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Identification of Genes for Biosynthesis of Antibacterial Compound from *Pseudomonas fluorescens* B16, and Its Activity Against *Ralstonia solanacearum*

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Abstract *Pseudomonas fluorescens* B16 is a plant growth-promoting rhizobacterium, which produces an antibacterial compound that is effective against plant root pathogens, such as *Agrobacterium tumefaciens* and *Ralstonia solanacearum*. We mutagenized the strain B16 with Omegon-Km and isolated six antibacterial-activity-deficient mutants. Two cosmid clones that hybridized with the mutant clones also were isolated from a genomic library of the parent strain. Using deletion and complementation analyses, it was found that the biosynthesis genes resided in a 4.3-kb *Sal*I-*Nar*I fragment. When a plasmid clone carrying the fragment was introduced into *P. fluorescens* strain 1855.344, which does not exhibit any antibacterial activity, the transconjugants exhibited antibacterial activity, indicating that the plasmid clone carried all the genes essential for production of the antibacterial compound. DNA sequence analysis of the fragment identified four putative open reading frames (ORFs): *orf1* through *orf4*. The deduced amino acid sequences of ORF1, ORF2, and ORF4 were similar to cystathionine gamma lyase, pyruvate formate-lyase activating enzyme, and transcriptional regulator, respectively, yet the amino acid sequence of ORF3 showed no similarities to any known proteins. It was also demonstrated that the antibacterial activity was responsible for biological control of the bacterial wilt caused by *R. solanacearum*.

Key words: *P. fluorescens*, biological control, antibacterial activity

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most destructive bacterial plant diseases. It causes significant damage to potatoes and tomatoes under favorable weather conditions, and affects economically important crops such as banana, eggplant, peanut, and tobacco, worldwide [10, 29]. Many agricultural chemicals have been used to prevent the disease, but their residues in the soil cause environmental problems. Therefore, alternative biological means have been employed to prevent bacterial wilt [1]. In general, biological controls using antagonistic microorganisms are safer and have longer-lasting effects than agricultural chemicals [1]. One of the most important criteria for choosing antagonistic microbial strains is the persistence of the organisms in the root systems of host plants [28].

We previously isolated *Pseudomonas fluorescens* strain B16 from the roots of graminaceous plants and found that the bacterium effectively colonizes the roots of various plants well [12, 15]. The strain also grows at a relatively wide range of temperatures, from 5 to 30°C. As such, these characteristics suggest that the strain would be a promising biological control agent, if it has any antimicrobial activity against important plant pathogens [12, 14, 20]. However, no detectable antifungal activity of the strain B16 has been found *in vitro* [15]. Conversely, the strain B16 promotes the growth of tomatoes, hot peppers, and barley in greenhouses and the field [17, 22]. Although the mechanism for growth promotion is unknown, it raises a question of whether the production of any antimicrobial compound by the strain B16 will affect plant growth.

Fluorescent pseudomonads produce a variety of antimicrobial compounds, and some of them are involved in the biological control of plant diseases [3, 13, 21, 27]. To show that an antimicrobial compound plays a primary role in biological control, one must provide genetic evidence or demonstrate the effects of the chemical compound. Recently, it was found that strain B16 inhibits the growth of bacterial plant pathogens, such as *Agrobacterium tumefaciens*, *R. solanacearum*, and other bacteria that colonize plant roots. Accordingly, this led to the identification

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of the genes responsible for the production of the antibacterial compound. In the present study, evidence of a four-gene cluster essential for the biosynthesis of the compound is presented, and its antibacterial activity to suppress bacterial wilt has been demonstrated.

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 the strains were cultured in Luria-Bertani (LB) medium. The *P. fluorescens* strain B16 and *Escherichia coli* were cultured at 28 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 antibiotic production assay was performed on *Agrobacterium* minimal (AB) medium [5], which contained per liter: 1.0 g NaH_2PO_4, 3.0 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.15 g KCl, 1.0 g NH_4Cl, 0.01 g $CaCl_2 \cdot 2H_2O$, and 2.5 mg $FeSO_4 \cdot 7H_2O$. Glucose was autoclaved separately and added to the AB medium at a 0.2% final concentration.

Table 1. Bacterial strains and plasmids.

Strain/plasmid	Characteristics[a]	Source/ reference
Escherichia coli		
DH5α	F^- Φ80d*lac*ZΔM15 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^- *mcr*B *mrr hsd*S20($r_B^- m_B^-$) *rec*A13 *leu are*-14 *pro*A2 *lac*Y1 *gal*K2 *xyl*-5 *mtl*-1 *rps*L20(Sm^r) *sup*E44λ^-	Gibco BRL
Pseudomonas fluorescens B16	wild-type, Rif^r	[12]
P. fluorescens 1855.344	wild-type, Rif^r	[6]
P. fluorescens B16		
K2-31	B16::Omegon-Km, antibacterial activity-enhanced mutant	This study
K4	B16::Omegon-Km, antibacterial activity-deficient mutant	This study
K5	B16::Omegon-Km, antibacterial activity-deficient mutant	This study
K6	B16::Omegon-Km, antibacterial activity-deficient mutant	This study
K7	B16::Omegon-Km, antibacterial activity-deficient mutant	This study
K14	B16::Omegon-Km, antibacterial activity-deficient mutant	This study
K18	B16::Omegon-Km, antibacterial activity-deficient mutant	This study
JWB100	B16 *orf1*::Tn*3-gus*	This study
JWB66	B16 *orf2*::Tn*3-gus*	This study
JWB118	B16 *orf2*::Tn*3-gus*	This study
JWB299	B16 *orf3*::Tn*3-gus*	This study
JWB354	B16 *orf3*::Tn*3-gus*	This study
JWB388	B16 *orf4*::Tn*3-gus*	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	[8]
pHoKmGus	promoterless β-glucuronidase gene, Km^r, Amp^r, *tnpA*	[4]
pSShe	Cm^r	[25]
pOK5	7.0-kb self-ligated *Eco*RI clone from K5	This study
pJWB1	20.6-kb DNA fragment from strain B16 cloned into pLAFR3	This study
pBO3	24.3-kb DNA fragment from strain B16 cloned into pLAFR3	This study
pBO5	27.8-kb DNA fragment from strain B16 cloned into pLAFR3	This study
pJWB4	9.3-kb *Pst*I fragment deleted from pJWB1	This study
pJWB39	4.3-kb *Sal*I-*Nar*I fragment cloned into pBluescript II SK(+)	This study
pJWB40	4.3-kb *Bam*HI-*Pst*I fragment from pJWB39 cloned into pLAFR3	This study
pJWB41	0.4-kb *Bam*HI-*Psh*AI fragment deleted from pJWB39	This study
pJWB42	3.9-kb *Bam*HI-*Pst*I fragment from pJWB41 cloned into pLAFR3	This study
pJWB44	0.75-kb *Eco*RI-*Nru*I fragment from pJWB40 cloned into pLAFR3	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.

Recombinant DNA Techniques

Chromosomal DNA from *P. fluorescens* strain B16 was isolated using the method of Sambrook *et al.* [23]. Isolation of small-scale plasmid DNA from *E. coli* was performed using QIAprep® Spin (Qiagen, Inc., Germany) according to the supplier's instructions, or by the alkaline lysis method [23]. Small-scale cosmid DNA was isolated from *E. coli* and *P. fluorescens* using the alkaline lysis method. Large-scale plasmid and cosmid DNA preparations were performed by alkaline lysis followed by cesium chloride density gradient centrifugation [23]. The restriction enzyme digestions were performed as recommended by the suppliers (Takara, Japan). The gel electrophoresis was performed in agarose gels (0.7% [wt/vol]), and Southern transfers were performed on Hybond-N™ nylon membranes (Amersham Biosciences, Uppsala, Sweden) as described by the manufacturer. For colony hybridization, all the procedures for preparing the probe DNA and hybridization were as described by the manufacturer. To construct a genomic library of strain B16, a 20 to 30 kb DNA insert was prepared by partial digestion of the total genomic DNA with *Sau*3AI, and the fragments ligated into the *Bam*HI site of pLAFR3. The ligated DNA was packaged into bacteriophage λ, as described by the manufacturer (Promega, Madison, U.S.A.), then transfected into *E. coli* HB101. All the pLAFR3 derivatives were mobilized into *P. fluorescens* B16 strains by triparental mating [8]. All the other basic molecular techniques were employed according to Sambrook *et al.* [23].

Transposon Mutagenesis

The suicide plasmid pJFF350 [7] was used to generate transposon insertions in the chromosome of the strain B16. The transconjugants were tested for their ability to produce antibacterial compounds. Since Omegon-Km carries an origin of replication and no *Eco*RI site, 1 µg of the total genomic DNA of the mutants was digested with *Eco*RI, self-ligated, and transformed into *E. coli* DH5α. Colonies carrying the flanking DNA fragments were isolated by selection on LB agar medium containing kanamycin. The flanking region was sequenced with the primer HR (5'-TGCTTCAATCAATCACCGG-3'). pJWB4 carrying all the essential biosynthesis genes of the antibacterial compound was mutagenized with Tn*3-gus*, as described by Bonas *et al.* [4]. The insertion site and orientation of Tn*3-gus* in each mutant were mapped by restriction enzyme digestion analysis and direct sequencing of the plasmid using the primer Tn3gus (5'-CCGGTCATCTGA-GACCATTAAAAGA-3'), which allows sequencing out of the Tn*3-gus*. The mutagenized plasmids that carried Tn*3-gus* insertions were introduced individually into the parent strain B16 by conjugation and marker-exchanged into strain B16, as described previously [4]. All marker-exchanges were confirmed by Southern hybridization analysis.

β-Glucuronidase Assay

The β-glucuronidase enzyme assay was performed as described previously, with some modifications [11]. The strains of *P. fluorescens* B16 were grown in an AB medium, centrifuged, resuspended in a GUS extraction buffer, and lysed by sonication with a VCX-400 sonicator (Sonics & Materials Inc., CT, U.S.A.). The extract was used in the β-glucuronidase enzyme assay with 4-methylumbelliferyl glucuronide as the substrate. The fluorescence was measured at 365-nm excitation and 460-nm emission in a TKO100 fluorometer (Hoefer Scientific Instruments, San Francisco, U.S.A.). One unit of β-glucuronidase was defined as 1 nmol of 4-methylumbelliferon released per bacterium per minute.

DNA Sequencing and Data Analysis

The DNA inserted in pJWB1 was digested with the appropriate restriction enzymes and subcloned into pBluescript II SK(+) before sequencing. Universal and reverse primers were used for the primary reactions, then synthetic primers were used to sequence both strands. All DNA sequencing reactions used to identify Omegon-Km insertion sites were carried out using the primer HR. The DNA sequence data were analyzed using the BLAST program at the National Center for Biotechnology Institute [9], MEGALIGN software (DNASTAR, U.S.A.), and GENETYX-WIN software (Software Development Inc., Japan).

Bioassay for Antibiotic Production

To 50 ml of AB containing 0.6 g of melted agar maintained at 45°C, 500 µl of an overnight culture of *R. solanacearum* were added. The culture was mixed thoroughly and immediately poured into culture plates. After the agar solidified, the strains to be tested for antibiotic production were spotted directly onto the surface of the plate. The plates were incubated at 28°C for 16 h to detect the inhibition zones.

Biological Control Assays

Hot peppers (*Capsicum annuum* L., cv. Nokkwang) were grown until the 4- or 5-leaf stage in a 50-cell plug tray filled with commercial peat mix soil (Baroco, Seoul Agriculture Materials Co., Seoul, Korea). The root systems of the seedlings were dipped in bacterial suspensions for bacterization, then transferred into plastic pots (15 cm in diameter) and kept in a greenhouse. After 7-days of incubation, the plants were inoculated with *R. solanacearum* which was grown on CPG (1 g hydrolysate, 10 g peptone, 5 g glucose, and 15 g agar/liter) agar medium for 2 days. The concentration of the cell suspension was adjusted to approximately 10^8 cells/ml using a spectrophotometer (Ultraspec 4000, Pharmacia Biotech, Cambridge, England), then 10 ml of the bacterial suspension was poured into each plastic pot with the transplanted hot pepper seedlings.

The diseased plants were recorded 10 days after inoculation. The root-colonizing ability was measured as described previously using the double layered-filter paper (DLF) method [2].

RESULTS

Isolation and Characterization of Antibacterial Activity-Deficient Mutants

After the mutagenesis of *P. fluorescens* B16 with Omegon-Km, two thousand prototrophic colonies were isolated and tested for antibiosis against *R. solanacearum* on an AB agar medium. Six antibacterial activity-deficient mutants (K4, K5, K6, K7, K14, and K18) and one mutant (K2-31) with enhanced antibacterial activity were isolated (Fig. 1).

To determine the insertion site of Omegon-Km in the mutants, the flanking DNA regions of the transposition element were cloned and sequenced. From BLAST search analyses with the limited DNA sequence data, mutants K4 and K7 exhibited a mutation in a gene homologous to the cystathionine gamma lyase (36% identity and 54% positives) of *Aeropyrum pernix*; mutants K5, K6, and K18 exhibited insertional mutations in a gene homologous to the pyruvate formate-lyase activating enzyme (39% identity and 60% positives) of *Thermotoga maritima*; mutant K14 exhibited a mutation in a hypothetical gene; and mutant K2-31 exhibited a mutation in a gene homologous to the nitrate/nitrite regulatory protein (36% identity and 59% positives) of *P. aeruginosa* (Table 2).

Isolation of Cosmid Clones Carrying Genes Involved in Antibiotic Biosynthesis

To isolate cosmid clones carrying the biosynthesis genes, the 3.2-kb *Hin*dIII fragment of pOK5, a rescued plasmid from mutant K5, was used as a probe in colony hybridization. Two overlapping clones were isolated: pJWB1 and pB03 (Fig. 2A). To determine whether these two clones contained essential genes responsible for the biosynthesis of the antibacterial compound, pB03 and pJWB1 were individually mobilized into *P. fluorescens* 1855.344, a heterologous host with no antibacterial activity. When the

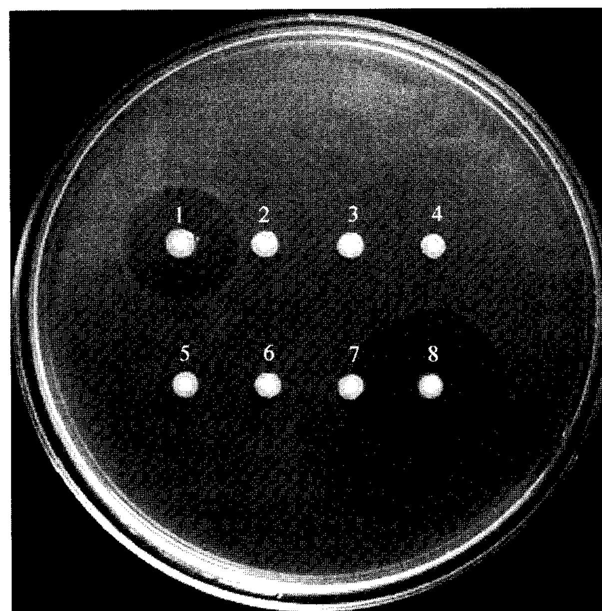

Fig. 1. Antibacterial activity of *P. fluorescens* B16 and its mutants against *R. solanacearum* on AB agar medium.
1, *P. fluorescens* B16; 2, K4; 3, K5; 4, K6; 5, K7; 6, K14; 7, K18; 8, K2-31.

transconjugants were tested for antibacterial activity against *R. solanacearum*, only pJWB1 conferred antibacterial activity to strain 1855.344 (Fig. 2A), indicating that pJWB1 contains genes that are essential for the biosynthesis of the antibacterial compound.

Complementation and DNA Sequence Analysis

When pB03 and pJWB1 were mobilized into K4, K5, K6, K7, K14, and K18, production of the antibacterial compound was recovered. pJWB1 complemented JWB100, JWB118, JWB354, and JWB388, while pB03 complemented JWB100, JWB118, and JWB354, yet not JWB388 (data not shown). The restriction map analysis showed that pJWB1 and pB03 overlapped over a 16.9-kb region, while an approximately 3.5-kb region that existed at the right end of pJWB1 was not present in pB03 (Fig. 2A), suggesting that the 3.5-kb region is critical for production of the antibiotic compound. Therefore, the 9.0-kb *Pst*I-*Eco*RI

Table 2. Characteristics of antibacterial compound biosynthesis genes and their products.

Gene	Base range[a]	Size (bp)	%GC	Amino acids (n)	Similar[b] to:
orf1	176-1,321	1,146	48.79	381	Cystathionine gamma lyase from *Aeropyrum pernix* (36%)[c]
orf2	1,318-2,802	1,485	47.75	494	Pyruvate formate-lyase activating enzyme from *Thermotoga maritime* (39%)
orf3	2,903-3,622	720	43.40	239	Unknown protein
orf4	3,823-4,254	432	49.47	143	Transcriptional regulator from *Pseudomonas aeruginosa* (36%)

[a] Nucleotide range within the 4.3-kb *Sal*I-*Nar*I fragment.
[b] Gene product in database with highest amino acid similarity.
[c] Identity based on predicted amino acid sequence.

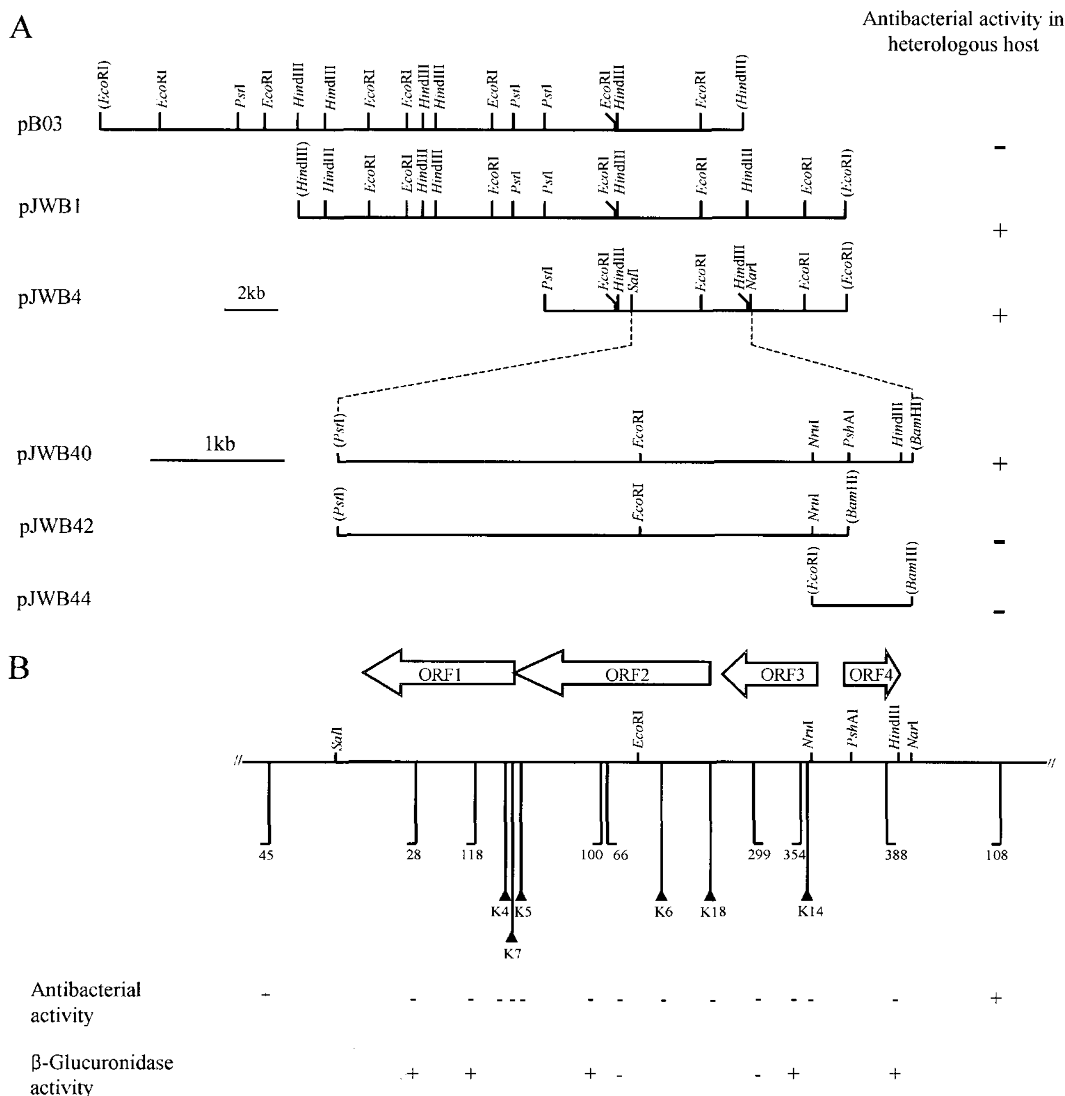

Fig. 2. Restriction enzyme maps of cosmids and subclones that exhibited antibacterial activity in a heterologous host, *P. fluorescens* 1855.344 (A). Genetic organization and restriction map of antibacterial compound biosynthesis gene locus of *P. fluorescens* B16, cloned in pJWB40 (B).

Open arrows indicate the positions and orientations of the antibacterial compound biosynthesis genes. Vertical bars in the map indicate the positions and orientations of the Tn*3*-*gus* insertions, and the major phenotypes of the mutants are represented below the restriction map. Vertical bars with arrowheads indicate the positions of the Omegon-Km insertions.

fragment encompassing the 3.5-kb fragment was subcloned into pBluescript II SK(+) for sequencing. There were four putative ORFs, ORF1 to 4, in the region (Fig. 2B). A computer analysis of the sequences revealed a set of three ORFs transcribed in the same direction from right to left (ORF1 to 3; Fig. 2B), while ORF4 was oriented in the opposite direction. ORF1 appeared to be translationally coupled with ORF2, overlapping by 4 bp. The predicted molecular masses of the ORFs 1-4 were 42.4, 55.9, 26.7, and 15.6 kDa, respectively.

A comparison of the deduced products of the ORFs with sequences in the databases did not reveal any similarities with proteins known to be involved in the synthesis of antimicrobial compounds. ORF1 and ORF2 showed the most significant similarities with cystathionine gamma lyase and a pyruvate formate-lyase activating enzyme, respectively (Table 2). The deduced product of ORF3 exhibited no significant similarity to any known proteins in the database, while ORF4 was similar to transcriptional regulators (36% identity) (Table 2). From the sequence analysis, it was found that pB03 had a truncated ORF4 missing 72 bp from the stop codon of *orf4* (Fig. 2A), suggesting that the putative transcriptional regulator plays an important role in producing the antibacterial compound in strain B16.

To prove the involvement of ORF4 in the production of the antibacterial compound, several clones were constructed

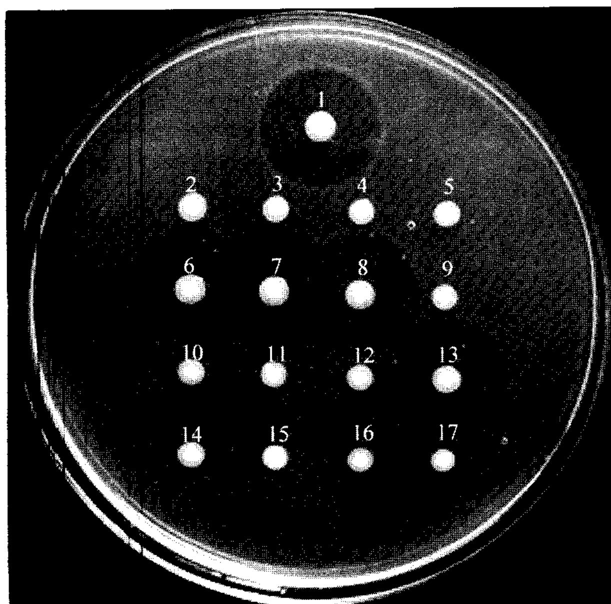

Fig. 3. Complementation of antibacterial activity-deficient mutants of *P. fluorescens* B16.
1, *P. fluorescens* B16; 2, JWB118; 3, JWB100; 4, JWB354; 5, JWB388; 6, JWB118(pJWB42); 7, JWB100(pJWB42); 8, JWB354(pJWB42); 9, JWB388(pJWB42); 10, JWB118(pJWB44); 11, JWB100(pJWB44); 12, JWB354(pJWB44); 13, JWB388(pJWB44); 14, JWB118(pLAFR3); 15, JWB100(pLAFR3); 16, JWB354(pLAFR3); 17, JWB388(pLAFR3).

(Fig. 2A). The 4.3-kb *Nar*I-*Sal*I fragment containing ORF1 to 4 was subcloned into pLAFR3 to generate pJWB40. pJWB42 carrying ORF1 to 3 was generated by deleting the 455-bp *Psh*AI-*Nar*I fragment from pJWB40 (Fig. 2A). The 748-bp *Nru*I-*Nar*I fragment of pJWB40 was subcloned into pLAFR3 to construct pJWB44, which only carried ORF4 (Table 1). These plasmids were then introduced into several antibacterial activity-deficient mutants, and the transconjugants were tested for antibacterial activity. pJWB40 restored antibacterial compound production in all the mutants, pJWB42 restored antibacterial activity in mutants JWB100, JWB118, and JWB354, yet not in JWB388, pJWB44 only restored the activity in mutant JWB388 (Fig. 3) and mutants JWB45 and JWB108 retained antibacterial activity (Fig. 2B). This indicates that the regions upstream from ORF1 and downstream from ORF4 are not important for production of the antibacterial compound. In addition, when pJWB40 was introduced into the heterologous host *P. fluorescens* 1855.344, the transconjugant exhibited antibacterial activity (Fig. 2A). Hence, all the genes essential for antibacterial compound biosynthesis in strain B16 lie within the 4.3-kb *Nar*I-*Sal*I fragment.

Expression of Genes Involved in Biosynthesis of Antibacterial Compound

To examine the expression of *orf1*-*orf4* from *P. fluorescens* B16, the expression levels of all transcriptional units, including the wild-type strain B16, were measured. All the ORFs were expressed better in AB medium than in LB medium (data not shown). The expression of *orf3* was the highest, while the expressions of *orf1*, *orf2*, and *orf4* were very low under the conditions used (Table 3). Strains with insertions oriented in the opposite direction did not exhibit any detectable β-glucuronidase activity on the same media (Fig. 2A). Together, our results indicate that all four ORFs from *P. fluorescens* B16 are expressed during the late growth stage after a stationary phase.

Table 3. β-Glucuronidase activity of *orf1/orf2/orf3/orf4*::Tn*3*-*gus* fusions in *P. fluorescens* B16 grown in AB minimal medium.

Strain	Tn*3*-*gus* insertion site	β-Glucuronidase activity (10^{-8} U/CFU)[a]		
		24 h[b]	60 h	120 h
B16	-	4.3	4.2	4.3
JWB118	*orf1*	4.2	4.6	31.3
JWB100	*orf2*	5.2	4.3	144.3
JWB354	*orf3*	107.2	351.6	662.2
JWB388	*orf4*	4.7	5.3	20.6

[a]One unit of β-glucuronidase was defined as one nmole of 4-methylumbelliferon released per bacterium per minute.
[b]The bacterial cells were harvested after incubation for 24, 60, or 120 h.

Sensitivity of Various Plant-Associated Bacteria to Antibiotic Substance

To determine the spectrum of antibiosis of strain B16, fourteen species or pathovars of plant pathogenic bacteria and biological control agents that are distantly related were tested (Table 4). Inhibition zones were formed on the lawns of *R. solanacearum*, *A. tumefaciens*, *P. syringae*, and *P. fluorescens* (Table 4). Within the same species or pathovar, sensitivity to the antibacterial substance was

Table 4. Antibiotic spectrum of antibacterial substance produced by *P. fluorescens* B16 and its mutants.

Strain	B16	K2-31	K5
Agrobacterium tumefaciens	++[a]	+++	-
Erwinia carotovora	-	-	-
E. chrysanthemi EC873	-	-	-
Escherichia coli	-	-	-
Paenibacillus polymyxa E681	-	-	-
Pseudomonas aeruginosa	-	-	-
P. aureofaciens 30-84	-	-	-
P. fluorescens 2-79	++	+++	-
P. fluorescens Cha94	++	+++	-
P. fluorescens 1855.344	-	-	-
P. putida PI	+	++	-
P. syringae Cit7	+	++	-
P. s. pv. *phaseolicola*	-	-	-
Ralstonia solanacearum	++	+++	-

[a]The degree of inhibition was divided by the range of the inhibition zone: +++: strong (>15 mm), ++: moderate (15-11 mm), +: weak (10-5 mm), -: no inhibition (<5 mm).

Fig. 4. Suppression of bacterial wilt by treatment with *P. fluorescens* B16.
1, Healthy control; 2, disease control; 3, *P. fluorescens* B16-treated plant; 4, antibacterial activity-deficient mutant K5-treated plant; 5, K5-carrying pB03-treated plant; 6, K5-carrying pLAFR3-treated plant. The photograph was taken 10 days after treatment.

dependent on the strain used. Other bacteria, such as *E. coli* and *P. aeruginosa*, were relatively insensitive to the antibiotic produced by strain B16 (Table 4).

Control of Bacterial Wilt in Hot Pepper

A question of whether the antibacterial activity of strain B16 is directly responsible for biological control activity was evaluated. Hot pepper seedlings were treated with the parental strain B16, antibacterial activity-deficient mutant K5, K5 carrying pB03, and K5 carrying pLAFR3, and the occurrence of bacterial wilt was then evaluated. More than 90% of the control plants wilted, yet no wilt symptoms were observed in the seedlings treated with strain B16 (Fig. 4). The antibacterial activity-deficient mutant K5 and strain K5 carrying pLAFR3 failed to suppress bacterial wilt in the hot pepper plants (Fig. 4). However, when pB03 was introduced into the K5 mutant, the transconjugant recovered its antibacterial activity. No *R. solanacearum* cells were found in the roots or stems of the plants treated with strain B16 and K5 carrying pB03, indicating bactericidal activity (data not shown).

Antibacterial Activity-Deficient Mutants Colonize Cucumber Roots

To determine whether the antibacterial activity-deficient mutants can maintain the ability to colonize roots, bacterial populations on cucumber roots were measured using the DLF method. The populations colonizing the parental strain B16, JWB100, JWB118, JWB354, and JWB388 on the last 1 cm of root segments were 2.6×10^3, 2.0×10^3, 3.4×10^3, 2.6×10^3, and 3.7×10^3 cfu/1 cm-root tip, respectively, indicating that the mutations in the genes from the antibacterial substance did not affect the root-colonizing activity of strain B16.

Nucleotide Sequence Accession Number

The complete DNA sequence of the antibacterial compound biosynthetic genes of *P. fluorescens* B16 was deposited in the GenBank database under accession number AY186595.

DISCUSSION

Antibiotics, bacteriocins, bacteriophages, and bdellovibrios are well-known causal agents of growth-inhibition zones. In addition, siderophores are also reported to form inhibition zones for some bacteria grown on media lacking Fe^{3+} [16, 18, 19]. Since inhibition zones were formed for indicator bacteria distantly related to strain B16, the factors causing the inhibition zones are thought to be neither bacteriocins nor bacteriophages. The causal factor is also unlikely to be a siderophore, because an inhibition zone appeared regardless of the Fe^{3+} concentration in the medium (data not shown). It is believed that the substance is bactericidal rather than bacteriostatic, because no *R. solanacearum* cells were recovered from the clear inhibition zones on the agar plates (data not shown). No *R. solanacearum* cells were found in the root tissues treated with strain B16 cells, also indicating bactericidal activity. Similar results have also been observed with plant tissues treated with *P. corrugata* IDVI [30]. The antibacterial activity was limited to plant pathogenic bacteria and rhizobacteria, indicating its narrow activity spectrum. This suggests that the antibacterial activity of strain B16 may be a way of maintaining ecological dominance among plant-associated bacteria.

All six antibacterial activity-deficient mutants generated with Omegon-Km were clustered in one region, and their phenotypes were complemented with proper clones. These genetic data prove that the isolated region is critical for the biosynthesis of antibacterial substances. The fact that pJWB40, which carried ORF1 to 4, conferred antibacterial activity to *P. fluorescens* 1855.344, whereas pJWB42 did not, indicates that the bacterium requires all four genes to produce the antibacterial substance. Three genes were found to encode proteins similar to known proteins. However, this information did not provide any clues to the chemical structure of the antibacterial substance. Since ORF4 was required for the biosynthesis of the antibacterial

substance and is similar to a transcriptional regulator, it is believed that ORF4 functions as a transcriptional activator rather than as a transcriptional repressor. The fact that the DNA sequence analyses of the biosynthesis genes did not reveal any significant homology with known genes involved in antimicrobial biosynthesis suggests two possibilities: either the genes are involved in the production of an uncharacterized antibacterial compound or the antibacterial compound is known. Nevertheless, we have discovered the genes responsible for its production.

Mutant K2-31 had a mutation in a gene homologous to a nitrite/nitrate regulatory protein and exhibited enhanced antibacterial activity, suggesting that the putative regulatory protein negatively regulates the genes that synthesize the antibacterial substance. However, it is still unclear how strain B16 responds to environmental signals. It is speculated that production of the antibacterial substance by strain B16 is affected by the nutrient conditions, as supported by the facts that no antibacterial activity was detected in nutrient-rich media such as LB and that the substance was produced only under minimal nutrient conditions (data not shown). Since a putative transcriptional activator was found in the gene cluster and a negative regulator in another region, it is suggested that the regulation of the biosynthesis genes is complex and that multiple factors regulate the antibiotic production.

The reduced suppressiveness of the 2,4-diacetylphloroglucinol-defective mutant of *P. fluorescens* cannot be attributed to its reduced ability to maintain effective populations in the rhizosphere, since the mutant and parental strain did not differ significantly in their root colonization ability [27]. This is in agreement with studies of other biocontrol rhizobacteria, in which the root colonization capacity of mutants deficient in antibiotic or siderophore production was not affected [3]. A similar phenomenon was observed when the bacterial populations of the parent strain and mutants were measured in the rhizosphere, indicating that the antibacterial activity does not affect the root colonization ability of strain B16.

Chemical controls of bacterial wilt caused by *R. solanacearum* are very ineffective in the field, thus an efficient way to control the disease has long been sought. Although a biological means may be an ideal way to control bacterial wilt, no microbial strains that can be used as biological control agents have yet been discovered. We believe that the strain B16 is a good biological candidate to control bacterial wilt, because it produces an antibacterial substance and is a good root colonizer.

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