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Isolation and Anti-fungal Activities of 2-Hydroxymethyl-chroman-4-one Produced by *Burkholderia* sp. MSSP

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In the course of screening for biological active compounds from microbial origins, various strains were isolated from roots of *Mimosa pudica* using standard dilution agar plate method. One isolate was selected for further studies of bacterial anti-fungal properties. It was designated MSSP and identified as *Burkholderia* sp. by many biological characteristics such as carbohydrate utilization, biochemical and physiological test and 16S rDNA sequence homology search. To investigate antagonistic principle, anti-fungal compounds were extracted and fractionated by different solvent systems. An anti-fungal compound was isolated from its culture filtrate using organic solvent extraction, column chromatography and thin layer chromatography and pure solid form compound (MSSP2) was obtained. The chemical structure of anti-fungal compound was identified as 2-Hydroxymethyl-chroman-4-one based on the data obtained from spectroscopic analysis such as mass spectral analyses and NMR spectral data. The compound 2-Hydroxymethyl-chroman-4-one exhibited good activities against phytopathogen such as *Pythium ultimum*, *Phytophthora capsici* and *Sclerotinia sclerotiorum*.

Various studies[1~3] reported that bacteria such as *Pseudomonas* sp. or *Bacillus* sp. might play a role in the reduction of plant diseases. Among them, *Burkholderia* (previously known as *Pseudomonas* sp.) can also antagonize and repress many soil-borne plant pathogens. Particularly, *B. cepacia* is known to be a ubiquitous inhabitant in soil which has been used as an effective biocontrol agent for *Pythium*-induced damping-off and *Aphanomyces*-induced root rot of pea[4], and *Rhizoctonia solani*-induced root rot of poinsettia[5]. *Burkholderia* sp. can provide an environmentally friendly alternative to potent and toxic fungicides, which might bring adverse effects to the environment. These antagonistic *Burkholderia* sp. produced the anti-fungal compounds such as pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin and phenazine[6]. Other *Pseudomonas* strains have also been reported to produce various antibiotics[7,8]. The antibiotics produced by the *Burkhoderia* sp. have been implicated as important mechanisms mediating phytopathogen suppression.

Most antibiotics isolated from *Burkholderia* culture filtrates, namely, phenazines, pyrrolnitrin-type antibiotics, pyoluterorin and indole derivatives, belong to the class of N-containing heterocycles and have been shown to originate from intermediates or end products of the aromatic amino acid biosynthetic pathways[9]. However, macrolides, polyenes, quinone-type antibiotics and hydroxyphenol have not been found so far among the secondary metabolites produced by the *pseudomonads*.

In this paper, the taxonomy of the producing strain, production, isolation and anti-fungal activities of 2-Hydroxymethyl-chroman-4-one produced from antagonistic soil bacteria *Burkholdera* sp. are reported.

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Materials and Methods

Identification of Anti-fungal Bacterium

A biological active compound-producing strain MSSP was isolated from roots of *Mimosa pudica*. Appropriate serial dilutions of soil suspension in sterile water were spread on a yeast-extract agar plate[10], and the plate was incubated at 28°C for 7 days. Single colonies inhibiting growth of microflora nearby were isolated and screened for anti-fungal activity using the petri plate assay[11,12]. The bacterial isolate MSSP was characterized by using physiological and biochemical tests as described in BERGEY's Manual of Systemic Bacteriology[13] and methods for general and molecular bacteriology[14]. The 16S rDNA was enzymatically amplified by Taq DNA polymerase using two oligonucleotides primers[15]. The 16S rDNA sequence of isolate MSSP was aligned with NCBI blast N database.

Production and Purification of Biological Active Compound

A single colony of strain MSSP was pre-cultured in a 100 ml Erlenmeyer flask containing 15 ml of LB broth (tryptone (Difco) 10 g, yeast extract (Difco) 5 g, and NaCl (Tedia) 5 g, 1 liter sterile distilled water) on a rotary shaker (140 rpm) at 30°C for 24 hours. For the production of anti-fungal compound(s), 3 ml of the seed culture was transferred into 1.2 liters of the YEMA medium (K_2HPO_4 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl (Tedia) 0.1 g, mannitol 10 g, and yeast extract (Difco) 1 g in 1 liter sterile distilled water) in a 3-liter Erlenmeyer flask, and cultivated for 5 days under the same culture conditions.

The culture broth (10 liters) was centrifuged at 7,000 rpm for 20 minutes to remove bacterial cells. The supernatant (10 liters) was extracted with ethyl acetate (10 liters). The ethyl acetate extracts were combined and evaporated to dryness to give a brown extract (2.5 g). The crude extracts were applied to a silica gel (silica gel $60F_{254}$, 230~400 mesh, Merck) column chromatography (f 20×400 mm) and eluted with stepwise gradients of hexane-ethyl acetate (50/1, 10/1, 1/1 to 0/1, V : V). By activities guided bioassay, the fractions (100 mg) exhibiting anti-fungal activity were combined and dried *in vacuo*. The residues were loaded onto a preparative TLC ($60\,GF_{254}$, size; 20×20 cm, thickness; 0.25 mm, Merck) and developed with hexane : ethyl acetate (3 : 2, V/V). The band showing anti-fungal activity was scrapped off the plate and eluted with ethyl acetate. The yield was *ca*. 20 mg from 10 liters of culture broth.

Structure Elucidation

The UV spectrum of the purified antibiotic was determined in a methanol solution with a Beckman DU-600 spectrophotometer. IR spectra were obtained with a Bruker IFS 66 spectrophotometer for samples as thin films on a KBr window. ^1H and ^{13}C NMR spectra were obtained with a Jeol JMM LA400 spectrometer. The sample was prepared as 25 mg/0.5 ml solution in $CDCl_3$ 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..

Measurement of Anti-fungal Activity

The serially diluted antibiotic was incorporated in 1/5 strength PDA plates at 0 to 150 ppm, and 5 mm agar plugs of phytopathogenic fungus were placed upside down in the center of the plates. The tested phytophatogenic fungi were followed; *P. ultimum*, *P. capsici*, *R. solani*, *Botrytis cinerea*, *Alternaria panax* and *S. sclerotiorum*. 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 Anti-fungal Bacterium

The anti-fungal bacterium MSSP was isolated from roots of *Mimosa pudica*. This strain was Gram-negative, rod and nonsporoforming aerobes. Colonies grown on LB agar or King's B medium at 30°C for 48 hours were milky pigmented and non-fluorescent. The strain was catalase, indol production and casein hydrolysis negative. This showed arginine dihydrolase, oxidase, fat (Tween 80) hydrolysis, and nitrate reduction positive. This and other physiological properties and carbon utilization of the isolate MSSP are shown in Table 1. We further analysed 16S rDNA sequence homology. The sequences of this isolate (accession number AY551271) showed over 99% identity with *Burkholderia* sp. CEB01056 (accession number AJ491304), *Burkholderia* sp. M356 (U96937), *B. glumae* (U96931), *B. vietnamiensis* (U96929) and *B. cepacia* (U96927). These characteristics indicate that it belongs to the genus *Burkholderia*.

Isolation and Structure Elucidation of Biological Active Compound

Active TLC eluates were concentrated to yield colorless solid, purified anti-fungal compound (20 mg), designated

Table 1. Comparison of bacterial characteristics of an isolate (MSSP) with *Burkholderia cepacia* ATCC 25416.

Test or characteristics	MSSP	*B. cepacia*
Gram stain	-	-
Cell type	Rod	Rod
Motility	+	+
Colony color (LB)	Milky	Yellow
Fluorescence in King's Medium B	-	+
Growth at 37℃	+	+
Hydrolysis of		
gelatin liquefaction	-	-
casein	-	+
fat(Tween 80)	+	+
starch	-	-
cellulose	-	-
Catalase	-	-
Oxydase	+	+
Indole production	-	-
Citrate utilization	+	+
Nitrate reduction	+	+
Denitrification	+	+
Arginine dihydrolase	+	+
Methyl-red test	-	-
Utilization of		
glucose	+	+
lactose	+	+
manitol	+	+
sucrose	+	+
sorbitol	+	+
arabinose	+	+
β-alanine	+	+
maltose	+	+

MSSP2. The Rf value was 0.42 on the silica gel 60 thin layer plate developed with hexane : ethyl acetate (3 : 2, v/v), and its anti-fungal activity against the phytopathogenic fungi in petri plate assay was confirmed. Physico-chemical properties of MSSP2 are shown in Table 2. Compound MSSP2 exhibited a molecular ion peak at *m/z* 178 in the low-resolution EI-MS, and the molecular formula was determined to be $C_{10}H_{10}O_3$ by EI-MS, ^1H and ^{13}C NMR data. Its IR spectrum exhibited characteristic absorption band at 3297 (OH), 2926 (C–H), 1618 (C=O) and 1595 (C=C) cm^{-1}. The structure of MSSP2 was inferred from the ^1H and ^{13}C NMR spectral data (Table 3) together with DEPT and 2D NMR experiments. The ^{13}C-NMR spectrum of compound MSSP2 showed ten carbons including one carbonyl carbon at δ 173.8 (C-4), two methylene carbon at δ 54.5 (C-1′), and 33.1 (C-3), a methane carbon at δ 78.1 (C-2), six aromatic carbons, of which four were methine carbons at δ 133.6 (C-6), 131.0 (C-5), 119.4 (C-7), 117.4 (C-8), and two were quaternary carbons at δ 159.4 (C-10), and 116.6 (C-9). The ^1H-NMR spectrum of this compound showed four *ortho*, *meta*-coupled aromatic protons at 7.39 (1H, dd, J=1.6, 7.8 Hz), 6.86 (1H, ddd, J=1.0, 7.5, 7.8 Hz), 6.98 (1H, dd, J=1.0, 8.3 Hz), and 7.34 (1H, ddd, J=1.6, 7.5, 8.3 Hz) that were assigned to H-5, H-6, H-8, and H-7, respectively, and oxymethine at δ 4.84. Furthermore, the ^1H-NMR spectrum showed two separated ABX systems at δ 3.95 (1H, dd, J=5.2, 11.3 Hz) and 3.82 (1H, dd, J=4.8, 11.3 Hz), and at δ 3.40 (1H, dd, J=8.9, 10.9 Hz) and 3.32 (1H, dd, J=8.2, 10.9 Hz). The ^1H-^1H COSY NMR spectrum of MSSP2 showed two spin-systems for this compound. Thus, aromatic proton H-5 at δ 7.39 was *ortho*-coupled to H-6 and *meta*-coupled to H-7, and H-7 also exhibited *ortho*-coupling with H-8 at δ 6.98. A second spin-system was observed starting with H-2 at δ 4.84, which exhibited a vicinal coupling with H-3 at δ 3.40 and 3.32, and H-1 at δ 3.95 and 3.82. The unassigned connection between C-9 and C-2 was determined on the basis of the HMBC correlation (Fig. 1). The carbonyl carbon resonating at δ 173.8 was assigned to C-4, because it showed HMBC cross peaks with the to H-5, H-3, and H-2 proton. Thus the structure of MSSP2 was determined as 2-Hydroxymethyl-chroman-4-one (Fig. 2).

Table 2. Physico-chemical properties of 2-Hydroxymethyl-chroman-4-one.

Appearance	**Colorless solid**
Molecular formula	**$C_{10}H_{10}O_3$**
EI-MS (*m/z*)	
(Calcd)	**178.06 (as $C_{10}H_{10}O_3$)**
(Found)	**178 (M^+)**
UV λ_{max} nm, (ε) in	
(MeOH)	**217 (4959), 277 (2446)**
IR ν_{max} (KBr) cm^{-1}	**3297, 2926, 1618, 1595**

Table 3. ^{13}C and ^{1}H NMR data for 2-Hydroxymethyl-chroman-4-one in $CDCl_3$.

Position	^{13}C	^{1}H
2	**78.1**	**4.84 (1H, m)**
3	**33.1**	**3.40(1H, dd, J=8.9, 10.9Hz)** **3.32(1H, dd, J=8.2, 10.9Hz)**
4	**173.8**	**Quaternary**
5	**131.0**	**7.39(1H, dd, J=1.6, 7.8Hz)**
6	**133.6**	**6.86(1H, ddd, J=1.0, 7.5, 7.8Hz)**
7	**119.4**	**7.34(1H, ddd, J=1.6, 7.5, 8.3 Hz)**
8	**117.4**	**6.98(1H, dd, J=1.0, 8.3 Hz)**
9	**116.6**	**Quaternary**
10	**159.4**	**Quaternary**
1′	**33.1**	**3.95(1H, dd, J=5.2, 11.3Hz)** **3.82(1H, dd, J=4.8, 11.3Hz)**

Fig. 1. Long-range couplings observed in the HMBC experiments.

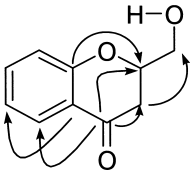
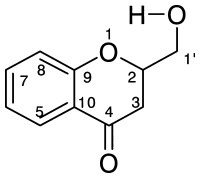


Fig. 2. Chemical structure of 2-Hydroxymethyl-chroman-4-one.



Antifungal Activity

The anti-fungal activity of 2-Hydroxymethyl-chroman-4-one was shown in Table 4. The biological active compound 2-Hydroxymethyl-chroman-4-one exhibited good activities against phycomycetes group of fungi such as *P. ultimum*,

Table 4. Antifungal activities of 2-Hydroxymethyl-chroman-4-one.

	ED_{50} (ppm)	Probit regression line		95% Limits	
		Slope	Intercept		
Pythium ultimum	54.99	0.24	2.73	48.54	63.77
Botrytis cinerea	98.55	1.55	1.73	65.99	237.98
Rhizoctonia solani	71.95	2.77	1.20	53.84	109.70
Phythophthora capsici	35.77	2.82	1.40	28.80	45.17
Alternaria panax	79.75	1.23	1.98	64.62	119.51
Sclerotinia sclerotiorum	52.03	2.99	1.17	38.45	83.03

P. capsici and *S. sclerotiorum*. ED_{50} values of the 2-Hydroxymethyl-chroman-4-one measured from degree of growth inhibition on PDA plates against *P. ultimum*, *P. capsici* and *S. scelotiorum* were 54.9, 35.7 and 52.1 ppm, respectively.

Discussion

B. cepacia is one of the most nutritionally versatile bacteria capable of using a number of carbohydrates and carbohydrate derivatives, organic acids and amino acid as carbon and energy sources[16]. These versatilities such as utilization of diverse nutrients, production of wide range of inhibitory compounds, and colonization and survival in the target area makes it very attractive for biocontrol use[17,18].

In this study, we have screened antagonistic bacteria and investigated the antagonistic activities against plant pathogenic fungi. The strain (MSSP) exhibited strong inhibitory activities against *P. ultimum*, *P. capsici*, *R. solani* and *B. cinerea* based on dual culture on a potato dextrose agar. On the basis of all the physiological and biochemical tests, we find that the characteristics indicate the species *Burkholderia*. The sequence homology of 16S rDNA was strikingly similar between the isolate and *Burkholderia* sp. CEB01056, *Burkholderia* sp. M356 (U96937), *B. glumae* (U96931), *B. vietnamiensis* (U96929) and *B. cepacia* (U96927). It can be categorized into *Burkholderia* sp. based on 16S rDNA sequences registered in nucleic acid database. But some of the tests such as colony color, production of fluorescence in King's B medium and ability of casein hydrolysis are different from *Burkholderia cepacia* ATCC 25416 as a reference isolate (Table 1).

This paper presents the isolation and characterization of a *Burkholderia* strain (MSSP) that secretes an anti-fungal compound against plant pathogenic fungi. The principal mode of action of the isolate of *Burkholderia* sp. in our studies appeared to be antagonism by the production of 2-Hydroxymethyl-chroman-4-one. The isolated anti-fungal compound 2-Hydroxymethyl-chroman-4-one exhibited activities against *Pythium*, *Phytophthora* and *Sclerotinia* (Table 4).

2-Hydroxymethyl-chroman-4-one was used to mediate for synthesis of benzopyranones[19].

However, there has no report on isolation and biological activities from bacterial strains of this species. This is the first report at the isolation and structural identification of 2-Hydroxymethyl-chroman-4-one produced by *Burkholderia* sp.

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Reference

1) Cook, R. J. & A. D. Rovira: The role of bacteria in the biological control of *Gaeumannomyces graminis* by suppressive soils. Soil. Biol. Biochem. 8: 269～273, 1976
2) Kommedahl, T. & I. P. Chang Mew: Biocontrol of corn root infection in the field by seed treatment with antagonists. Phytopathology 65: 296～300, 1975
3) Howell, C. R. & R. D. Stipanovic: Suppression of

Pythium ultimum-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. Phytopathology 70: 712～715, 1980

4) KING, E. B. & J. L. PARKE: Biocontrol of *Aphanomyces* root rot and *Pythium* damping-off by *Pseudomonas cepacia* AMMD on four pea cultivars. Plant Dis. 77: 1185～1188, 1993
5) CARTWRIGHT, D. K. & D. M. BENSON: Comparison of *Pseudomonas* species and application techniques for biocontrol of *Rhizoctonia* stem rot of poinsettia. Plant Dis. 79: 309～313, 1995
6) ROSALES, A. M.; L. THOMASHOW, R. J. COOK & T. W. MEW: Isolation and identification of anti-fungal metabolites produced by rice-associated antagonistic *Pseudomonas* sp. Phytopathology 85: 1028～1032, 1995
7) PARKER, W. L.; M. L. RATHNUM, V. SEINER, W. H. TREJO, P. A. PRINCIPE & R. B. SYKES: Cepacin A and cepacin B, two new antibiotics produced by *Pseudomonas cepacia*. J. Antibiotics 37: 431～440, 1984
8) LEE, C.; S. KIM, B. HYUN, J. SUH, C. YON, C. KIM, Y. LIM & C. KIM: Cepacidine A, a novel anti-fungal antibiotic produced by *Pseudomonas cepacia*. J. Antibiotics 47: 1402～1418, 1994
9) LEISINGER, T. & R. MARGRAFF: Sencondary metabolites of the fluorescent Pseudomonads. Microbiol. Rev. 43: 422～442, 1979
10) STEVENSON, I. L. & J. W. ROUATT: Qualitative studies of soil microorganisms. Can. J. Botany. 31: 438～447, 1953
11) KIM, K. K.; J. G. KANG, Y. L. CHOI, H. D. YUN, H. S. HA & K. Y. KANG: Characterization of an anti-fungal compound isolated from an antagonistic fungus *Aspergillus terreus* against phytopathogenic fungi. Kor. J. Pesti. Sci. 2: 40～45, 1998
12) KIM, K. K.; J. G. KANG, S. S. MOON & K. Y. KANG: Isolation and identification of anti-fungal N-butylbenzenesulphonamide produced by *Pseudomonas* sp. AB2. J. Antibiotics 53: 131～136, 2000
13) PALLERONI, N. J.: Family I. *Pseudomonadaceae*, *In* BERGEY's Manual Systemic Bacteriology. Volume 1. *Ed*., N. R. KRIEG *et al*., pp. 141～218, Williams & Wilkins, 1989
14) SMIBERT, R. M. & N. R. KRIEG: Phenotypic Characterization, *In* Methods for General and Molecular Bacteriology. *Ed*. P. GERHARDT, W. A. WOOD, N. R. KRIEG, pp. 607～654, American Society for Microbiology, Washington, D. C., 1994
15) KANG, J. G.; S. T. KIM, K. Y. KANG: Production of the anti-fungal compound phenylacetic acid by antagonistic bacterium *Pseudomonas* sp. Agric. Chem. Biotechnol. 42: 197～201, 1999
16) BALLARD, R. W.; N. J. PALLERONI, M. DOUDOROFF, R. Y. STANIER & M. MANDEL: Taxonomy of the aerobic pseudomonad: *Psueudomonas cepacia*, *P. marginiata*, *P. alliicola* and *P. caryophylli*. J. Gen. Microbiol. 60: 199～214, 1970
17) JANISIEWIEZ, W. J. & J. ROITMAN: Biological control of blue mold and gray mold on apple and pear with *Pseudomonas cepacia*. Phytopathology 78: 1697～1700, 1988
18) WELLER, D. M: Biological control of soil borne pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathol. 26: 379～407, 1988
19) SASCHA, R.; A. DANILO, K. STEPHAN, E. MONIKA, L. THIERRY, C. PETER, E. F. GERHARD & R. N. CHRISTIAN: In silico screening with benzofurane- and benzopyrane-type MDR modulators. Il Farmaco. 58: 185～191, 2003