

concentrations: kanamycin at 50 μg/mL and ampicillin at 50 μg/mL.

Genomic sequencing, gene annotation and bioinformatic analysis

The complete genome sequence of *P. protegens* FD6 was performed with Pacific Biosciences RS II (Pacific Biosciences, Menlo Park, CA, USA) sequencing platform, providing a coverage depth of approximately 175. De novo assembly of PacBio Reads was carried out with the RS_HGAP_Assembly v3.0 protocol included in the SMRT analysis pipeline v2.3, and additional assembly was executed by Minimus2. Open reading frames were identified and annotated automatically with GeneMarks and proceeded with manual curation. RNAs were predicted using ARAGORN, tRNAscan-SE, RNAmmer and Barrnap (Laslett and Canback 2004; Lowe and Eddy 1997; Lagesen et al. 2007). Other non-coding RNAs were predicted and classified by Infernal and Rfam (Nawrocki and Eddy 2013; Nawrocki et al. 2015). The genome sequence of strain FD6 was retrieved from the GenBank database and submitted to RAST and AntiSMASH 4.0 (Weber et al. 2015) for genomic analysis of secondary metabolites. The orthologous coding sequences (CDSs) of each group were identified using Inparanoid software.

DNA manipulations and sequence analysis

Plasmid DNA isolation and other molecular assays were performed using standard protocols (Russell and Sambrook 2001). Plasmids were introduced into *Pseudomonas* strains via electroporation (Wei and Zhang 2006). DNA sequencing was performed by Nanjing Qing Ke Biological Technology Co., Ltd. (Nanjing, China). Nucleotide sequences were analysed with BLASTn databases.

Construction of *P. protegens* mutations in strain FD6

To delete *pltD* from the chromosome of strain FD6, two DNA fragments flanking the *pltD* gene were PCR amplified using primers pltF1–240/pltR1–1291 and pltF2–2164/pltR2–2748 (Table 1). These two fragments were fused together by PCR and digested using *Bam*HI/*Hin*dIII to generate a 1635 bp DNA fragment containing *pltD* with an 873 bp internal deletion. This DNA fragment was ligated to pEX18Km to create the construct pEX18-ΔpltD. This plasmid was integrated into the chromosome of strain FD6 by electroporation. A second cross-over mutant was isolated by loss of kanamycin resistance. An analogous gene replacement strategy was performed to obtain the 541 bp ΔphlC deletion mutant. To generate the *phlC* mutant ΔphlC, two DNA fragments flanking the *phlC* gene were PCR amplified using primers phlA138-F1/phlC1222-R1 and phlC1763-F2/phlB2743-R2 (Table 1). These two fragments were fused together by PCR and digested using *Bam*HI/*Hin*dIII to generate a 2064 bp DNA fragment containing *phlC* with a 1084 bp internal deletion. The transformation and selection of this deletion construct, pEX18-ΔphlC, were performed as described above. The deletion of *phlC* and *pltD* was confirmed by PCR using primers pltF1–240/pltR2–2748 and phlA138-F1/phlB2743-R2, respectively. The resulting PCR products were also sequenced to confirm that the products were incorporated into the *pltD* and *phlC* genes.

Antifungal assays

Radial diffusion assays were performed to assess fungal inhibition in vitro. A 5 mm fungal plug of *B. cinerea* or *M. fructicola* was placed on the centre of a plate, and aliquots (3 μL) of overnight bacterial cultures were spotted on potato sucrose agar plates, 2.5 cm away from the fungal plug. The plates were incubated at 25 °C, and the antifungal activity was assessed after 5–7 d by measuring the distance between the edges of the colony and the fungal mycelium. Three replicates were performed for each strain, and the antifungal assays were repeated twice.

Extraction and detection of PLT, 2,4-DAPG and its phloroglucinol

Extraction and HPLC (high-performance liquid chromatography) detection of 2,4-DAPG and PLT from *P. protegens* FD6 were performed as described previously (Liu et al. 2010; Costa et al. 2009; Zhang et al. 2015). Antibiotics extracted from the culture suspension were analysed by C18 reverse-phase high-performance liquid chromatography (HITACHI L-2000). Aliquots of 10 μL were injected directly into the HPLC system for the determination of PLT and 2,4-DAPG. Three replicates were used for each treatment, and the experiment was performed twice. In the HPLC analysis, standard 2,4-DAPG and PLT were used as controls. PG, a 2,4-DAPG intermediate, quantification was performed as previously described (Kidarsa et al. 2011). Briefly, 150 μL bacterial supernatants was collected, and then 50 μL concentrated HCl and 200 μL 0.2% 4-hydroxy-3-methoxy-cinnamaldehyde were added. The mixture was allowed to react for 5 min, then recording the absorbance at 600 nm.

Detection of other extracellular metabolites

Extracellular protease was assessed using skim milk agar plates (Reimmann et al. 1997). Assays for HCN production on KB agar medium were performed qualitatively using HCN-indicator paper (Castric and Castric 1983). Qualitative siderophore assays were performed on a siderophore detection plate as previously described (Schwyn and Neilands 1987). These tests were repeated twice.

Efficacy of *P. protegens* FD6 and its derivatives in controlling tomato grey mould disease

Tomato cultivars "Sufen" were harvested from Shatou Greenhouse in Yangzhou and quickly brought to the laboratory. To evaluate the biocontrol effect of *P. protegens* FD6 and its derivatives on controlling postharvest decay, we determined the development of tomato grey mould disease after treatment with different bacterial cultures as previously described by Zhang et al. (2018). There were three replicate trials of two fruits. The test was repeated twice.

GenBank accession

The complete annotated genomic sequence of *P. protegens* FD6 is available under GenBank accession number CP031396. Accession numbers for the complete genome sequences of Pf-5 and 2P24 are CP000076 and NZ_CP025542, respectively.

Statistical analysis

Data were analysed and compared by performing Fisher's least significant difference (LSD) test ($p < 0.05$ was considered significant) using SPSS software.

Results

Genome features and comparative genomics

General characteristics of the genomes of *P. protegens* FD6 and Pf-5, and *Pseudomonas fluorescens* 2P24

The *P. protegens* FD6 genome is composed of one circular chromosome of 6.7 Mb. A total of 6264 CDSs were identified within the *P. protegens* FD6 genome. The genome of *P. protegens* FD6 is larger than that of the biocontrol strain *P. fluorescens* 2P24 isolated from wheat take-all decline soil (Zhang et al. 2014). However, the genome of *P. protegens* FD6 is smaller than that of *P. fluorescens* Pf-5 isolated from soil in College Station, Texas, USA (Kraus and Loper 1995). The complete genome sequence included one phage region, and no insertion element was identified. No genomic islands or CRISPR units are present in the genome. A total of seven 5S rRNAs are present on the FD6 chromosome, whereas the genomes of both Pf-5 and 2P24 contain six 5S rRNAs (Table 2). The complete genomes of Pf-5 and 2P24 were performed by Sanger sequencing and early Pacbio sequencing. These two sequencing systems may misassemble genomes due to read length limitations and poor assembly accuracy for series repeats. Three-generation PacBio sequencing was applied in the study of the complete genome sequence of FD6. All protein sequences from the three species were clustered into 3431 orthologous groups through multiparanoid. Based on additional analyses, 511 genes unique to *P. protegens* FD6 were identified that participate in many biological processes, such as signal transduction and amino acid and carbohydrate metabolism (Fig. 1, Fig. S1).

Secondary metabolite analysis

The results from antiSMASH 4.0 showed twelve secondary metabolite gene clusters in the genome of *P. protegens* FD6 (Fig. 2). These include gene clusters for PLT, 2,4-DAPG, PRN and HCN. The gene cluster responsible for the biosynthesis of the antibiotic 2,4-DAPG, *phlHGFACBDE*, was identified in the FD6 genome. The structural genes *pltLABCDEFG* are also present in the PLT biosynthetic gene cluster. Two gene clusters for the siderophores pyoverdine and pyochelin are present, and these genes are conserved in some *Pseudomonas* spp. isolates (Gross and Loper 2009). Two previously unknown gene clusters encoding secondary metabolites were identified.

Table 2 General characteristics of the genomes of *Pseudomonas fluorescens* 2P24 and *P. protegens* Pf-5 and FD6

Strain	Pf-5	FD6	2P24
Number of bases	7,074,893 bp	6,667,995 bp	6,611,149 bp
G + C content (%)	63.3	62.5	60.8
Pseudogenes	75	113	480
Genes	6358	6470	6018
CDSs	6325	6380	5932
Coding (%)	89.2	89.0	89.3
rRNA (5S rRNA, 16S rRNA, 23S rRNA) genes	16 (6, 5, 5)	17 (7, 5, 5)	16 (6, 5, 5)
tmRNA genes	1	1	2
tRNA genes	70	77	66
misc_RNA genes	NA	70	83

NA not analysed

Gene annotations were obtained from a *Pseudomonas* Genome database (http://www.pseudomonas.com/)

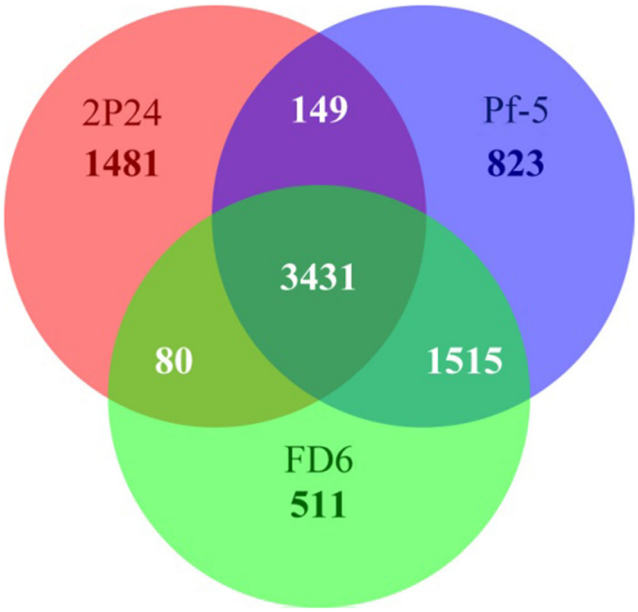

Fig. 1 Venn diagram showing the number of predicted proteins in *Pseudonmonas protegens* FD6 with significant homology to the predicted proteins from the sequenced genomes of *P. protegens* Pf-5 and *P. fluorescens* 2P24. The orthologous CDSs for each group were identified using Inparanoid software. This diagram was created using Venn Diagram

Orfamide synthases, which contain three structural genes named *ofaA*, *ofaB* and *ofaC*, are present in the whole genome of *P. protegens* FD6. Orfamide lipopeptides produced by *P. protegens* show different biological activities against phytopathogenic fungi (Ma et al. 2016). Another 5964 bp gene cluster that contains six ORFs, *MbnTPHABC*, was identified as the methanobactin gene cluster. The operon includes the genes *mbnA*, *mbnB* and *mbnC*, which are involved in the biosynthesis of methanobactin, *mbnT*, which is involved in export of methanobactin, and *mbnP* and *mbnH* of unknown function (Kenney et al. 2018). Dispirito et al. (2007) reported that methanobactin isolated from methanotrophic bacteria had antagonistic activity against gram-positive bacteria (Dispirito et al. 2007). However, orfamide and methanobactin have not been extracted from *P. protegens* FD6 culture filtrate or cells. *P. protegens* FD6 and Pf-5 had seven common compound biosynthesis clusters, and the gene clusters for methanobactin and rhizoxins are unique to FD6 and Pf-5, respectively. Rhizoxins from cultures of *P. fluorescens* Pf-5 have demonstrated antifungal, phytotoxic, and cytotoxic properties (Loper et al. 2008). The gene clusters for syringomycin, fragin and mupirocin are unique to *P. fluorescens* 2P24 (Table S1).

Genes involved in plant growth promotion

Pseudomonas sp. UW4 is a plant growth-promoting bacterium that can grow under different environmental stresses. A number of genes involved in plant growth promotion were identified in *Pseudomonas* sp. UW4, such as indole-3-acetic acid (IAA) synthesis and acetoin biosynthesis (Duan et al. 2013). A search for FD6-, 2P24- or Pf-5-like IAA pathway-related genes revealed the presence of four orthologous genes in these three strains, suggesting similar pathways in comparison to UW4. Certain *Pseudomonas* isolates promote plant growth through the production of a volatile compound, acetoin (Ryu et al. 2003). The associated genes, including acetolactate synthase and zinc-containing alcohol dehydrogenase, were identified in the genomes of FD6, Pf-5, and 2P24. Pyrroloquinoline quinine (PQQ) is a plant-growth promotion factor, and biosynthetic genes for PQQ are clustered in an operon *pqqABCDEF* (Choi et al. 2008). This complete operon was present in the genomes of Pf-5, FD6, and 2P24. The deaminase 1-aminocyclopropane-1-carboxylate (ACC) produced by *Pseudomonas* strains promotes root elongation and controls plant diseases (Wang et al. 2000). The genome of 2P24 contained the putative ACC deaminase structural gene *acdS* and its regulatory gene *acdR*, whereas these two genes are absent from both the Pf-5 and FD6 genomes (Table 3).

Rhizosphere colonization

Biocontrol agents usually show certain competitive colonization traits, including motility and the ability to attach to the root surface. Many genes related to chemotaxis and motility were found in the genomes of the three *Pseudomonas* isolates. FD6 contained eight genes associated with chemotaxis traits. In this study, we also found eight genes responsible for flagella biosynthesis, such as the *fli* and *flh* operons in the genomes of Pf-5, FD6, and 2P24. Attachment to the root surface is another competitive colonization trait (Shen et al. 2013; Periasamy et al. 2015). A large number of genes involved in attachment were predicted in the pseudomonad genomes, such as genes associated with type IV pili, adhesion, and agglutination (Table S2).

Construction and isolation of *P. protegens* FD6 derivatives

The details of the construction of the *pltD* and *phlC* gene mutations are shown in Fig. 3. The 1051 bp upstream and 584 bp downstream regions of *pltD* were amplified by PCR from chromosomal DNA of *P. protegens* FD6 and fused together by overlap PCR. The resulting fragment was used to construct the pEX18-ΔpltD recombinant plasmid (Fig. 3a). More than 4000 transconjugants were screened for in-frame deletions in *pltD* on LB agar medium and LB agar medium supplemented with kanamycin. From this library, one mutant, ΔpltD, was obtained and identified by PCR. Using the primers pltF1–240 and pltR2–2748, a 1635 bp fragment with an 873 bp in-frame deletion of *pltD* was obtained as expected.

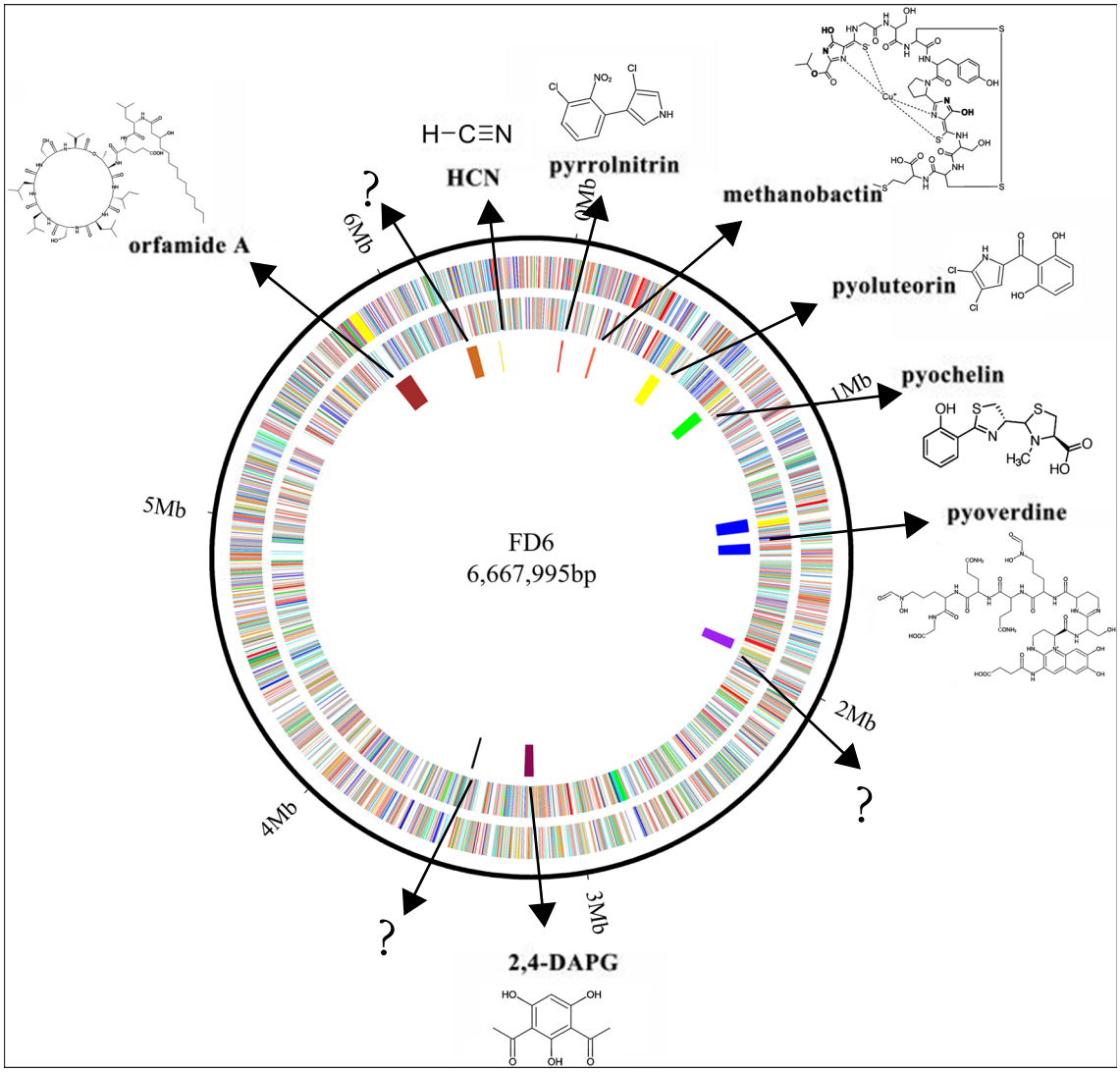

Fig. 2 Circular representation of the genome of *Pseudomonas protegens* FD6. The outer scale designates coordinates in base pairs. The two circles show predicted genes on the plus strand and minus strand. The third circle shows twelve secondary metabolite biosynthesis gene clusters, with the structures and names of the corresponding compound indicated with arrows. Three gene clusters whose metabolic products are unknown are indicated with a question mark. The genomic analysis of secondary metabolites was performed using AntiSMASH bacterial version (https://antismash.secondarymetabolites.org/#!/start), and the artwork was created by Circos software

An analogous gene replacement strategy was performed to screen the *phlC* deletion mutant (Fig. 3b). A ΔphlC deletion mutant was obtained from 12,000 transconjugants. Using the primers phlA138-F1 and phlB2743-R2, a 2064 bp fragment with a 541 bp in-frame deletion of *phlC* was obtained as expected.

Role of PLT and 2,4-DAPG in growth inhibition of phytopathogenic fungi

To further clarify the relative importance of 2,4-DAPG and PLT production by the FD6 strain in the inhibition of fungal growth, the ΔpltD and ΔphlC mutants were assayed for growth inhibition of the pathogens *B. cinerea* and *M. fructicola*. The results showed that the ΔphlC mutant showed reduced inhibition of *M. fructicola.* However, the ΔpltD mutant had reduced antagonistic activity compared to the wild-type strain, and no fungal inhibition was observed suggesting that pyoluteorin is required for inhibition (Fig. 4).

Detection of antifungal compound production in tested *Pseudomonas* strains

As shown in Fig. 5, we clearly observed that ΔphlC did not biosynthesize 2,4-DAPG in the PSA medium, whereas the PLT yield markedly increased by more than sixtyfold compared with wild-type FD6. The PLT-deficient strain ΔpltD could not produce PLT, and 2,4-DAPG production dropped by approximately sixteenfold compared to strain FD6 (Fig. 5a, b). Also, the PG production of mutant ΔpltD and ΔphlC

Table 3 Putative genes involved in plant growth promotion in *Pseudomonas* spp. genomes

Gene	Product name	Pf-5 ORF ID PFL_	FD6 ORF ID DWF74_	2P24 ORF ID C0J56_
iaaM	tryptophan synthase subunit alpha	PFL_5670	DWF74_RS16175	C0J56_RS27395
aux	auxin efflux carrier	PFL_0889	DWF74_RS03070	C0J56_RS04820
ami	amidase	PFL_0088	DWF74_RS16435	C0J56_RS00225
phe	phenyl acetaldoxime dehydratase	PFL_4132	DWF74_RS04154	C0J56_RS23725
acdS	1-aminocyclopropane-1-carboxylate deaminase	–	–	C0J56_RS04240
acdR	leucine-responsive regulatory protein	–	–	C0J56_RS04235
ilvB	acetolactate synthase 3 catalytic subunit	PFL_5255	DWF74_RS10920	C0J56_RS25230
ilvH	acetolactate synthase 3 regulatory subunit	PFL_5254	DWF74_RS10915	C0J56_RS25225
adh	zinc-containing alcohol dehydrogenase	PFL_1816	DWF74_RS25120	C0J56_RS20810
pqqA	pyrroloquinoline quinone biosynthesis protein PqqA	PFL_5673	DWF74_RS12945	C0J56_RS27410
pqqB1	pyrroloquinoline quinone biosynthesis protein PqqB	PFL_2224	DWF74_RS26975	–
pqqB2	pyrroloquinoline quinone biosynthesis protein PqqB	PFL_5674	DWF74_RS12950	C0J56_RS27415
pqqC	pyrroloquinoline quinone biosynthesis protein PqqC	PFL_5675	DWF74_RS12955	C0J56_RS27420
pqqD	pyrroloquinoline quinone biosynthesis protein PqqD	PFL_5676	DWF74_RS12960	C0J56_RS27425
pqqE	pyrroloquinoline quinone biosynthesis protein PqqE	PFL_5677	DWF74_RS12965	C0J56_RS27430
pqqF	coenzyme PQQ biosynthesis protein PqqF	PFL_5672	DWF74_RS12940	C0J56_RS27405

[a] Homologous genes were identified at a 60% identity threshold by searching the NCBI GenBank

[b] "-" No homologs were present in the compared genome

was significantly decreased compared with wild-type FD6 (Fig. 5c).

In addition to secondary metabolite biosynthesis, other biocontrol factors were detected, which are presented in Table 4. The results showed that there was no difference in the production of HCN, proteases or siderophores between ΔpltD and ΔphlC compared with strain FD6.

Efficacy of strain FD6 and its derivatives in controlling grey mould of tomato

Treatment of bacterial cultures of strain FD6 showed an obvious effect for controlling grey mould in tomato fruit. The largest lesion diameters were observed on tomato treated with LB broth. There were no visible disease lesions at 5 days post inoculation after treatment with strain FD6. The lesion diameters of grey mould were inhibited by 65% and 30% after treatment with ΔphlC and ΔpltD bacterial cultures, respectively (Fig. 6).

Discussion

A wide range of biocontrol agents, such as *P. fluorescens* A506 and 1629RS, has been in commercial production to control fungal and bacterial diseases in crops over the past decade (O'Brien 2017). At least seven types of compounds, including pyoluteorin, phloroglucinols, phenazines, pyrrolnitrin, lipopeptides, siderophores, and hydrogen cyanide, which were

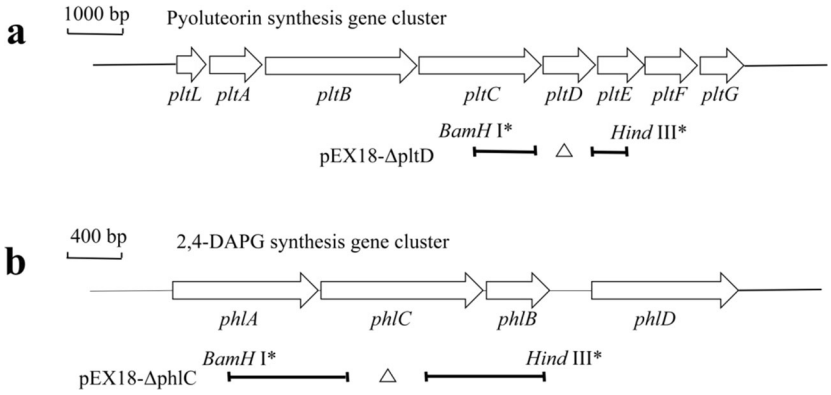

Fig. 3 Construction scheme of recombinant plasmids pEX18-pltD and pEX18-phlC. The bars designate the fragments cloned into the vector pEX18 to give pEX18-ΔpltD and pEX18-ΔphlC. *, restriction enzyme; Δ, gene deletion

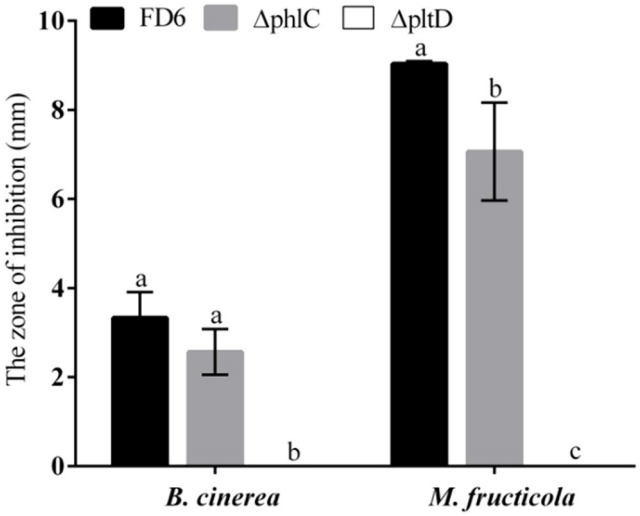

Fig. 4 Inhibitory activity of *Pseudomonas protegens* FD6 and its derivatives against *Botrytis cinerea* and *Monilinia fructicola*. Error bars represent standard deviation, and different letters indicate statistically significant differences ($p < 0.05$). FD6 is the wild strain of *P. protegens* FD6, ΔpltD is the mutant defective in production of pyoluteorin, and ΔphlC is the mutant defective in production of 2,4-diacetylphloroglucinol

obtained from some isolates of *Pseudomonas* spp., have received attention because they naturally suppress multiple fungal pathogens (Liu et al. 2015; Yan et al. 2017). Both 2,4-DAPG and PRN have been found to exist alone in one antibiotic-producing bacterium so far as we know. However, PLT is produced with other antibiotics in several isolates, and no isolate has been obtained that only produces PLT (Liu et al. 2006).

Our previous study showed that ΔprnA mutant was as effective as the wild-type strain FD6 at inhibiting hyphal growth, suggesting that inhibition is not due to production of pyrrolnitrin (Zhang et al. 2016). In addition to pyrrolnitrin, 2,4-DAPG is another antibiotic produced by *P. protegens* FD6. To determine the role of PLT and 2,4-DAPG in strain FD6, we generated ΔpltD and ΔphlC mutants with an in-frame deletion of *pltD* and *phlC*, respectively. The HPLC data indicated that the PLT yield in ΔphlC was markedly increased compared with strain FD6. The *pltD* gene mutation led to a decrease in 2,4-DAPG production compared with strain FD6. In agreement with the above research on the quantity of antibiotics, ΔphlC mutant still displayed weak inhibition of *M. fructicola and B. cinerea* due to overproduction of pyoluteorin. It has been demonstrated that PG is converted into PG-Cl and PG-Cl_2, which serves as signals to activate the expression of pyoluteorin biosynthetic genes (Yan et al. 2017).

The results of the HPLC analysis show that both ΔpltD and ΔphlC mutants produce less phloroglucinols. The low level of phloroglucinols biosynthesis was also observed in the ΔphlA mutant, a derivative of Pf-5. The decrease of PG production in the ΔphlC mutant might be attributed to its inability to acetylate PG to MAPG and DAPG, along with the overproduction of pyoluteorin, leading to loss of auto-induction of the phlACBD operon by DAPG (Kidarsa et al. 2011). These results suggested that the absence of PLT influenced the production of another antibiotic: 2,4-DAPG in strain FD6. The *pltD* mutant which produces no pyoluteorin or 2,4-DAPG, still exhibits certain biological control activity against tomato grey mould disease. We supposed *P. protegens* FD6 promotes the biocontrol activity through other mechanisms, such as induction of systemic resistance and competition.

In some biocontrol agents that produce a number of antibiotics, mutants lacking one antibiotic might not lose their inhibitory ability due to homeostatic regulatory systems or metabolic co-regulation. Metabolic co-regulation is usually observed in microorganisms and plays a great role in microbial interactions. Moreover, there is a metabolic co-regulation between the biosynthetic pathways for 2,4-DAPG and PLT in *P. protegens* Pf-5. Phloroglucinol, as an intermediate in the 2,4-DAPG biosynthetic pathway, is converted into mono-

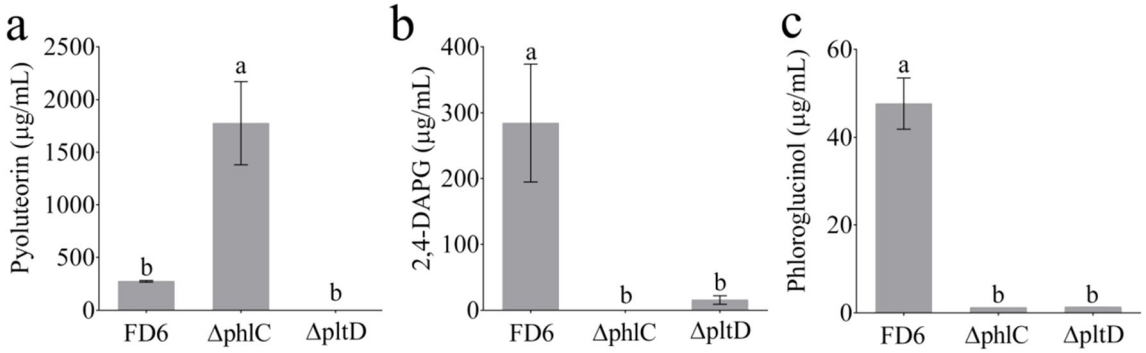

Fig. 5 Detection of pyoluteorin (**a**), 2,4-DAPG (**b**) and phloroglucinol (**c**) production in *Pseudomonas* strains. *P. protegens* FD6 and variants were assayed for 2,4-DAPG and pyoluteorin concentration by HPLC (Zhang et al. 2015). Phloroglucinol was quantified by colorimetric method (Kidarsa et al. 2011). The experiment was repeated twice. Error bars represent standard deviation, and different letters indicate statistically significant differences ($p < 0.05$). FD6 is the wild strain of *P. protegens* FD6, ΔpltD is the mutant defective in production of pyoluteorin, and ΔphlC is the mutant defective in production of 2,4-diacetylphloroglucinol

Table 4 Phenotypic characterization of *Pseudomonas protegens* FD6 and its derivatives

Strain	Character	Extracellular metabolite activity			
		Antifungal	Protease	Siderophore	HCN
FD6	Wild type	++	++	++	++
ΔpltD	*pltD*⁻	–	++	++	++
ΔphlC	*phlC*⁻	+	++	++	++

Extracellular proteases and siderophores were assessed using skim milk agar plates and siderophore detection plates, respectively (Reimmann et al. 1997; Schwyn and Neilands 1987). Assays for HCN on KB agar medium were performed qualitatively using HCN-indicator paper (Castric and Castric 1983). The experiment was repeated twice. ++, metabolite detected; +, reduced activity; −, no metabolite detected

and di-chlorinated phloroglucinols by a halogenase that is encoded in one of the pyoluteorin gene clusters. The chlorinated phloroglucinols serve as signals that induce the expression of PLT biosynthetic genes (Yan et al. 2017). In short, 2,4-DAPG repressed the biosynthesis of PLT in *P. protegens* FD6. However, it is not clear why the *pltD* mutation led to the reduction in 2,4-DAPG production. The current study indicated that 2,4-DAPG and PLT play a crucial role in *P. protegens* FD6 biocontrol traits.

Pseudomonads promote their biocontrol activities through the production of multiple secondary metabolites with distinct roles in competitive and cooperative microbial interactions (O'Brien 2017). Orfamide-type cyclic lipopeptides consist of 10 amino acids and three fatty acid residues, and show different biological activities against phytopathogenic fungi (Gross and Loper 2007; Ma et al. 2016). BLAST searches revealed that *P. protegens* FD6 may be a potential producer of orfamide and methanobactin, but this prediction has yet to be experimentally verified. In addition, *P. protegens* FD6 displayed stronger antagonistic activity against *B. cinerea* in potato agar than in potato dextrose agar or potato sucrose agar. The MALDI-TOF analysis showed that *P. protegens* FD6 may produce another antifungal lipopeptide (data not shown). More research on this compound is underway.

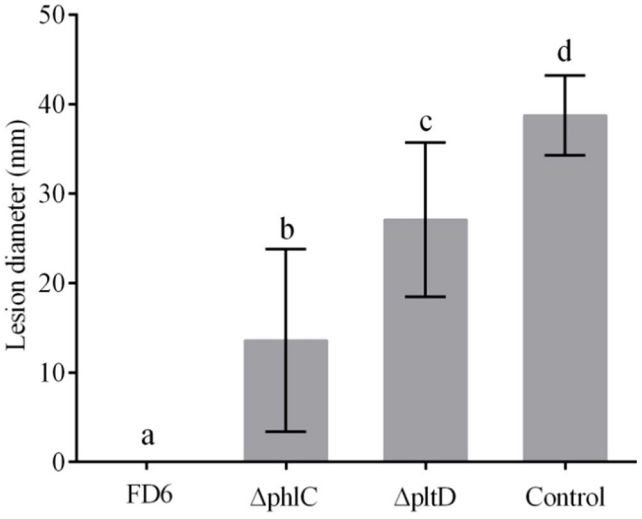

Fig. 6 Biocontrol effect of *Pseudomonas protegens* FD6 and its derivatives on *Botrytis cinerea*. Each treatment was composed by three replicates with two fruits per replicate. Error bars represent standard deviation, and different letters indicate statistically significant differences ($p < 0.05$). FD6 is the wild strain of *P. protegens* FD6, ΔpltD is the mutant defective in production of pyoluteorin, and ΔphlC is the mutant defective in production of 2,4-diacetylphloroglucinol

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