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Identification of Fluorescent Pseudomonads Producing Siderophore and Construction of Siderophore Biosynthesis Defective Mutant

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The present study was performed to isolate the fluorescent pseudomonads from Kwang-Ju soil and to construct a mutant strain defective in siderophore biosynthesis. The siderophore-secreting pseudomonads were screened on Blue agar (Chrome Azuol S agar) plates and one strain of them was designated to *Pseudomonas fluorescens (P. fluorescens)* PY002. To construct a mutant defective in siderophore biosynthesis, *P. fluorescens* PY002 was randomly mutagenized with a transposon Tn5. The location of Tn5 integrated into chromosomal of the mutants strain was determined by Southern blot analysis. The mutagenized strain showed non-fluorescent on a King's B agar plate and were defective in iron (III) acquisition ability.

KEY WORDS □ **Siderophore, CAS medium Tn5, Southern hybridization**

Siderophores are low-molecular-weight iron (III) chelators which are secreted in response to iron limitation and which facilitate iron uptake in conjunction with specific transport systems (3, 7). Bacterial grwoth, in turn, depends on the availability of iron, and essential nutrient that participates in many biological process, inculding electron transport system, and that is a cofactor of enzymes of intermediary metabolism (12, 14). Treatment of e.g., seed tubers with isolates of rhizosphere-colonizing *Pseudomonas putida* and *Pseudomonas fluorescens* resulted in stimulation of plant growth and increased in crop yields (4). *Pseudomonas* spp. are of considerable impotrance in agriculture (9, 10). Two important siderophore-mediated iron uptake systems have been found in these bacteria : one is the fluorescent siderphore (pyoverdin) (11, 12, 15) and the other is the sierophore pyochelin.

In this study, we isolated *Pseudomonas fluorescens* PY002 (*P. fluorescens*) from cropping rhizosphere soil in Kwangju city and its suburbs. The strain isolated was mutagenized with transposon Tn5 and its insertion site was identified by Southern blotting.

MATERIALS AND METHODS

Bacterials strain and plasmids

The bacterial strains and plasmids used in this study are summarized in Table 1.

Media and growth condition

The basal medium for screening or fluorescent pseudomonads was used King's B (KB) medium (6) and cetrimide agar. Blue agar (CAS agar) was used for the siderophore detection (16). *E. coli* strains harboring Tn5 were grown in LB medium containing kanamycin (50 μg/ml) at 37℃. All antibiotics were purchased from Sigma Co. and used in the following concentrations : kanamycin (50 μg/ml), chloramphenicol (50 μg/ml), ampicillin (50 μg/ml) and tetracycline (50 μg/ml).

Identification of the *Pseudomonas fluorescens* PY002

P. fluorescens PY002 was isolated as described by Macfaddin (8), Starr *et al.* (18) and Butler (2).

Detection of siderophore

A method to detect the siderophore was according to the procedure of Schwyn and Neilands (16). Detection of siderophores is base on their affinity for iron (III). The following chemical equation explains the principle : $FeDye^{3}$ $^{a}+L^{b-}\rightarrow FeL^{3-b}+Dye^{a-}$ (1, 19). A strong ligand L (e.g., a siderophore) is added to a highly colored iron dye complex. When the iron ligand complex is formed the release of the free dye is accompained by a color change.

Tn5 mutagenesis

Tn5-mediated mutagenesis were carried out as described by Simon *et al.* with slightly modification (17). The *E. coli* cells harboring Tn5

as the donor and the *P. fluorescens* PY002 as recipient were grown to late-log phase (1×10^9/ml), respectively and mixed in a test tube (ratio 1 : 1). The mating mixture was spread onto a Millipore filter on a prewarmed LB agar plate. After incubation for 3 to 4 hrs at 30℃, cells were suspended in 1 ml of 0.1 M phosphate buffer, pH 7.0, and then 100 μg/ml, chloramphenicol (50 μg/ml), and $FeCl_3$ (100 μM). Colonies appeared on the plates after incubation for 48 hrs at 30℃. Colonies were then screened for nonfluorescence, for their inability of growth under iron limitation, and for autotrophy by replica plating on KB medium, KB medium with 2,2-bipyridyl, and minimal medium, respectively. The plates were incubated overnight at 30℃.

Preparation of genomic DNA

Genomic DNA from wild type and mutants were isolated from stationary culture by Murry and Thompson's method (13).

Southern blot analysis

Southern blot hybridization was carried out as described in "Nonradioactive DIG-DNA Labeling and Detection Kit manual" (Boehringer Mannheim). Genomic DNA was digested with EcoRI endonuclease and separated by electrophoresis on 0.8% agarose gel in Tris-borate buffer, pH 8.0. After transfer to Nytran membrane (Schleicher and Schnull), the blots were hybridized with pSUP5011 (pBR325::Tn5) DNA which was labeled with DIG-dUTP.

RESULTS AND DISCUSSION

Identification of *P. fluorescens* PY002

The isolated *P. fluorescens* PY002 was negative in gram staining, rod shaped, oxidase positive, catalase positive, nonfermentative, and motile by flagella. The strain isolated reduced nitrate to nitrite and did not hydrolyze starch : it produced

Table 1. *Bacterial strain and plasmids used.*

Strain and plasmids	Relevant characteristics of genotypes
P. fluorescens PY0002	Wild type
Plasmid	
pBEE 132	pRK2013::Tn5-132(tet) Km^r
pSUP 5011	pBR325 Tet^s::Tn5-mob Ap^r Cm^r Km^r
pSUP 10141	pACYC mob::Tn5+tet from RP4 Cm^r Tet^r Km^r
pRZ 102	ColE1::Tn5 Km^r
pRK 2013	Km^r Mob$^+$ Tra$^+$

*Abbreviations : Ap, ampicillin; Cm, chloramphenicol; Tra$^+$, self transmissible; Mob$^+$, mobilizable.

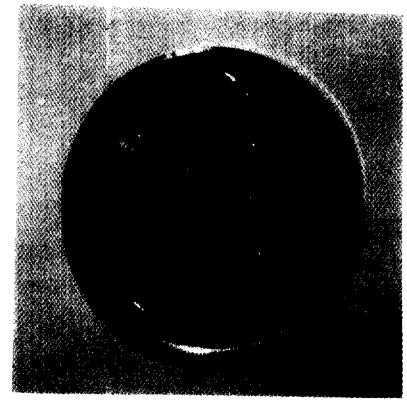

Fig. 1. *Screening of siderophore-producing P. fluorescens on the Blue agar.*

Table 2. *Biochemical properties of P. fluorescens PY002.*

Items	PY002	Items	PY002	Items	PY002
Fluorescent:		Hydrolysis:		Utilization of:	
King's A medium	−	Starch	−	Aer-Gluc[c]	+
King's B medium	+	Gelatin	+	Sucrose	−
Motility	+	Voges-Proskauer	−	Mannitol	−
Shape	rod	Citrate	+	Ana-Gluc[d]	−
Gram staining	−	Indole test	−	Lactose	−
Catalase	+	H_2S	−	Inositol	−
Arginine dehydrolase	+	ONPG[a]	−	Rhamnose	−
Tryptophane deaminase	−	Urease	+	Arabinose	+
Ornithine decarboxylase	−	Oxy. Ferm test	O[b]	Amygdalin	−
Lysine decarboxylase	+	Denitrification	+	Sorbitol	−
Oxidase	+			Melibiose	+
				Xylose	+

[a]Ortho nitro phenyl-β-D-galactoside; [b]oxidation; [c]aerobic glucose; [d]anaerobic glucose.

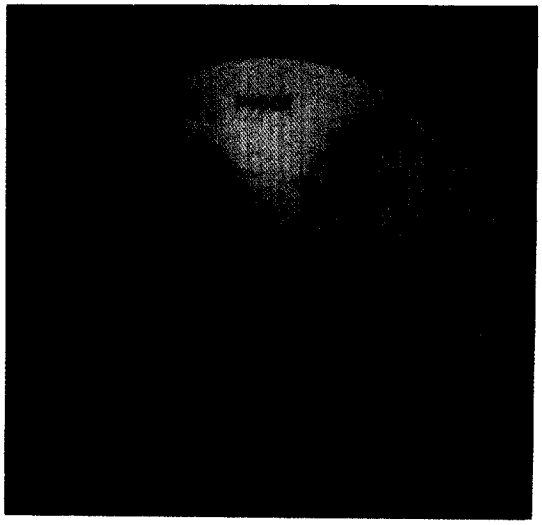

Fig. 2. *Screening of fluorescent siderophore-defective mutants of siderophore production.*
PY002, Wild type (*P. fluorescens*) : MP111, 113, 114, 115 and 116, The Tn5-mutagenized mutant strains.

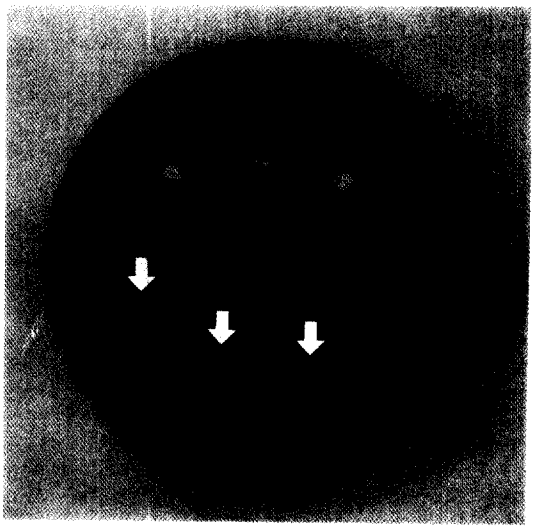

Fig. 3. *Screening of siderophore biosynthesis-defective mutants.*
Arrow indicates mutants.

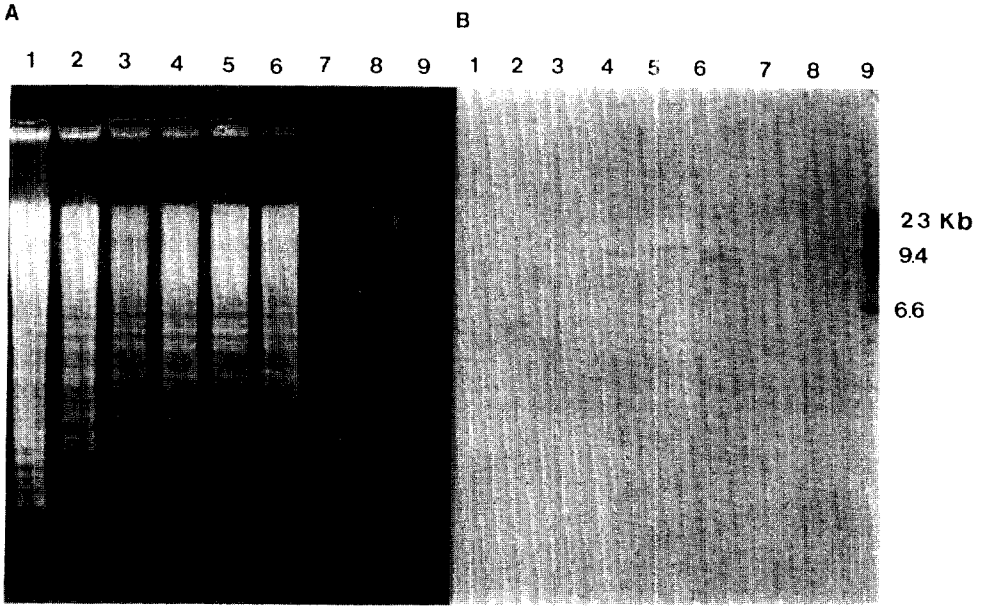

Fig. 4. *Identification of Tn5 sequence integrated into chromosomal DNA of Tn5-induced mutants by Southern blot analysis.*
A. Chromosomal DNA from Tn5-induced mutant, which was digested with EcoRI endonuclease and then separated by electrophoresis on 0.8% agarose gel. Lane 1 : *P. fluorescens* PY002 (Wild type). Lane 2-8 : Constructed mutants. Lane 9 : λ/HindIII markers; B. Southern blot hybridization. The fractionated DNA on the agarose gel was bloted onto Nytan filter and probed with DIG-dUTP-labeled pSUP5011 DNA.

yellow green pigment and hydrolyzed gelatin. The following additional tests or reactions were positive : Aer-glucose, xylose, melibiose, arabinose. The following tests or reactions were negative : H_2S production, indole. Voges-Proskauer, sucrose, mannitol, sorbitol, amygdalin, inositol, rhamnose. The overall biochemical properties were summarized in Table 2.

Detection of siderophores

Fig. 1 shows that the *P. flurescens* PY002 produced the siderophore on Blue agar (CAS agar) plate (16). The 10 μM Fe(III) tints the agar with blue color, while the concentration of siderophores excreted by iron-starved microorganism generally exceeds control level, by which the color was completly changed from blue to orange.

Mutants defective in siderophore biosynthesis

Transposon Tn5 was introduced into *P. fluorescens* PY002 with the RP4-derived mobilization system developed by Simon *et al.* (17). In the prescence of recipient cells, Tn5 mobilized in the chromosomal DNA. Since the vector is unable to replicate in *P. fluorescens* PY 002, recipient cells that have acquired the Tn5-associated antibiotic resistance should harbor Tn5 which had been integrated in the host chromosome. The Tn5 mutagenesis resulted in mutant colony bank of *P. fluorescens* PY002 with kanamycin-resistant transconjugants. The mutants defective in biosynthesis of yellow-green fluorecent siderophore were isolated from the mutagenized colony bank on a Blue agar plate (Fig. 2). The mutants constructed grew much more slowly than the wild type strain on an iron-starved plate (Fig. 3).

Detection of Tn5 insertion region by Southern hybridization

Southern blot hybridization was performed to verify the Tn5 insertion of transconjugants. Chromosomal DNA isolated from transconjugants was digested with EcoRI endonuclease and fractionated on 0.8% agarose gel. The DNA fragments were transferred to Nytran filter and hybridized with DIG-dUTP-labeled pSUP5011 containing the kanamycin resistance gene of Tn5. The labeled probe strongly hybridized with the fractionated DNA (Fig. 4). This result shows that the Tn5 transposon is integrated into chromosomal DNA of the constructed mutants and that the kanamycin-resistancy mutants are due to the integrated transposons.

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초 록: Siderophore를 생성하는 Fluorescent Pseudomonads의 분리, 동정 및 돌연변이 유기
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광주근교 지역의 근권 토양으로부터 cetrimide agar medium을 이용하여 형광성 pseudomonads를 분리하였고, CAS medium에서 siderophore의 생성능력이 우수한 pseudomonads만을 분리하여 생리화학적인 실험을 수행하였다. Kanamycin-sensitive pseudomonads를 Tn5를 이용한 mutagenesis를 실시하여 kanamycin에 내성을 갖는 transconjugants를 선별하였고, siderophore 생합성을 하지 못하는 돌연변이주를 선별하기 위하여 CAS medium에서 yellow hallow를 형성하지 못하거나 King's B medium에서 형광성을 나타내지 못하는 colony를 선별하였다. 선별된 mutants들의 genomic DNA에 Tn5가 삽입되었는지를 확인하기 위하여 Southern blot hybridization을 실시한 결과 intact Tn5에 homology를 나타내는 하나의 single band를 확인하였다.