Isolation and Identification of Antifungal *N*-Butylbenzenesulphonamide Produced by *Pseudomonas* sp. AB2

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An antifungal bacterial strain, isolated from a greenhouse soil sample, inhibits growth of microflora nearby. It was selected for further studies of bacterial antifungal properties. This isolate was identified as a *Pseudomonas* sp. based on carbohydrate utilization, and other biochemical and physiological tests. Petri plate assay revealed that the *Pseudomonas* sp. exhibited antifungal activity against the plant pathogens, *Pythium ultimum*, *Rhizoctonia solani*, *Phytophthora capsici*, *Botrytis cinerea* and *Fusarium oxysporum*. Using direct inhibition bioassay on TLC plates after ethyl acetate extraction of the culture filtrate, we correlated antifungal activity with production of antifungal compounds. An antifungal antibiotic was isolated from the culture filtrate and was identified as *N*-butylbenzenesulphonamide. ED_{50} values of the *N*-butylbenzenesulphonamide against *P. ultimum*, *P. capsici*, *R. solani*, and *B. cinerea* were 73, 41, 33 and 102 ppm, respectively.

Each year, fungal diseases cause millions of dollars worth of crop damage all over the world despite the extensive use of pesticides[1]. Also, environmental concerns and development of resistance in target populations have reduced the availability of effective fungicides. Among alternative measures to cope with those problems, the vast array of antimicrobial molecules produced by diverse soil microbes remain as a reservoir of new and potentially safer biopesticides. For these and other reasons, a heightened interest in biocontrol has arisen[2~4].

Biological control has potential for the management of soil-inhabiting fungi. A variety of soil microorganisms have demonstrated activity for the control of various soil-borne plant pathogens. Biological control fungi, such as *Trichoderma* and *Gliocladium* spp., have been used to control a variety of fungal pathogens, including *Rhizoctonia*, *Pythium*, *Sclerotinia*, *Sclerotium* and *Fusarium* spp.[5,6]. Also, certain strains of fluorescent *Pseudomonas*, when applied to planting material or soil, can provide biological control of root pathogens[7,8].

There are numerous reports of the production of antibiotic compounds by *Pseudomonas* spp. Some of these antibiotics have been characterized chemically[9]. HOWELL and STIPANOVIC[10,11] provided evidences that different isolates of *P. fluorescens* were antagonistic to pathogens of cotton seedlings because of the production of the antibiotics pyrrolnitrin and pyoluteorin. Another antibiotic, produced by a *Pseudomonas* strain that can suppress take-all disease in wheat, was identified as a dimer of phenazine-1-carboxylate[12]. Other workers have reported a *Pseudomonas* isolate produced an unidentified antibiotic that was able to inhibit the fungus causing Dutch elm disease[13].

Support for the role of antibiotics in biological control has come mainly from studies that have shown correlations between bacterial inhibition of pathogens *in vitro* and disease suppression in the soil. Studies on the formation of Pseudomonad secondary metabolites will contribute to our knowledge, and ultimately to the control, of certain plant diseases.

This study aimed to isolate and identify biocontrol agents

from greenhouse soils. An antifungal compound was identified and its biological activity against several plant pathogens was measured.

Materials and Methods

Isolation and Taxonomic Identification of Antifungal Bacterium

An antifungal compound producing strain (AB2) was isolated from a soil sample collected from a greenhouse in Chinju, Korea. Appropriate serial dilutions of soil suspension in sterile H_2O were spread on a yeast-extract agar plate[14], and the plate was 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[15]. The bacterial isolate AB2 was characterized by using physiological and biochemical tests as described in BERGEY's Manual of Systemic Bacteriology[16].

Production and Isolation of Antifungal Compound

A single colony of strain AB2 was inoculated into an 100 ml Erlenmeyer flask containing 15 ml of culture medium consisting of tryptone (Difco) 10g, yeast extract (Difco) 5 g, and NaCl (Tedia) 5 g in 1 liter sterile H_2O. The flask was shaken on a rotary shaker (140 rpm) at 30°C for 24 hours. This seed culture (3 ml) was transferred into 3 liter Erlenmeyer flask containing 1.2 liter of the same medium. Production of antifungal compound was carried out at 30°C for 5 days on a rotary shaker.

The culture broth (10 liter) was centrifuged at 8,000 rpm for 10 minutes to remove bacterial cells. The supernatant (9 liter) was extracted with ethyl acetate (9 liter). The ethyl acetate extracts were combined and evaporated to dryness to give a brown extract (2.5 g). This extract was applied to a silica gel (230~400 mesh) chromatography column (20×400 mm), and eluted with chloroform-acetone step gradients. The fraction which exhibited biological activity was applied to a silica gel (230~400 mesh) column (10×150 mm), and eluted with cyclohexane-ethyl acetate step gradients from 1/0, 100/1, 50/1, 10/1, 1/1 to 0/1. Fifteen milliliter eluate fractions were collected. Ten-microliter aliquots from each fraction were spotted on a silica gel TLC plate (60 GF254, 20×20 cm, 0.25 mm, Merck), and developed with cyclohexane-diethyl ether (6 : 4, v/v). Direct inhibition bioassay was performed by using a developed TLC plate sprayed with a spore suspension of *Botrytis cinerea* in 1% potato dextrose agar (Difco) until the silica gel began to appear translucent. The sprayed plate was incubated at 28°C in a moistened plastic box under white light with 12-hour photoperiod. After 4~5 days, fungal growth was visible; zones of inhibition due to antifungal compounds on the plate appeared as white areas where fungal growth was absent. The identified active fractions were concentrated *in vacuo* to dryness. The residues were loaded onto preparative TLC and developed with cyclohexane-diethyl ether (6 : 4, v/v).

Structure Elucidation

The UV spectrum of the purified antibiotic was determined in methanol solution with a Beckman DU-600 spectrophotometer. Elemental analysis was determined by Vario EL analyzer. IR spectra were obtained with a Bruker IFS 66 spectrophotometer for samples as thin films on a KBr window. Proton and ^{13}C NMR spectra were obtained with a BRUKER AW500 spectrometer. The sample was prepared as 25 mg/0.5 ml solution in CD_3OD with tetramethylsilane (Me_4Si) as an internal reference. The mass spectrum was obtained on a JEOL JMS-DX300 spectrometer by the direct probe method, with electron impact ionization at 70 eV.

Biological Activity

The serially diluted antibiotic was incorporated in 1/5 strength PDA plates at 0 to 200 ppm, and 5 mm agar plugs of five phytopathogenic fungi, *Pythium ultimum*, *Phytophthora capsici*, *Rhizoctonia solani*, *Botrytis cinerea* and *Fusarium oxysporum* were placed onto the center of the plates. The diameters of mycelial growth were measured from 3 replicates of each treatment. The ED_{50} values of the mycelial growth inhibition were calculated by a probit analysis.

Results

Taxonomy of the Antifungal Bacteria

The antifungal bacterium AB2 was isolated from greenhouse soil, Chinju, Korea. This strain grew at 20 to 37°C but not at 41°C. Colonies grown on LB agar or King B medium at 30°C for 48 hours were yellow pigmented and nonfluorescent. The strain was oxidase, arginine dihydrolase and denitrification negative, and catalase, polyhydroxybutyrate accumulation and indole production positive. The physiological properties and carbon utilization of strain AB2 are shown in Table 1. These

Table 1. Physiological properties and carbon utilization of strain AB2.

Test or characteristic	Result	Test or characteristic	Result
Gram stain reaction	-	Indole production	+
Cell type	Rod (many in pairs)	Citrate utilization	+
Size	0.6μm wide,	Nitrate reduction	-
	2.8μm long	Denitrification	-
Motility	-	PHB accumulation	+
Flagella	-	Arginine dihydrolase	-
Colony color (LB)	+ (yellow)	Methyl-red test	-
Fluorescence in	nonfluorescent	Utilization of	
King's Medium B		cellobiose	+
Growth		β-alanine	+
37℃	+	manitol	-
41℃	-	tartrate	+
Hydrolysis		sorbitol	+
gelatin liquefaction	+	arabinose	+
casein	+		
fat (Tween 80)	+		
starch	+		
cellulose	+		

Table 2. Physico-chemical properties of *N*-butylbenzenesulphonamide.

Appearance	Oily
Molecular formula	$C_{10}H_{15}NSO_2$
EI-MS (*m/z*)	
Calcd:	213.30 (as $C_{10}H_{15}NSO_2$)
Found:	213 (M^+)
UV λ_{max} nm, (log ε) in	
MeOH	209 (4.52), 220 (4.62), 262 (3.50)
IR ν_{max} (KBr) cm^{-1}	3300, 3100, 2950, 2300, 1325, 1140

characteristics of strain AB2 revealed that it belongs to the genus *Pseudomonas* sp.

Isolation of Antifungal Compound

Active TLC eluates were concentrated to yield oily purified antifungal compound (4.6 mg), designated AB2K1. The Rf value of AB2K1 was 0.24 on the silica gel 60 thin-layer plate developed with cyclohexane - diethyl ether (6 : 4, v/v), and its antifungal activity against the phytopathogenic fungi in petri-plate assay was confirmed.

Structure Elucidation

Physico-chemical properties of AB2K1 are shown in Table 2. The compound was soluble in most organic solvents such as acetone, ethyl acetate, methanol, chloroform and diethyl ether, but insoluble in hexane and water. Elemental analysis revealed that this compound contained 56.8% carbon, 7.2% hydrogen, 14.8% sulfur, 6.5% nitrogen and 15.1% oxygen (caled. 56.31% carbon, 7.09% hydrogen, 15.03% sulfur, 6.57% nitrogen and 15% oxygen). The molecular formula for AB2K1 was established as $C_{10}H_{15}NSO_2$ by elemental analysis, EI-MS

and NMR spectra. AB2K1 gave a positive color reaction with ninhydrin. The IR spectrum showed a strong absorption band at 3300 cm^{-1} (N−H), 1325 and 1140 cm^{-1} (S = O) characteristic of a sulphonamide absorption. The ^{1}H and ^{13}C NMR data of AB2K1 are shown in Table 3. The ^{13}C NMR data and DEPT experiment revealed the presence of one methyl carbon, three methylene carbons, three methine carbons and one quaternary carbon signal. In the ^{1}H NMR spectrum, a proton signal at δ_H 4.68 was observed, indicating the presence of an NH proton. ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY experiments of AB2K1 revealed two partial structures. The ^{1}H-^{1}H COSY spectrum showed that 4′-H (δ_H 0.85) was correlated to 3′-H (δ_H 1.29), which in turn was correlated to 2′-H (δ_H 1.44) and then to 1′-H (δ_H 2.96). This indicated the presence of a butyl group. The NH proton (δ_H 4.68) was correlated to 1′-H (δ_H 2.96) in ^{1}H-^{1}H COSY spectrum. In the ^{1}H–^{13}C-NMR spectrum, the symmetric protons (δ_H 7.89) 2-H and 6-H were correlated to one carbon at δ_C 127, and the two protons (δ_H 7.52) of 3-H and 5-H were correlated to one carbon at δ_C 129, which indicated the presence of a benzene ring. Thus the structure of AB2K1 was determined as *N*-butylbenzenesulphonamide (Fig. 1).

Table 3. ^{13}C and ^{1}H NMR data for *N*-butylbenzenesulphonamide.

	^{13}C	^{1}H
2+6	127.01	7.89 (2H, m)
3+5	129.04	7.52 (2H, m)
4	132.51	7.57 (1H, m)
1′	42.92	2.96 (2H, q)
2′	31.57	1.44 (2H, m)
3′	19.63	1.29 (2H, m)
4′	13.45	0.85 (3H, t, *J*=7.3 Hz)
NH		4.68 (1H, t)

Biological Activity

The antifungal activity of *N*-butylbenzenesulphonamide is shown in Table 4. This antibiotic showed broad activities against several phytopathogenic fungal species. ED_{50} values of the *N*-butylbenzenesulphonamide measured from degree of growth inhibition on PDA plates against *P. ultimum*, *P. capsici*, *R. solani* and *B. cinerea* were 73, 41, 33 and 102 ppm, respectively. The slope and intercept of the probit regression line indicated that *R. solani* exhibited higher sensitivity to increasing concentrations of the antibiotic.

Discussion

In recent years, biological control of soil-borne pathogens has received increasing attention as a promising

Fig. 1. Structure of *N*-butylbenzenesulphonamide.

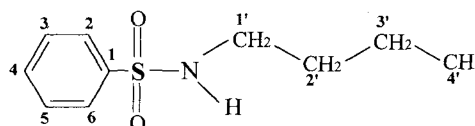

Table 4. Antifungal activities of *N*-butylbenzenesulphonamide.

	ED_{50} (μg/ml)	Probit regression line		95% Limits	
		Slope	Intercept		
Pythium ultimum	73	2.8	1.2	56	92
Phytophthora capsici	41	3.0	1.3	0.0	99
Rhizoctonia solani	33	3.9	0.7	19	49
Botrytis cinerea	101	2.7	1.2	79	139
Fusarium oxysporum	n. d*	n. d	n. d	n. d	

* not detected

alternative to chemical control. To improve efficacy of biological control, however, understanding of the mechanisms of action, nutrition, and ecology of biocontrol agents is needed. Such knowledge will lead to substantial progress in selection of superior strains, mass production, and appropriate formulation of biocontrol organisms. Disease suppression by microorganisms in agriculturally important crops has been studied in detail[7,17~19].

We have initiated a study of screening and investigating the antagonistic microorganisms of phytopathogenic fungi in the greenhouse. This paper presents the isolation and characterization of a *Pseudomonas* strain (AB2) that secretes an antifungal compound against phytopathogenic fungi. On the basis of all the physiological and biochemical tests, we find that the characteristics indicate the species *Pseudomonas*, although some of the tests described for *Pseudomonas* in BERGEY's Manual Systemic Bacteriology were negative[16]. The strain (AB2) exhibited strong inhibitory activities against *P. ultimum*, *P. capsici*, *R. solani*, *B. cinerea* and *F. oxysporum* based on dual culture on potato dextrose agar.

Using direct inhibition bioassay on TLC, we correlated antifungal activity with production of an antifungal compound[20]. The isolated antifungal compound, *N*-butylbenzenesulphonamide, was active against all of the fungi that were tested except *F. oxysporum* (Table 4). This is the first report at the isolation and structural identification of *N*-butylbenzenesulphonamide produced by *Pseudomonas* sp.

N-Butylbenzenesulphonamide was synthesized from benzenesulfonyl chloride and butylamine. The natural product and synthetic compound agree in physical and chemical data as well as biological activities. Although it has relatively low antifungal activity, broad action spectrum of the compound could lead to the chemical synthesis of this compound and analogs for use in the suppression of the destructive pathogens in the field as well as in grain storage.

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