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Molecular Characterization of Biosynthetic Genes of an Antifungal Compound Produced by *Pseudomonas fluorescens* MC07

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Abstract *Pseudomonas fluorescens* MC07 is a growth-promoting rhizobacterium that suppresses mycelial growth in fungi such as *Rhizoctonia solani*, *Pythium ultimum*, *Fusarium oxysporum*, and *Phytophthora capsici*. To determine the role of the bacterium's antifungal activity in disease suppression, we screened 2,500 colonies generated by Tn*5lacZ* insertions, and isolated a mutant 157 that had lost antifungal activity. The EcoRI fragment carrying Tn*5lacZ* was cloned into pBluescript II SK(+) and used as a probe to isolate wild-type clones from a genomic library of the parent strain, MC07. Two overlapping cosmid clones, pEH4 and pEH5, that had hybridized with the mutant clone were isolated. pEH4 conferred antifungal activity to the heterologous host *P. fluorescens* strain 1855.344, whereas pEH5 did not. Through transposon mutagenesis of pEH4 and complementation analyses, we delineated the 14.7-kb DNA region that is responsible for the biosynthesis of an antifungal compound. DNA sequence analysis of the region identified 11 possible open reading frames (ORF), ORF1 through ORF11. A BLAST search of each putative protein implied that the proteins may be involved in an antifungal activity similar to polyketides.

Key words: *Pseudomonas fluorescens*, soilborne fungal diseases, antifungal activity, biosynthetic genes of antifungal compound

Pseudomonads show antimicrobial activity toward plant pathogens, and are potential biological control agents [27, 30]. Important features of biocontrol agents by microorganisms include effective root colonization, production of antimicrobial compounds, and induction of systemic resistance in plants [27, 29, 31]. The suppression of soilborne plant diseases by pseudomonads is carried out by multiple biotic and abiotic factors.

A common mechanism underlying the antagonistic activity of biocontrol agents is the production of antifungal compounds produced by pseudomonads [28]. There are many well-known antifungal compounds, such as phenazine-1-carboxylic acid (PCA) [29], 2,4-diacetylphloroglucinol (Phl) [14], pyrrolnitrin (Prn) [10], hydrogen cyanide (HCN) [33], and siderophores [2, 18, 23]. These compounds have been chemically characterized, and their biosynthetic genes are known [1, 3, 8, 11, 17, 19, 32]. Some pseudomonad strains that produce these compounds have been used as biocontrol agents against various soilborne diseases [2, 9, 10, 12–16, 20, 29]. The structural genes for the biosynthesis of PCA, Phl, Prn, and HCN are organized in a cluster, probably as operons [1, 3, 8, 17, 19, 32]. However, the enzymatic activity of proteins involved in the biosynthesis of these compounds and their biosynthetic intermediates are known only for Prn [3, 8, 17]: There are four enzymes involved in converting L-tryptophan to 7-chlorotryptophan, monodechloroaminopyrrolnitrin, aminopyrrolnitrin, and Prn [3, 8, 17]. Phl and Plt are polyketides, but it is not clear how they are enzymatically assembled [1, 3, 28]. It has been suggested that acetyl-CoA and malonyl-CoA would be derived from central carbohydrate or fatty acid metabolism [2, 3]. Like HCN, Plt is thought to be derived from amino acid metabolism, because it contains a proline moiety [3, 11, 28].

Previous studies indicated that *P. fluorescens* MC07 is a potential biological control agent, because it possesses antifungal activity, colonizes a variety of crop roots, and grows at a wide range of temperatures [15, 34]. It was demonstrated that the bacterium effectively suppresses the damping-off of cucumber caused by *Pythium ultimum*, significantly enhancing cucumber growth [15, 34]. The strain MC07 inhibited mycelial growth in *Rhizoctonia solani*, *P.*

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ultimum, *Fusarium oxysporum*, and *Phytothora capsici* on potato dextrose agar (PDA) and other media [15, 34]. Mutational analysis of the strain MC07 by Omegon-Km showed that the ability to produce antifungal compounds is directly related to the biological control effects of the damping-off of cucumber [15, 34].

In this study, we cloned the genes involved in the biosynthesis of an antifungal compound produced by strain MC07, and then, through Tn*3-gusA* mutagenesis and marker-exchange experiments, defined the 14.7-kb region essential for exhibiting antifungal activity. Analysis of the DNA sequences identified 11 possible open reading frames (ORF). Based on the homology of each putative protein with proteins contained in the database, we predicted that each putative protein would be involved in the biosynthesis of an antifungal compound similar to the polyketides. We found that both the production of the antifungal compound and the root-colonizing ability of the strain MC07 play important roles in suppressing the damping-off of cucumber.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All of the strains were cultured in Luria-Bertani (LB) medium. *P. fluorescens* strain MC07 and *Escherichia coli* were cultured at 28°C and 37°C, respectively. Antibiotics at the following concentrations were used: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; gentamycin, 50 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml; rifampicin, 50 μg/ml; spectinomycin, 25 μg/ml; and tetracycline, 10 μg/ml. The pathogenic fungi, *Pythium ultimum*, *Fusarium oxysporum*, and *Rhizoctonia solani*, were grown at 28°C on potato-dextrose agar (PDA; Difco Lab., Detroit, U.S.A).

Recombinant DNA Techniques

Chromosomal DNA from *P. fluorescens* strain MC07 was isolated according to Sambrook *et al.* [22]. Small-scale plasmid DNA was isolated from *E. coli* using QIAprep® Spin (Qiagen, Inc., Germany) according to the supplier's instructions or by the alkaline lysis method [22]. Small-scale cosmid DNA was isolated from *E. coli* and *P. fluorescens* using the alkaline lysis method, and large-scale plasmid and cosmid DNA were prepared by alkaline lysis followed by cesium chloride density gradient centrifugation [22]. Restriction enzyme digestions were performed as recommended by the suppliers (Takara, Japan). Gel electrophoresis was performed in 0.7% (w/v) agarose gels, and Southern transfers were done on Hybond-N™ nylon membranes (Amersham Biosciences, Uppsala, Sweden) as described by the manufacturer. For colony hybridization, all of the procedures for preparing probe DNA and hybridization followed the manufacturer's instructions. To construct a genomic library of strain MC07, 20- to 30-kb segments of insert DNA were prepared by partially digesting the total genomic DNA with Sau3A1, and ligating the

Tabl 1. Bacterial strains and plasmids.

Strain/plasmid	Characteristics[a]	Source/ reference
Escherichia coli		
DH5α	F⁻ Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *endA1 deoR recA1 hsdR17* ($r_K^-m_K^+$) *phoA supE44* λ- *thi-1 gyrA96 relA1*	Gibco BRL
S17-1	Tra⁺, *recA;* Sp^r	[24]
C2110	*polA*, Nal^r	[25]
HB101	F⁻ *mcrB mrr hsd*S20($r_B^-m_B^-$) *rec*A1386 *leu ara*-14 *pro*A2 *lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 rpsL20(Sm^r) *sup*E44 λ⁻	Gibco BRL
Pseudomonas fluorescens		
MC07	Wild-type, Rif^r	[15]
1855.344	Wild-type, Rif^r	[5]
157	MC07::Tn*5lacZ*, antifungal activity-deficient mutant	This study
Plasmids		
pBluescript II SK(+)	Cloning vehicle; phagemid, pUC derivative, Amp^r	Stratagene
pLAFR3	Tra⁻, Mob⁺, RK2 replicon, Tet^r	[26]
pRK2013	Helper plasmid; Tra⁺, ColE1 replicon, Km^r	[6]
pHoKmGus	Promoterless β-glucuronidase gene, Km^r, Amp^r,	[4]
pSShe	Cm^r	[25]
pEH4	19.2-kb DNA fragment from strain MC07 cloned into pLAFR3	This study
pEH5	20.6-kb DNA fragment from strain MC07 cloned into pLAFR3	This study
pYK701	7.2-kb *Eco*RI fragment from strain 157 cloned into pBluescript II SK(+)	This study

[a]Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance; Rif^r, rifampicin resistance; Sp^r, spectinomycin resistance; Tet^r, tetracycline resistance.

fragments into the BamHI site of pLAFR3. The ligated DNA was packaged into bacteriophage l as described by the manufacturer (Promega, Madison, U.S.A.), and then transfected into *E. coli* HB101. All of the pLAFR3 derivatives were mobilized in *P. fluorescens* MC07 strains by triparental mating [6]. All other basic molecular techniques were performed according to Sambrook *et al.* [22].

Transposon Mutagenesis

The suicide plasmid pSUP102 [24] was used to generate transposon insertions in the chromosome of strain MC07, as previously described [15]. To determine the insertion site of Tn*5lacZ* in the chromosome, 1 μg of total genomic DNA was digested with EcoRI and transformed into *E. coli* DH5α, followed by selecting kanamycin-resistant colonies. pEH4 was mutagenized with Tn*3-gusA*, as previously described [4]. The insertion site and orientation of Tn*3-gusA* in each mutant were determined by restriction enzyme digestion analysis and by direct sequencing of the plasmid using primer Tn3gus (5'-CCGGTCATCTGAGACCATTA-AAAGA-3'), which allows to sequence out the Tn*3-gusA*. Mutagenized plasmids carrying the Tn*3-gusA* insertion were introduced individually into parent strain MC07 by conjugation, and marker-exchanged into strain MC07 as described previously [4]. All marker exchanges were confirmed by Southern hybridization analysis.

DNA Sequencing and Data Analysis

The DNA inserted into pEH4 was digested with appropriate restriction enzymes and subcloned into pBluescript II SK(+). Universal and reverse primers were used for the primary reactions, and then synthetic primers were used to completely sequence both strands. The DNA sequence data were analyzed using the BLAST program at the National Center for Biotechnology Institute [7], MEGALIGN software (DNASTAR, U.S.A.), and GENETYX-WIN software (Software Development Inc., Japan).

Bioassay for Production of Antifungal Compound

Two-thousand colonies carrying Tn*5lacZ* were picked and inoculated with an equal spacing around mycelial disks (1-cm diameter) of *P. ultimum* and *R. solani*, which were grown on Potato-Dextrose-Peptone Agar (PDP; 10 g Difco potato-dextrose broth, 5 g Difco proteose peptone No. 3, 15 g agar, 1 l distilled water) at 25°C for 3 and 6 days, respectively. Inhibitory activity was measured as the width of the clear zone between the bacterial colony and fungal pathogens.

Root Colonization Assay

Cucumber seeds were disinfected with 1% NaOCl and inoculated with bacterial cells as previously described [12, 15]. The root-colonizing abilities of the test isolates were examined according to Ahmad and Baker [21, 34]. After the seeds had germinated, roots were cut into 1-cm segments with a sterile scalpel, and the first, middle, and last 1-cm segments were used for population analyses.

Inoculation and Seed Treatment

Cucumber seeds (*Cucumis sativas* L. cv. 'Shinpung', Hungnong Seed Co.) were soaked for 1 h in an overnight culture of bacterial cells resuspended in 0.1 M $MgSO_4$. The treated seeds were air-dried at room temperature for 30 min and then sown in plastic pots (10 cm in diameter) filled with commercial peat mix soil (Baroco, Seoul Agriculture Materials Co., Seoul, Korea) that had been inoculated with *P. ultimum*. The inoculumn was prepared as previously described [34]. After 2 days of incubation at 28°C in a dark chamber, the pots were moved to a greenhouse. The plants were examined daily for the development of damping-off, and the number of infected plants and emergence rates were scored.

RESULTS AND DISCUSSION

Isolation and Characterization of Antifungal Activity-Deficient Mutants

After mutagenizing *P. fluorescens* MC07 with Tn*5lacZ*, 2,000 prototrophic colonies were isolated and tested for antibiosis against *P. ultimum* on PDA medium. One antifungal activity-deficient mutant, 157, was isolated (Table 1). To determine the insertion site of Tn*5lacZ* in the mutant, the mutant clone carrying the flanking DNA regions was cloned, resulting in pYK701 (Table 1). BLAST search analyses of the flanking region sequences revealed that the mutant 157 has an insertion in a gene homologous to 3-oxoacyl-(acyl-carrier-protein) synthase II (70% identity and 79% positives) of *P. putida* (Table 2).

Isolation of Cosmid Clones Carrying Genes Involved in the Biosynthesis of an Antifungal Compound

Two cosmid clones that hybridized with the 2-kb EcoRI fragment of pYK701 were isolated and mobilized in the mutant 157. Transconjugants were assayed for antifungal activity against *P. ultimum* (Figs. 1 and 2). pEH4 complemented the antifungal activity of the mutant 157, whereas the overlapping clone pEH5 did not (Figs. 1 and 2). Restriction map analysis showed that most of the insert DNA of pEH4 and pEH5 are overlapped, but that the 2.3-kb EcoRI-BamHI fragment in pEH4 is missing in pEH5 (Fig. 1).

To determine whether these clones contain all of the essential genes responsible for the biosynthesis of the antifungal compound, pEH4 and pEH5 were individually mobilized in *P. fluorescens* 1855.344, a heterologous host having no antifungal activity. When transconjugants were tested for antifungal activity against *P. ultimum*, pEH4 conferred antifungal activity to the strain 1855.344 (Fig. 1).

Table 2. Characteristics of putative ORFs involved in an antifungal compound biosynthesis.

ORF	ORF length (bp)	Protein mol mass (kDa)	Protein with the highest sequence similarity [Organism] (accession number)	BLASTX E-value
ORF1	571	20.4	Acyl carrier protein, putative [*P. putida*] (AAN68385)	$5e^{-74}$
ORF2	1,275	45.8	3-Oxoacyl carrier protein synthase II, putative [*P. putida*] (AAN68386)	e^{-147}
ORF3	1,059	36.9	Beta-ketoacyl synthase, putative [*P. putida*] (AAN68387)	$9e^{-98}$
ORF4	1,296	45.8	3-Oxoacyl carrier protein synthase II, putative [*P. putida*] (AAN68388)	0.0
ORF5	1,209	41.9	Beta-ketoacyl synthase, putative [*P. putida*] (AAN68389)	e^{-116}
ORF6	2,880	105	Pyridoxalphosphate dependent aminotransferase, classIII, putative [*P. putida*] (AAN68390)	0.0
ORF7	738	25.9	3-Ketoacyl ACP reductase, putative [*P. putida*] (AAN68391)	e^{-106}
ORF8	765	27.4	Oxidoreductase, putative [*P. putida*] (AAN68392)	e^{-101}
ORF9	1,297	48.4	Hypothetical protein [*P. putida*] (AAN68393)	0.0
ORF10	837	31.9	Hypothetical protein [*P. putida*] (AAN68394)	e^{-126}
ORF11	2,622	97.4	Transporter, putative [*P. putida* KT2440] (AAN68395)	0.0

This indicates that pEH4 contains all of the essential genes for biosynthesis of the antifungal compound, and that the 2.3-kb EcoRI-BamHI fragment missing in pEH5 plays an important role in the production of the compound.

Delineation of the Biosynthetic Region by Transposon Mutagenesis

To determine the minimum DNA region required for the biosynthesis of the antifungal compound in pEH4, the plasmid was mutagenized with Tn*3-gusA*. The mutant plasmids were transferred to *P. fluorescens* 1855.344, and transconjugants were assayed for antifungal activity against *P. ultimum*. As shown in Fig. 1, the 14.7-kb region in pEH4 was found to be responsible for the biosynthesis of the antifungal compound, based on the bioassay results of each Tn*3-gusA* insertion into *P. fluorescens* 1855.344. Each marker-exchange mutant in the strain MC07 showed the same result as that observed in *P. fluorescens* 1855.344 (Fig. 1).

DNA Sequence Analysis of the Biosynthetic Region Essential for Antifungal Activity

DNA sequencing of the 14.7-kb region and subsequent computer analysis identified 11 putative ORFs, ORF1 through ORF11 (Fig. 1). ORF1 through ORF6 exhibited high homologies with acyl carrier protein, 3-oxoacyl-acyl carrier protein synthase II, beta-ketoacyl synthase, 3-oxoacyl-acyl carrier protein synthase II, beta-ketoacyl synthase, and pyridoxalphosphate-dependent aminotransferase class III of *P. putida*, respectively (Table 2). ORF7 and ORF8 were similar to 3-ketoacyl ACP reductase and oxidoreductase of *P. putida*, respectively (Table 2). ORF9 and ORF10 showed high homologies with hypothetical proteins of *P. putida* (Table 2). Based on homologies with proteins involved in polyketide compounds, it is very likely that the antifungal compound produced by the strain MC07 is a polyketide-like compound. ORF11 was nearly identical to a transporter protein of *P. putida*, indicating that ORF11 might be responsible for transporting the antifungal compound

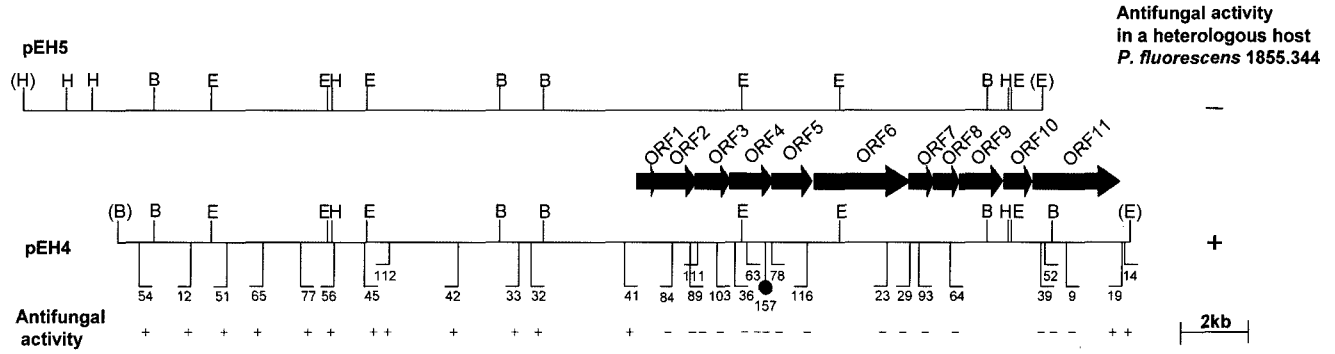

Fig. 1. Restriction enzyme maps of pEH4 and pEH5.

Arrows mark the positions and orientations of the antifungal compound biosynthesis genes. Vertical bars in the map indicate the positions and orientations of the Tn*3-gusA* insertions. The ability of mutants to produce antifungal activity is represented below the restriction map. A vertical bar with closed circle indicates the position of the Tn*5lacZ* insertion. B, BamHI; E, EcoRI; H, HindIII.

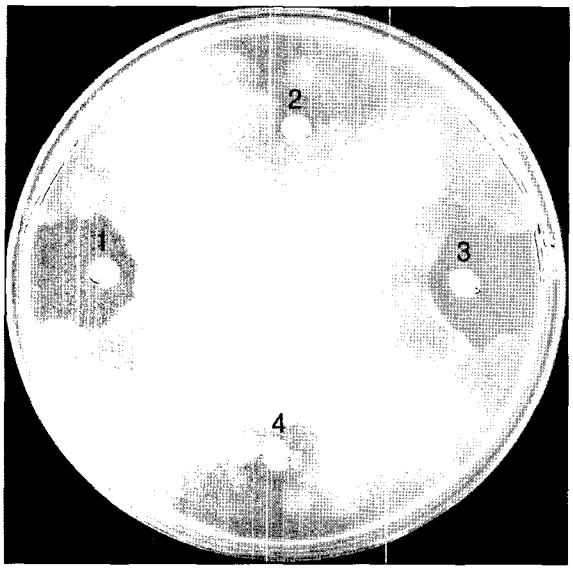

Fig. 2. Antifungal activity of *P. fluorescens* MC07 and its derivatives against *P. ultimum* on PDP agar medium.
1, *P. fluorescens* MC07; 2, the mutant 157; 3, the mutant 157 carrying pEH4; 4, the mutant 157 carrying pEH5.

after synthesis. This is consistent with the observation that pEH5 lacking ORF11 did not confer antifungal activity to a heterologous host, *P. fluorescens* 1855.344.

Antifungal Activity of the Strain MC07 is Directly Responsible for Biological Control

To determine whether the antifungal activity of the strain MC07 is directly responsible for the biological control activity, cucumber seedlings were treated with the parental strain MC07, the mutant 157, the mutant 157 carrying pEH4, and the mutant 157 carrying pEH5, and the occurrence of damping-off was then evaluated in inoculated soil with *P. ultimum*. Although more than 90% of the cucumber plants treated with the mutant 157 and water control were diseased, no damping-off symptoms were observed in the seedlings treated with the strain MC07 (Fig. 3). The mutant 157 carrying pEH4 suppressed damping-off at the same level as did the parent strain MC07, however, the strain 157 carrying pEH5 failed (Fig. 3). This indicates that the antifungal activity of the strain MC07 directly contributes to the biological control effect.

Fig. 3. Suppression of damping-off by treatment with *P. fluorescens* MC07 in cucumber.
Each pot contains cucumber plants treated with 1, *P. fluorescens* MC07; 2, the antifungal activity-deficient mutant 157; 3, the mutant 157 carrying pEH4; 4, the mutant 157 carrying pEH5.

Table 3. The differences of population densities of *P. fluorescens* MC07 and antifungal-deficient mutant 157 that colonized on each part of cucumber roots as analyzed by the Ahmad and Baker methods.

Strain	Population density on cucumber root ($\times 10^4$ cfu/cm)[a]		
	First 1-cm	Middle 1-cm	Last 1-cm
MC07	275±5	8.9±0.2	3.7±0.1
157	236±4	3.9±0.1	1.9±0.1

[a]The bacterial populations were determined using a dilution plate method on King's B (KB) agar plate supplemented with 50 μg/ml of rifampicin and 100 μg/ml of cyclohexamide. Values are mean±SDs of three replicates.

Antifungal Activity-Deficient Mutant Colonizes Less Effectively

To determine whether the antifungal activity-deficient mutant maintains the ability to colonize roots, bacterial populations on cucumber roots were measured. The populations of the wild-type strain MC07 and the mutant 157 on the first 1-cm root segment were 2.7×10^6 and 2.3×10^6 cfu/1-cm root, respectively (Table 3). However, the colonizing populations of MC07 and 157 on the last 1-cm root segment were 3.7×10^4 and 1.9×10^4 cfu/1-cm root tip, respectively (Table 3). These results indicate that the antifungal activity-deficient mutant 157 colonizes cucumber roots less effectively than the wild-type strain.

Taken together, we conclude that both the production of an antifungal compound and the ability to colonize roots play important roles in suppressing the damping-off of cucumber. However, it is not clear how antifungal activity and root-colonization ability are related.

Nucleotide Sequence Accession Number

The complete DNA sequence of the antifungal compound biosynthetic genes of *P. fluorescens* MC07 was deposited in the GenBank database under the accession number DQ119108.

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