Production of the Antifungal Compound Phenylacetic Acid by Antagonistic Bacterium *Pseudomonas* sp.

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Antagonistic bacteria active against phytopathogenic fungi, *Phytophthora capsici*, *Pythium ultimum*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Fusarium oxysporum* were isolated from greenhouse soils. An antifungal compound was extracted by ethyl acetate from acidified culture filtrate and purified through column chromatography and thin layer chromatography. Activity-guided bioassay was followed throughout the purification steps using *Pythium ultimum* as a test organism. The purified antifungal compound was identified as phenylacetic acid (PAA) based on the data obtained from IR, EI/MS, ^{1}H-NMR, and ^{13}C-NMR. Two different isolates, which had vast differences in differential characteristics except 16S rDNA sequence homology, produced the same compound, phenylacetic acid. ED_{50} values of the phenylacetic acid against *P. ultimum*, *P. capsici*, *R. solani*, *B. cinerea*, and *F. oxysporum* were 45, 21, 318, 360, and 226 ppm, respectively.

Key words: *antagonistic fungus,* Pseudomonas *sp., phenylacetic acid, rDNA sequence.*

Recently, there has been an increasing interest among researchers in the field of pest management to exploit beneficial microorganisms that protect plants from phytopathogens.[1,2] Strains of *Pseudomonas* spp. have been studied extensively as biocontrol agent of plant diseases. The efficacy of biocontrol microbes in greenhouses and fields was frequently attributed to the production of antifungal compounds. Some of these antibiotics have been characterized chemically.[3]

Fluorescent pseudomonads, particularly *P. fluorescens* and *P. putida*, frequent habitants of plant rhizosphere, produce either antibiotics such as phenazine,[4] 2,4-diacetylphloroglucinol,[5,6] pyoluteorin,[7] pyrrolinitrin,[8] or siderophores.[9]

Burkholderia (*Pseudomonas*) *cepacia* is also a ubiquitous soil organism which can be used as an effective biocontrol agent for *Pythium* induced damping-off, *Aphanomyces*-induced root rot of pea,[10] *Botrytis*-induced gray mold of apple,[11] and *Rhizoctonia solani*-induced root rot of poinsettia[12] and cotton.[13] Other strains have also been reported to produce various antibiotics[14-16] and siderophores.[17]

This report describes first isolation and identification of phenylacetic acid antifungal compound from antagonistic soil bacteria *Pseudomonas* sp. as a part of the study for development of integrated disease management system in greenhouse. The bacterium's fungicidal activity against phytopathogens was evaluated. Two different isolates of *Pseudomonas* species with big differences in characteristics produced the same phenylacetic acid. However, the 16S rDNA sequences of both isolates matched over 98% with *B.* (*Pseudomonas*) *cepacia* registered earlier in nuclei acid database.

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Abbreviation: PAA, phenylacetic acid.

Materials and Methods

Isolation and taxonomic identification of antifungal bacteria. Antifungal compound-producing strains, AB2 and AB101, were isolated from greenhouse soils, Chinju, Korea. Appropriate serial dilutions of soil suspension in sterile H_2O were spreaded on yeast extract agar plate, and the plates were incubated at 28°C for 7 days. Single colonies inhibiting growth of microflora nearby were isolated and screened for antifungal activity using the petri plate assay.[18] The bacterial isolates AB2 and AB101 were characterized by using physiological and biochemical tests described in Bergey's manual of systemic bacteriology[19] and methods for general and molecular bacteriology.[20]

16S rDNA sequencing. The 16S rDNA was enzymatically amplified by *Taq* DNA polymerase using two oligonucleotide primers. The sequences of the two primers are 5'-TATGGATCCTTCTACGGAGAGTTTGATCC-3' and 5'-TATGGATCCCACCTTCCGGTACGGCTACC-3'. The temperature cycle was at 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min 30 sec for 30 cycles and 5 min at 72°C for extension. The PCR products were gel purified and cloned into pGEM®-T easy vector (Promega Co. Madison, Wisconsin, USA). The nucleotide sequences of both orientations of these clones were determined by the automated DNA sequencer (LI-COR, Lincoln, Nebraska,

Table 1. Physiological properties and carbon utilization of strains AB2 and AB101.

Tests or characteristics	Result		Tests or characteristics	Result	
	AB2	AB101		AB2	AB101
Gram stain	-	-	Motility	-	+
Cell type	Rod	Rod	Hydrolysis		
Size	0.6 μm wide		gelatin liquefaction	+	-
	2.8 μm long		casein	+	+
Fluorescence in King's B Medium	non-fluorescent	fluorescent(noncolor)	starch	+	+
Growth			fat(Tween 80)	+	+
37°C	+	+	cellulose	+	-
41°C	-	+	O/F-test	aerobic	aerobic
Oxidase	-	-	Utilization		
Catalase	+	+	citrate	+	+
Nitrate reduction	-	-	cellobiose	+	+
Denitrification	-	+	mannitol	-	+
Indole production	+	+	tartarate	+	+
PHB accumulation	+	+	sorbitol	+	+
Arginine dihydrolase	-	-	arabinose	+	+
Methyl-red	-	-	β-alanine	+	+

USA, model 4200L). The 16S rDNA sequences of 1518 bp of both isolates were aligned with NCBI BlastN database.

Examination of antifungal spectrum of the isolated bacterium. The isolated bacterial strains AB2 and AB101 were initially screened for the ability to inhibit growth of several plant pathogens on 1/5 strength of PDA plate. Culture suspensions of the isolated bacterium strain were placed at the center of plates, and phytopathogenic fungi were placed at distance around the perimeter of each plate. The plates were incubated for 1~6 days at 28°C depending on the growth rate of plant pathogens tested and examined for growth inhibition by the antagonistic bacteria.[21] The plant pathogenic fungi tested were *P. ultimum, R. solani, P. capsici, B. cinerea,* and *F. oxysporum*.

Isolation and purification of antifungal compound. An antifungal compound was isolated from the culture broth of the antagonistic bacterium strains AB2 and AB101 grown in a LB medium at 32°C in a shaking incubator for 5 days. The culture broth (10 liters) was centrifuged at 6,000 rpm for 10 min at 4°C. The supernatant was acidified (adjusted to pH 2 with HCl) and extracted with ethyl acetate (10 liters). The ethyl acetate extract was combined and concentrated *in vacuo*. The concentrate (2 g) was then applied on a silica gel (230-400 mesh) chromatography column (20×400 mm) and eluted with cyclohexane-ethly acetate step gradients (200 ml) of 50/1, 25/1, 10/1, 1/1, and 0/1. The fraction (60 mg) exhibiting biological activity was applied on a silica gel (230 -400 mesh ASTM) column (10×150 mm) and eluted with cyclohexane-ethylacetate step gradients from 1/0, 100/1, 50/1, 10/1, 1/1 to 0/1. Fractions containing inhibitory activity were combined and dried *in vacuo*. The residues were loaded onto preparative TLC (60 GF_{254}, 20×20 cm, 0.25 mm, Merck) and developed with cyclohexane : ethyl ether (3:2). The band showing antifungal activity was purified with preparative TLC, and the active fraction was scratched and eluted with diethyl ether. The purified antifungal compound (4.5 mg) obtained was analyzed.

Table 2. Antifungal activity of Phenylacetic acid.

	ED_{50} (ppm)	Probit regression	
		Slope	Intercept
Pythium ultimum	45.0	0.72	2.59
Phytophthora capsici	21.6	3.45	1.16
Rhizoctonia solani	318.5	1.42	1.43
Botrytis cinerea	360.2	0.49	1.76
Fusarium oxysporum	226.1	0.99	1.70

Structural elucidation of antifungal compound. The ultraviolet spectrum of purified antibiotic was determined in methanol solution with Beckman DU-600 spectophotometer. Infrared spectrum was obtained with Bruker IFS 66 spectophotometer. Sample was prepared as thin films on KBr window. Proton and ^{13}C nuclear magnetic resonance spectra were obtained with JEOL JMM LA-400 spectrometer. The sample was prepared in a 5-mm tube as 18 mg/0.5 ml solution in $CDCl_3$ with tetramethylsilane (Me_4Si) as an internal reference. Mass spectrum was obtained on JEOL JMS-DX300 spectrometer. Sample was introduced into the instrument by direct probe method. Ionization was made by electron impact at 70 eV.

Determination of antifungal activity. The serially diluted antibiotic was amended in 1/5 strength of PDA plate as 0 to 200 ppm, and a 5-mm agar plug of five phytopathogenic fungi were placed onto the center of the plate. The diameters of mycelial growth were measured from three replicates of each treatment. The ED_{50} values of the mycelial growth inhibition were calculated by a probit analysis.

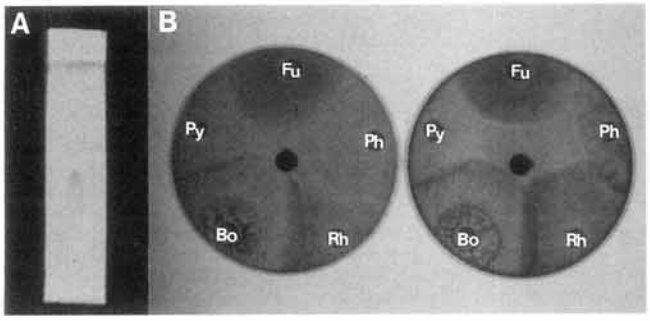

Fig. 1. The purified antifungal compound and its antifungal activity in a petri plate assay. A: Purified antifungal compound identified on a TLC plate. Developing solvent, cyclohexane:diethyl ether = 1:1. Rf, 0.38. B: Antifungal activity of purified antifungal compound on Phytopathogenic fungi (100/disc) was applied, Py, *Pythium ultimum*; Ph, *Phytophthora capsici*; Rh, *Rhizoctonia solani*; Bo, *Botrytis cinerea*; Fu, *Fusarium oxysporum*. Left, Untreated control; Right, Purified antifungal compound treated.

Results

Taxonomy of the antifungal bacteria. The antifungal bacterium strains AB2 and AB101 were isolated from greenhouse soil. Both strains were gram negative, nonsporoforming aerobes which accumulated poly-β-hydroxybutyrate as carbon reserve material. These characteristics indicate that they belong to the genus *Pseudomonas* (Table 1). Both isolates showed catalase positive, oxidase negative, arginine dihydrolase negative, indole production, and casein and fat hydrolyzing activities. However, characterestics such as fluorescense, growth at 41°C, denitrification, motility, gelatin liquefaction, and cellulose and mannitol utilization were different from each other. Since both isolates produced the same antifungal compound phenylacetic acid, we further analyzed 16S rDNA sequence homology. Among the 1518 sequenced bp of the two isolates, six bases were found to be different. (Sequences of 16S rDNAs are to be registered in NCBI data base: AB2, AF219125; AB101, AF219126.) The sequences of both isolates showed over 98% identity with *B.* (*Pseudomonas*) *cepacia* registered (EMBL, accession number X87275).

Isolation of antifungal compound. By the activity-guided bioassay, the antifungal compound was found in the acidic fraction. The acidic fraction was analyzed by silica gel column chromatography using solvent system, cyclohexane-ethyl acetate. The active fraction was separately rechromatographed on a silica gel column with the same solvent system and preparative TLC. It was then scratched and eluted with diethyl ether. The purified antifungal compound (4.5 mg) was obtained and was designated AB2K2. The *Rf* value of AB2K2 was 0.38 on the silica gel thin-layer plate developed with cyclohexane-diethyl ether (1:1, v/v), and its antifungal activity on the phytopathogenic fungi in petri-plate assay was confirmed (Fig. 1). Antifungal compound produced by starin AB101 was prepared by a similar scheme to that of strain AB2 purification and was designated AB101AE1. Physico-chemical properties of AB101AE1 were identical with AB2K2.

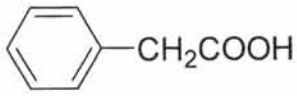

Fig. 2. Chemical structure of antifungal compound, Phenylacetic acid.

Structural elucidation. Physico-chemical properties of antifungal compound are determined. The compound was soluble in most organic solvents such as chloroform, ethyl acetate, acetone, methanol, and diethyl ether, but insoluble in hexane and water. By comparing the data with the reference,[22] this antifungal compound was determined as phenylacetic acid (Fig. 2). Copies of original spectra are obtainable from the author upon request.

Antifungal activity. The antifungal activity of phenylacetic acid are shown in Table 2. ED_{50} values of the phenylacetic acid measured from degree of growth inhibition on PDA plates against *P. ultimum, P. capsici, R. solani, B. cinerea*, and *F. oxysporum* were 45, 21, 318, 360, and 226 ppm, respectively. The slope and intercept of the probit regression line indicated that *P. capsici* exhibited higher sensitivity to the increasing concentration of the antibiotic.

Discussion

PAA is a compound which is widely distributed in nature. It has been known as a natural auxin found in shoots of plants.[23-24] PAA induced organogenesis in tissue cultured cells,[25] with other biological actions including antibacterial activity[26,27] and phytotoxicity.[22,28] PAA was also well studied as a precursor of penicillin G.[29] Recently, Burkhead *et al.*[30] reported that a biological control bacterium *Enterobacter cloacae*, effective against *Fusarium* dry rot of potatoes[31] and preemergence-damping off of pea and cucumber seedlings incited by *Pythium* species,[32] produced the antifungal compound phenylacetic acid in maltose broth culture.

Previous study showed that the antagonistic bacterium exhibited two different inhibiting zones on TLC-autobiography test.[33] One was identified as *N*-butylbenzenesulfonamide and the other as phenylacetic acid. We isolated an antifungal compound from another isolate AB101 which produced the same phenylacetic acid. First, we thought that two isolates were different species of *Pseudomonas* which showed different characteristics in general key for species differentiation (Table 1). However, the sequence homology of 16S rDNA was strikingly similar (over 98%) between the two isolates and can be categorized into *B.* (*Pseudomonas*) *cepacia* based on 16S rDNA sequences registered in nucleic acid database. But several key physiological properties have shown some discrepancies

to those of *P. cepacia*[19] such as in fluorescence, oxidase, nitrate reduction, and motility. *P. cepacia* is known as one of the most nutritionally diverse bacteria which can use over 200 different organic compounds as its carbon source.[34] This competence may contribute to its ability to survive and colonize roots and rhizosphere. This might indicate that diverse environment drive this organism to adapt its physiological characters more to its niches but perhaps less in structural gene such as 16S rDNA.

Phenylacetic acid is known as a plant growth regulator[23,24] and phytotoxic[22,28] and antifungal compound.[30] In this study, phenylacetic acid exhibited good activities against phycomycetes group of fungi such as *Pythium* and *Phytophthora* but low against others such as *Fusarium*, *Rhizoctonia*, and *Botrytis*. The concentration and nature of ecosystem can make possible the exploitation of this compound as a plant growth regulator or fungicide, and further studies are needed to evaluate this possibility.

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