Characterization of Complemented Mutants in *Pseudomonas fluorescens* and Cloning of the DNA Region Related in Antibiotic Biosynthesis

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길항세균 *Pseudomonas fluorescens*의 Complemented Mutant에 대한 특성조사 및 길항물질 유전자 Cloning

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ABSTRACT : *Pseudomonas fluorescens* produces the antibiotic, 2,4-diacetylphloroglucinol (Phl), which promotes plant growth by inhibiting bacteria and fungi. Cosmids (genomic library) were mobilized into Phl-nonproducing mutants through the triparental matings with pRK2013 as the helper plasmid at the frequency of 8.37×10^{-4}. Complemented mutants that showed antibiotic activity were selected among about 2,000 transconjugants. The complemented mutants were confirmed by acquired drug resistances (kanamycin and tetracycline). The antibiotic substances of wild type and complemented mutants showed the most excellent anti-bacterial activity. Inhibitory effects of complemented *P. fluorescens* against phytopathogenic fungi were equal to the parental strain. Complemented mutant and wild type of *P. fluorescens* were causal microbes of fungal morphological abnormalities. Complemented mutants in potato dextrose agar supplemented with bromothymol blue also showed restoration of glucose utilization as wild type. Plasmids of complemented mutants were isolated from transcojugants and transformed into competent cells of *E. coli* DH5α. The plasmid DNA was reisolated from transformed *E. coli* DH5α.

Key words : *Pseudomonas fluorescens*, **complemented mutant, transformation, nonantagonistic mutant, pepper blight.**

Fluorescent pseudomonads are common soil microorganisms, often found on plant root surfaces. Cook and Rovira suggested that antibiotic-producing, root-colonizing pseudomonads might be useful for controlling take-all of wheat (2, 5). *Pseudomonas putida* and *P. fluorescens* include strains able to promote plant growth and suppress plant pathogens through a production of 2,4-diacetylphloroglucinol (Phl) and phenazine (1).

In the last few years numerous genes involved in the biosynthesis of antibiotics, pigments, and other secondary metabolites have been cloned (13). In some cases it appears that an antibiotic resistance gene is a part of a cluster containing at least some of the corresponding biosynthesis genes (15).

Understanding expression of these genes, which are not essential for growth, is of utmost importance for establishing how they have evolved as compared to operons of primary biosynthetic genes.

Genetic analysis of several *Pseudomonas* strains has established a positive correlation between antibiotic production and disease suppression (4). Futhermore, knowledge of the regulatory mechanisms controlling gene expression has relevance for industrial overproduction of these metabolites (9). Douglas and Gutterson showed that glucose stimulated the production of some antibiotics by a strain of *P. fluorescens*, whereas the production of other antibiotics was inhibited by glucose (3, 12). As yet, relatively little is known of the control mechanisms which regulate expression of genes of antibiotic synthesis or modification. Many antibiotics from

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Pseudomonas species were identified and chemically characterized (6).

In this study, complemented mutants of *P. fluorescens* were obtained through conjugation and used for cloning of a gene related in Phl biosynthesis. On the other hand, we also examined the characterized complemented mutants by chemical and genetic analyses.

MATERIALS AND METHODS

Organisms and culture conditions. Antagonistic *P. fluorescens* was provided from Kim *et al.* (6). The strain of nonantagonistic mutant was obtained from Lee *et al.* and the strain was rifampicin and kanamycin resistant (7). Cosmid pLAFR3 in *Escherichia coli* HB101 and nonantagonistic mutant were used to make complemented mutants. *Phytophthora capsici, Colletotrichum gloeosporioides, Fusarium oxysporum* f. sp. *cucumerinum, Rhizoctonia solani, Pythium* spp., *Agrobacterium tumefaciens* and *Pseudomonas solanacearum* were used for the bioassay of biological activity of complementation in *P. fluorescens*. Plant pathogenic bacteria and *E. coli* were cultured at 30° C and 37°C, respectively. All fungi were grown on potato dextrose agar (PDA) at 25°C. The concentrations of antibiotics were as follow: rifampicin, 100 µg/ml; kanamycin, 25 µg/ml; tetracycline, 15 µg/ml.

Transformation. Transformation of *E. coli* was done as described by Sambrook *et al.* (11).

Complementation. The mobilization of broad host range cosmid pLAFR3 (genomic library) into *P. fluorescens* strain was done by using the triparental mating with *E. coli* HB101 pRK2013 as a helper plasmid (11).

Selection of complemented mutants of *P. fluorescens* were selected through the ability of inhibiting growth of fungi on PDA plate. Paper disc containing culture of complemented mutants was placed on PDA plate and incubated at 30°C for 24 hrs. The fungi were transferred then to the center of PDA plate and incubated for 3~4 days.

Biological activity of complemented and nonantagonistic mutants. The antibacterial activity of purified antibiotics from wild type, nonantagonistic mutant and complemented mutants (C1-C4) of *P. fluorescens* was tested by using a paper disc method for *A. tumefaciens* and *P. solanacearum*. Each compound was dissolved in ethyl acetate and bioassayed agai-

nst two bacteria. Paper disc (8 mm, φ) soaked with each solution were placed on LB agar and PS agar. Ethyl acetate was used as control. Inhibition zone was measured after incubation at 30°C for 24 hrs. The method for *P. capsici, C. gloeosporioides, F. oxysporum* f. sp. *cucumerinum, R. solani* and *Pythium* spp. was just the same with selection of complemented mutants. In this case, L broth was used as control. Morphological changes of fungal mycelia by 2,4-diacetylphloroglucinol of *P. fluorescens* strains were observed under the light microscope. The agar blocks of the inhibition zone were cut and observed under the microscope.

Detection of antibiotics (Phl). The crude extract was concentrated into 1 ml of the volume and analyzed by thin layer chromatography (TLC). Each crude extract and the standard antibiotic solution in ethyl acetate were chromatographed on a silica gel 60 F254 plate. The plate was developed in chloroform-methanol (9 : 1) solvent system and was dried. By spraying with 0.5% *p*-anisaldehyde, each antibiotic was observed after charring the TLC plates (110°C, 5 min).

Restoration of glucose utilization. For restoration of glucose utilization, wild type, nonantagonistic and complemented mutants were toothpicked onto PDA supplemented with 0.15 g bromothymol blue per liter. The plates were incubated at 30°C for 72 hrs (8).

DNA digestions and agarose gel electrophoresis. For agarose gel electrophoresis, horizontal slab gels containing 0.8% agarose were prepared in TBE (0.045 M Tris-borate and 0.001 M EDTA) buffer. DNA samples containing dye mixture (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% Ficoll (Type 400)) were loaded into the gel. Electrophoresis was carried out at 30 V. The gels were stained for 30 min in 2 µg/ml of ethidium bromide solution and visualized with UV transilluminator (254 nm). Lamda DNA digested with *Hin*dIII were used as size markers.

RESULT

Restoration of antibiotic production in complemented mutant. While nonantagonistic mutant did not exhibit yellow halo within 60 to 72 hrs, 4 complemented mutants did show yellow halo on the medium indicating the conversion of glucose into glu-

conate. Mutant strains deficient in glucose dehydro-genase failed to grow on glucose, but complemented mutants exhibited glucose utilization as wild type. Complemented mutants were regarded as restoration of biosynthesis of the 2,4-diacetylphloroglucinol (Phl) (Fig. 1). To detect the production of Phl in a complemented mutant, the solution was analyzed by a thin layer chromatography (TLC). Compounds of a complemented mutant and wild type exhibited brown spots when sprayed with reagents (Fig. 2). However, nonantagonistic mutants did not show brown spot.

Biological activity of a complemented mutant. When the paper discs containing Phl of complemented mutants were placed on medium of *Agrobacterium tumefaciens* and *Pseudomonas solanacearum*, inhibition zones were observed (Table 1). But mu-

tant did not show an activity against *A. tumefaciens* and *P. solanacearum*. Especially complemented *P. fluorescens* against *P. solanacearum* showed greater antibacterial activity than it did against *A. tumefaciens*. A complemented mutant of *P. fluorescens* showed an antifungal activity against *C. gloeosporioides*,
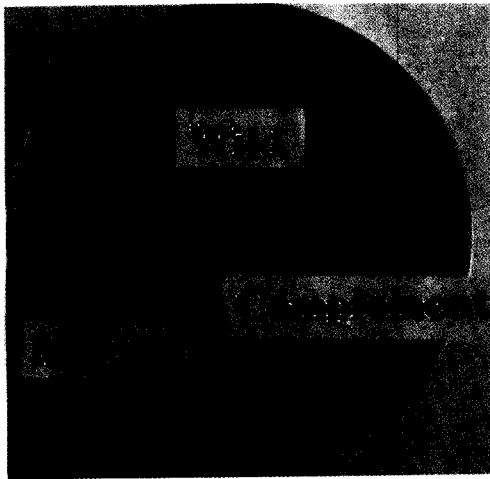

Fig. 1. Restoration of glucose utilization in a complemented mutant (C1) of *P. fluorescens* on PDA (pH 7.0) plus 0.015% bromothymol blue.
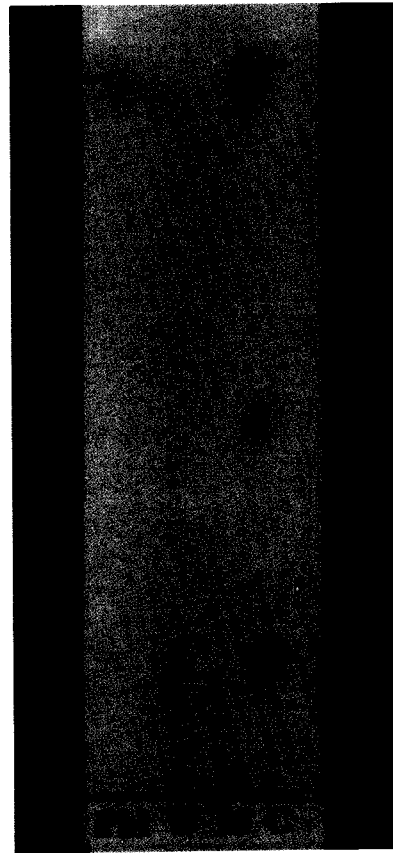

Fig. 2. Thin layer chromatogram of antibiotics produced by *P. fluorescens*. Phl; 2,4-diacetylphloroglucinol, W; Wild type, M; Mutant, C; Complemented mutant.

Table 1. Inhibitory effect of complemented *Pseudomonas fluorescens* against microorganisms

Microorganisms	Control	Wild type (*P. fluorescens*)	Mutant	Complemented mutant (C1)
Agrobacterium tumefaciens	−	+	−	+
Pseudomonas solanacearum	−	+	−	+
Colletotricum gloeosporioides	−	+	−	+
Fusarium oxysporum f. sp. *cucumerinum*	−	+	−	+
Rhizoctonia solani	−	+	−	+
Phytophthora capsici	−	+	−	+
Pythium spp.	−	+	−	+

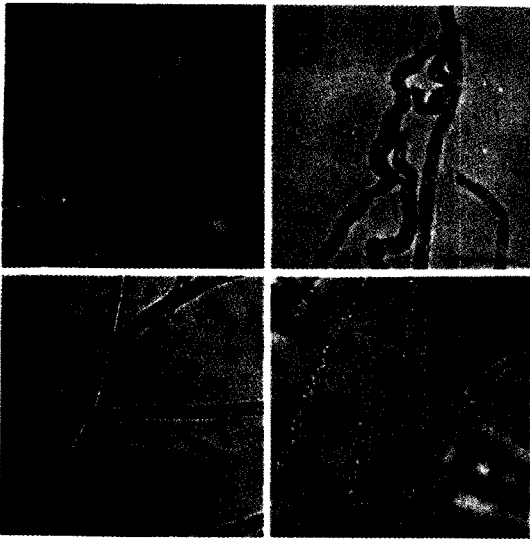− : No inhibition zone, + : inhibition zone, Control : ethylacetate or L broth.

Fig. 3. Fungal morphological abnormalities induced by *P. fluorescens.* A: Normal mycelia of *Pythium* spp., B: Abnormal mycelia induced by wild type of *P. fluorescens,* C: Normal mycelia in the presence of a mutant of *P. fluorescens,* D: Abnormal mycelia induced by a complemented mutant of *P. fluorescens.* The scale bars represent 50 μm (A, D) and 25 μm (B, C), respectively.

Fig. 4. Digestion of complemented plasmid (C1-C3) with *Eco*RI. Electrophoresis was carried out for 4 hrs at 30 V on 0.8% agarose gel. Lane 1 : λDNA *Hind*III, size marker; Lanes 2, 4 and 6 : Undigested DNA; Lanes 3, 5 and 7 : Digested DNA; Lanes 2 and 3 : C1; Lanes 4 and 5 : C2; Lanes 6 and 7 : C3.

F. oxysporum f. sp. *cucumerinum, R. solani, P. capsici* and *Pythium* spp. While complemented mutants of *P. fluorescens* showed inhibition zones as wild type, a nonantagonistic mutant did not exhibit inhibitory effect.

Morphological abnormalities of *Pythium* spp. induced by a complemented mutant. The effect of Phl in complemented mutants suppressed myceilal growth of *Pythium* spp. The agar blocks were observed under microscope and the antifungal substance produced by complemented mutants were found to cause a hyphal curling and swelling (Fig. 3D). This effect was similar to wild type (Fig. 3B), but nonantagonistic mutants did not show a hyphal swelling (Fig. 3C). Assay plate without Phl showed normal mycelial growth of *Pythium* spp. (Fig. 3A).

Complementation and transformation. Complementation by triparental mating was accomplished with frequency of 8.37×10^{-4}. All 11,011 transconjugants containing genomic DNA were screened. Four complemented mutants, C1, C2, C3 and C4 were rifampicin, kanamycin and tetracycline resistant and restored fungal inhibition activity fully. Such mutants were believed to be complemented to the level
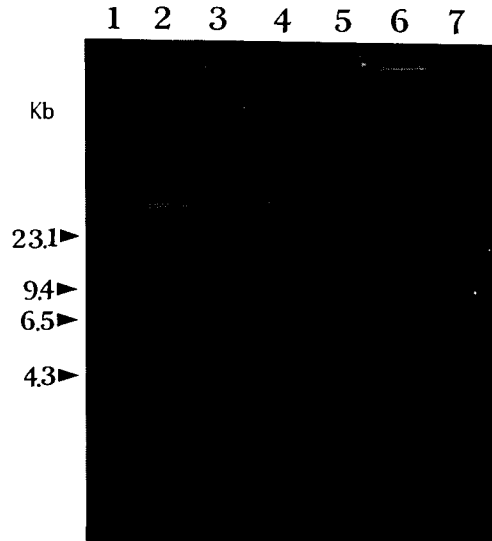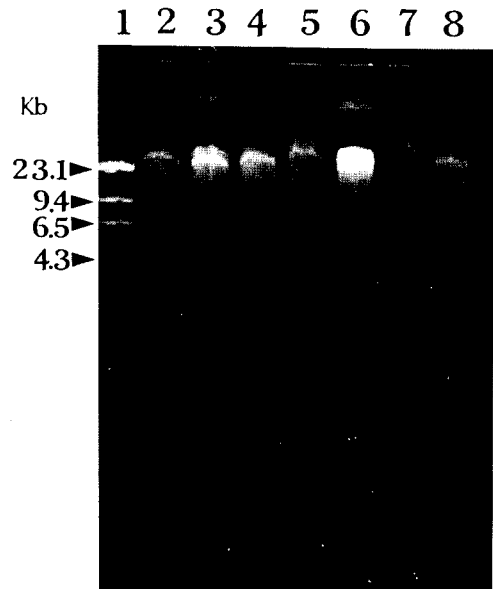

Fig. 5. Profiles of transformed plasmid DNA. Electrophoresis was carried out for 4 hrs at 30 V on 0.8% agarose gel. Lane 1 : λDNA *Hind*III, size marker; Lane 2, 3, 4, 5, 6 and 7 : Transformed plasmid DNA.

of wild type. Cosmid clones were isolated and were digested with *Eco*RI (Fig. 4). The average size of co-

smid clones were about 8 Kb. It will relate to producing antibiotics and inhibitory effects of antagonists. The cosmid clone from that transconjugant was isolated and transferred into *E. coli* DH5α by transformation. The transfomants were tetracycline resistant and the plasmid DNA was reisolated (Fig. 5).

DISCUSSION

P. fluorescens protects pepper seedlings from pepper blight caused by *Phytophthora capsici* (10). However, *Rhizoctonia solani* was used to test the inhibitory effect of antifungal substance in disease protection in this study, because of its faster growth rate than any other fungi.

The broad toxic activity of Phl is well documented. The mode of action of Phl is unclear and little is known about the genetics of fungal inhibition by pseudomonads, however (4). This study showed that *P. fluorecens* was amenable to genetic analysis and to study the synthesis of antifungal substance at the molecular level.

Molecular evidence for a key role of antibiosis in disease suppression has also come from rescent studies on Tn5 mutants of *P. fluorescens* and *P. aureofaciens*, which are deficient in the synthesis of a phenazine antibiotics. These mutants exhibited significantly less protection against take-all disease of wheat than do their parental strains (13). Complementation of the mutants with cloned homologous sequences from a library of wild-type DNA coordinately restored antibiotic synthesis, fungal inhibition and control of pepper-blight on pepper seedlings to wild-type levels (data not shown).

P. fluorescens does not contain significant levels of the Entner-Doudoroff (ED) pathway enzymes, 6-phosphogluconate (6-PG) dehydratase and 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase when grown in the absence of either glucose or gluconate. ED pathway enzymes in *P. fluorescens* are induced by gluconate and that such induction was occured in the presence of glucose because of the metabolic production of inducer.

In this study, TLC and PDA supplemented with bromothymol blue analysis showed the restoration of glucose utilization in complemented mutant. It will suppose that one or both of the genetic loci in the mutants could also regulate Phl production either directly or by modulating the activity of peripheral pathways that affect antibiotic synthesis. Cloning of genes involved in antibiotic synthesis has opened the possibility of designing superior biocontrol agents by incorporating desirable traits from several strains into a single strain (14). Cosmid clones of complemented mutants were isolated and digested with *Eco*RI. The average sizes were about 8 Kb and were suggested to link to antibiotic production with fungal disease suppression. For the stable acquisition of genes, transfomation was performed. In addition, the cloned gene will be avaiable to explore regulation of gene expression and sequencing in *P. fluorescens*.

요   약

항생물질 2,4-diacetylphloroglucinol(Phl)을 생성하는 *Pseudomonas fluorescens*는 식물병원성 세균과 진균의 생육을 억제하여 식물의 생장을 촉진한다. Helper plasmid인 pRK2013을 이용하여 triparental mating 방법으로 Phl을 생성하지 못하는 돌연변이체 내로 cosmid(genomic library)를 전이시켰으며 그 빈도는 8.37×10^{-4}이었다. 길항능력을 회복한 complemented mutant들은 약 2,000개의 transconjugant 로부터 bioassay 방법을 이용하여 선발되었다. 이러한 complemented mutant들은 kanamycin과 tetracycline에 저항성을 나타내었다. 모균주와 complemented mutant의 항생물질은 식물병원 세균에 대해 우수한 항세균 활성을 나타내었으며 식물병원성 진균에 대한 저해효과는 모균주와 complemented mutant 모두 동일하였다. 또한 모균주와 complemented mutant은 진균 균사의 비정상적인 성장을 유도하였다. Complemented mutant의 glucose 이용 능력의 회복은 bromothymol blue가 첨가된 PDA 배지에서 확인되었으며 이용 능력은 모균주와 같은 수준으로 회복되었다. Complemented mutant의 plasmid를 분리한 다음 *E. coli* DH5α로 형질전환 시켰다. 형질전환된 *E. coli* DH5α로부터 plasmid DNA를 재분리할 수 있었다. 분리된 plasmid는 *P. fluorescens*에서 Phl 생합성에 관련된 주요한 유전인자임을 알 수 있었다.

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