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Phylogenetic Analysis on the Bacteria Producing Non-Volatile Fungistatic Substances

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This study characterized the soil bacteria producing non-volatile fungistatic substances. Among the 2,100 colonies of soil bacteria randomly isolated from seven agricultural soil samples, 518 isolates (24.67% of total) showed fungistatic activity toward nematophagous fungi *Paecilomyces lilacinus* and *Trichoderma viride* by producing non-volatile substances. A phylogenetic analysis based on amplified ribosomal DNA restriction analysis (ARDRA) and 16S rDNA sequence placed the 518 bacteria in three groups of the domain *Bacteria*: *Actinomycetales*, *Bacillales*, and *Gammaproteobacteria*. Three genera, *Arthrobacter*, *Bacillus*, and *Pseudomonas*, were the most frequently encountered groups.

***Keywords*:** soil fungistasis, bacteria, nematophagous fungi

In recent years, a great many chemical pesticides have been or are being phased out (e.g., organochlorine insecticides and methyl bromide) either because of potential human health risks, environmental pollution, effects on non-target organisms or the development of pest resistance. This provides an opportunity for development of biological control agents (BCAs) as environmental friendly alternative for stimulation of plant productivity. BCAs could be used where chemical pesticides are banned or being phased out or where pests have developed resistance to conventional pesticides. In recent years, a number of fungi, e.g. mycoparasites such as *Trichoderma* spp., *Gliocladium* spp., *Fusarium* spp., *Phlebiopsis gignatea*, entomogenous fungi such as *Metarhizium anisopliae*, *Beauveria bassiana*, nematophagous fungi such as *Myrothecium verrucaria* and fungal pathogen of weed (*Alternaria* spp. and *Colletotrichum* spp.), have been developed into commercial BCAs and used widely in agriculture for plant protection (Butt *et al*., 2001). However, the BCAs still face a number of obstacles, including the inconsistent performance in the field. The control effect of BCAs in fields could be affected by considerable factors, ranging from agent quality to biological and abiotic factors in soils. To control targets, a prerequisite is that the BCAs should establish their populations in fields. Unfortunately, most BCAs could not germinate and grow normally in soils due to the soil fungistasis (Zhou and Mo, 2002). The negative effects of fungistasis on fungal agents should be given more attention. In our previous studies, bacteria producing fungistatic volatiles had been reported (Xu *et al*., 2004; Zou *et al*., 2007). The objective of this study was to characterize the fungistatic bacteria producing non-volatile substances by 16S rDNA sequence analysis and ARDRA.

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Materials and Methods

Soil sampling

Soils were collected from seven agricultural fields respectively located in seven counties of Yunnan Province, China (Table 1). In a field, about 2 kg of soil was sampled randomly from the top layer (2~15 cm) over an area of more than 100 m^2. After being sieved through a 2 mm sieve, the samples were stored at 4°C until used.

Measurement of soil fungistasis and isolation of fungistatic bacteria

Fungistasis of soil was determined according to Xu *et al*. (2004). Soil bacteria were randomly isolated from soils following Zou *et al*. (2007). To isolate the fungistatic bacteria, on a water agar medium, a 15 mm BPSA (0.37 g beef extract, 1.2 g peptone, 0.6 g NaCl, 1 L soil extract, and 15 g agar). To prepare soil extract, 1 kg of soil from each corresponding site was suspended into 1 L of tap water and shaken for 30 min at 200 rpm. After filtration, the soil suspension was adjusted to 1 L and autoclaved. Disc removed from the actively growing colony of individual isolate was co-cultured with a 7 mm Potato Dextrose Agar (PDA) disc of target fungi, *Paecilomyces lilacinus* (CGMCC No. 0241) and *Trichoderma viride* (CGMCC No. T6-12). *P. lilacinus* (a nematophaogus fungus) and *T. viride* (a mycoparasite) have been developed into bio-agents respectively for some species of parasitic nematodes and phytopathgens (Chen *et al*., 2004; Liu *et al*., 2004). For their sensitivity to soil fungistasis, these two fungi were selected as targets. A same size of BPSA disc was used to replace the bacterial culture as the control. Three duplicates were done for each strain. After incubation of 72 h at 28°C, the diameter of fungal colony

Table 1. Soil samples and their fungistatic intensities

Sample	Sampling location	Vegetation	Growth inhibition (%)	
			T. viride	*P. lilacinus*
I	Yuxi county (24°14'N,102°68'E)	Cabbage	91.0±1.2	92.5±1.7
II	Honghe county (24°13'N, 103°39'E)	Sugarcane	94.7±0.3	90.3±2.3
III	Mengzi county (23°32'N, 103°44'E)	Megranate	93.9±0.2	90.1±1.0
IV	Jianshui county (23°46'N, 103°24'E)	Sweet potato	94.5±0.3	92.9±3.6
V	Yiliang county (24°38'N, 103°43'E)	Grape	84.1±4.7	87.1±0.5
VI	Jianshui county (23°46'N, 103°24'E)	Maize	90.5±2.4	88.9±0.4
VII	Kaiyuan county (24°23'N, 102°55'E)	Tobacco	85.1±1.8	83.3±0.2

Table 2. 16S rDNA ARDRA types of bacteria identified from different samples

ARDRA type	Isolate number and origin							Total
	I	II	III	IV	V	VI	VII	
Z9	6	5	6	9	15	15		56
Z7	6	11	13	6	3	6	7	52
I2	9	9	2	11	9	3	6	49
I3	3	11	9	3	6	8	6	46
I1	15	3	7	3	3	4	9	44
Z10		3	6	7	2		8	26
R4		3	6	6		3	6	24
R5	3			3	6	8	3	23
B		3	3	7	6	3		22
Z8		3			6	6	6	21
F1	3			7			2	12
F3	3		1	8				12
Z2	3			3	6			12
R1		5	3			3		11
S1		5				6		11
T2	3		2		3		3	11
R2		6			4			10
Z6		3			3	3		9
F2	5			3				8
Z11		1		2			4	7
F4		3					3	6
K2						3	3	6
R6	4					1		5
O2				2	3			5
M						4		4
Z3			3					3
Z5		3						3
W2						3		3
D		2					1	3
Z1		1	1					2
Z4							2	2
R3					2			2
O1					1	1		2
P					2			2
K1		1				1		2
S2		1						1
C				1				1

was measured and the growth inhibition (GI) formed by the bacteria was calculated (Zou *et al*., 2007). After examining production of volatile fungistatic substances by applying the three-compartment Petri dish method (Zou *et al*., 2007) those bacteria producing such compounds were excluded from further studies.

DNA extraction and PCR amplification

Genomic DNA of bacteria was extracted using a bacterial genomic DNA Extraction Kit (BioTeke Corporation, China, Cat#:DP2001). Bacterial 16S rRNA genes were amplified by PCR using the combination of universal primer 1492r and bacterial primer 27f (Lane, 1991). PCR amplification was carried out in a BIO-RAD PCR Mycycler thermal cycler (Eastwin Life Science Inc.). Initial DNA denaturation and enzyme activation steps were performed at 95°C for 4 min, followed by 30 cycles of denaturation at 94°C for 40 sec, annealing at 62°C for 40 sec, and extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. The amplified products for amplified rDNA restriction analysis (ARDRA) were purified with Agarose Gel DNA Purification Kit (TaKaRa, code DV805A).

ARDRA analysis of 16S rDNA sequence of fungistatic bacteria

To select isolates for sequencing, the purification products were analyzed by ARDRA (Lagace *et al*., 2004) using separate enzymatic digestions with *Rsa*I (Promega), *Alu*I (TaKaRa), and *Hae*III (TaKaRa) endonucleases following the manufacturer's instructions. The digested DNA fragments were electrophoresed in 4.4% agarose gels. After staining with ethidium bromide, the gels were photographed using an image-capture system UVITEC DBT-08, and scanning image analyses were performed manually.

DNA sequencing and phylogenetic analysis

One to six representative clones from each unique ARDRA type were submitted to Beijng Genomics Institute for sequencing. The resulting sequences of 16S rRNA gene (about 1,380 bp in length) were compared with those available in GenBank by use of the BLAST network service to determine their phylogenetic affiliation. Phylogenetic analysis was performed using the MEGA software packages (Version 3.1) (Kumar *et al*., 2004) after multiple alignment of data by CLUSTAL X (Thompson *et al*., 1997), with gaps treated as missing data. Clustering was performed by using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 resamplings (Felsenstein *et al*., 1985).

The 16S rRNA gene sequences have been deposited in the GenBank database under accession numbers (EU182830-EU182901).

Data analysis and statistics

Data were analyzed using analysis of variance (ANOVA), and means were compared by the test of least significant difference (LSD) at $P=0.05$ using SPSS 11.0 for Windows (SPSS Inc., USA).

Results

Measurement of soil fungistasis and isolation of fungistatic bacteria

Soil fungistasis was evaluated in terms of reduced radial growth of the tested fungi. All of the 7 soil samples strongly inhibited the mycelial growth of *T. viride* and *P. lilacinus* with GI of 84.1%~94.7% and 83.3%~92.9%, respectively. Though there were significant differences in fungistatic intensities among the samples ($P<0.01$), the value of fungistatis higher than 80% indicated that soil fungistasis was one of the greatest obstacles for fungi to grow in natural soil environment.

Totally, 2,100 colonies of soil bacteria were randomly isolated from the seven soil samples (300 from each sample). After fungistatic test, the 518 isolates (24.67% of total) could reduce mycelial growth of targets, in which the proportion of bacterial isolates showing strong (GI > 75%), moderate (75% > GI > 50%), low (50% > GI > 25%) and slight (25% > GI > 10%) fungistatic activities were 19.3%, 58.05%, 20.1%, and 2.13%, respectively. Of the 300 evaluated isolates of each sample, the proportion of fungistatic bacteria ranged from 25.7% (sample III) to 41.0% (sample VI). This study only focused on the 518 isolates producing non-volatile fungistatic substances, while 322 isolates in the total 2, 100 isolates showed production of volatile inhibitors. Among the fungistatic isolates of the genera *Pseudomonas* and *Bacillus*, the proportions of isolates producing both non-volatile and volatile substances were 85.1% and 42.7%, respectively.

ARDRA analysis

The 16S rRNA genes of the 518 fungistatic bacteria were successfully amplified using primer pair of 1492r and 27f, resulting in a characteristic single band of about 1,500 bp in length. All PCR products were analyzed by ARDRA using separate enzymatic digestions with endonucleases *Rsa*I, *Alu*I, and *Hae*III. Totally, 37 different ARDRA types were generated (Table 2). The five largest groups, type Z9, Z7, I2, I3, and I1, together accounted for 47.7% (10.8%, 10.0%, 9.6%, 8.9%, and 8.5% respectively from each) of the isolates analyzed. The groups of Z10, R4, R5, B, and Z8, which included 21~26 isolates, were also relatively large. The remaining 27 ARDRA patterns were represented by 1~16 isolates (Table 2).

Phylogenetic analysis

Based on ARDRA pattern, 72 representative isolates, at least one from each unique ARDRA type, were selected for 16S rDNA sequencing. The resulting sequences, about 1380 nucleotides, were compared with those available in GenBank by using the BLAST network service to determine their approximate phylogenetic affiliation (Table 3 and Fig. 1). It was established that all the sequences were identical to 16S rDNA sequences available in GenBank as of October 2007, and all were closely related to the cultivated bacteria members (sequence similarity of 96~100%). Phylogenetic analyses placed the 518 fungistatic isolates in the following three groups of the domain *Bacteria* (Fig. 1): *Actinomycetales* (17.2% out of 518 isolates), *Bacillales* (42.3%), and *Gammaproteobacteria* (40.5%).

Of the three groups, the *Bacillales* was the dominant one. This group contained 219 isolates represented by 18 ARDRA types (Z1-Z11, C, D, P, S1, S2, K1, and K2) and were phylogenetically associated with six different genera of the *Bacillales* (97∼100% similarities): *Bacillus* (208 isolates, 11 types), *Exiguobacterium* (12, 2), *Paenibacillus* (8, 2), *Lysinibacillus* (2, 1), *Sporosarcina* (3, 1), and *Staphylococcus* (1, 1) (Table 2 and 3). The members of types Z9 (56 isolates) and Z7 (52 isolates) were the most frequently encountered. They were found phylogenetically associated with the species *Bacillus subtilis* and *B. pumilus*, respectively.

The *Gammaproteobacteria* represented the second dominant group. Members of this group (210 isolates) could be divided into 9 ARDRA types (F1, F2, F3, F4, I1, I2, I3,

Table 3. List of bacterial isolates obtained in this study representing each ARDRA group and their closest affiliation according to the partical 16S rRNA gene

Isolate	GI[a] value	ARDRA group	The closest relative in the database [accession no.]	Sequence similarity (%)
MH38	96.2±2.0	F1	*Acinetobacter calcoaceticus* [AF417872]	99
MH40	72.4±3.5	F1	*Acinetobacter calcoaceticus* [AF417872]	100
MH04	58.7±4.0	F2	*Acinetobacter haemolyticus* [AY047216]	97
MH27	20.7±3.5	F3	*Acinetobacter johnsonii* CAI-6 [DQ257426]	96
MH03	41.5±1.5	F3	*Acinetobacter johnsonii* P152 [AF188300]	99
MH07	20.3±4.0	F4	*Aeromonas hydrophila* AE-53 [AY987732]	100
MH09	25.9±1.5	R1	*Arthrobacter arilaitensis* 8ME [DQ361023]	99
MH13	47.0±5.0	R2	*Arthrobacter dextranolyticus* T6 [AB117515]	99
MH64	22.9±3.5	R3	*Arthrobacter globiformis* TUT1005 [AB098573]	100
MH30	95.2±2.7	R4	*Arthrobacter nitroguajacolicus* CCM 4924T [AJ512504]	98
MH61	81.1±4.5	R5	*Arthrobacter oxydans* CF-46 [AJ243423]	99
MH14	94.9±3.5	R6	*Arthrobacter polychromogenes* c311 [AB167181]	98
MH47	45.2±2.5	Z1	*Bacillus barbaricus* V2-BIII-A2 [AJ422145]	97
MH12	12.8±2.5	Z2	*Bacillus cereus* 39240 [DQ152243]	99
MH66	80.4±4.5	Z3	*Bacillus horikoshii* DSM 8719 [X76443]	99
MH10	93.5±0.5	Z4	*Bacillus koguryoae* SMC 4352-2 [AY904033]	98
MH39	15.5±1.5	Z5	*Bacillus luciferensis* KSC_SF5b [DQ870692]	99
MH52	27.3±1.5	Z6	*Bacillus megaterium* A10-2 [AB244298]	100
MH02	59.6±3.5	Z7	*Bacillus pumilus* A14-2 [AB244427]	99
MH56	34.7±3.5	Z7	*Bacillus pumilus* A14-2 [AB244427]	97
MH06	88.9±3.5	Z8	*Bacillus simplex* WN570 [DQ275175]	100
MH46	23.6±4.5	Z9	*Bacillus subtilis* BCRC 10058 [DQ993674]	99
MH11	66.9±5.0	Z10	*Bacillus thuringiensis* ATCC10792 [AF290545]	99
MH29	15.6±2.5	Z11	*Bacillus velezensis* C6-1 [AB244462]	100
MH41	76.3±4.0	W2	*Brachybacterium fresconis* LMG 20333 [AJ415379]	97
MH21	63.8±5.0	S2	*Exiguobacterium acetylicum* DSM 20416 [DQ019167]	99
MH70	24.1±4.5	S1	*Exiguobacterium antarcticum* DSM 14480 [DQ019164]	98
MH49	62.5±4.0	O2	*Kocuria himachalensis* K07-05 [AY987383]	100
MH26	47.9±4.0	O1	*Kocuria rhizophila* DSM 11926 [Y16264]	98
MH33	77.6±2.5	P	*Lysinibacillus fusiformis* DSM 2898T [AJ310083]	100
MH24	87.7±1.5	T2	*Lysobacter gummosus* KCTC 12132 [AB161361]	97
MH54	78.1±3.5	M	*Micrococcus luteus* [AB167385]	100
MH74	48.5±1.0	K2	*Paenibacillus barcinonensis* BP-23[AJ716019]	100
MH67	44.3±0.5	K1	*Paenibacillus chondroitinus* DSMZ5051T [D82064]	97
MH05	94.7±3.5	I3	*Pseudomonas fluorescens* A1XB1-4 [AY512614]	99
MH36	77.7±0.5	I2	*Pseudomonas putida* IA2XCDB [AY512613]	99
MH73	75.9±5.0	I2	*Pseudomonas putida* IH-2000 [AB029257]	99
MH08	88.5±2.5	I1	*Pseudomonas stutzeri* KC [AF067960]	99
MH75	55.7±3.0	D	*Sporosarcina saromensis* HG711 [AB243864]	99
MH37	41.9±0.5	C	*Staphylococcus epidermidis* KL-004 [AY030340]	99
MH58	29.0±4.0	B	*Stenotrophomonas maltophilia* Es2-5 [AY367030]	99
MH34	44.1±3.5	B	*Stenotrophomonas maltophilia* LMG 10883 [AJ131906]	99

[a] GI: growth inhibition of bacteria to target fungi, this value was calculated following the formulation of Zou *et al*. (2007)

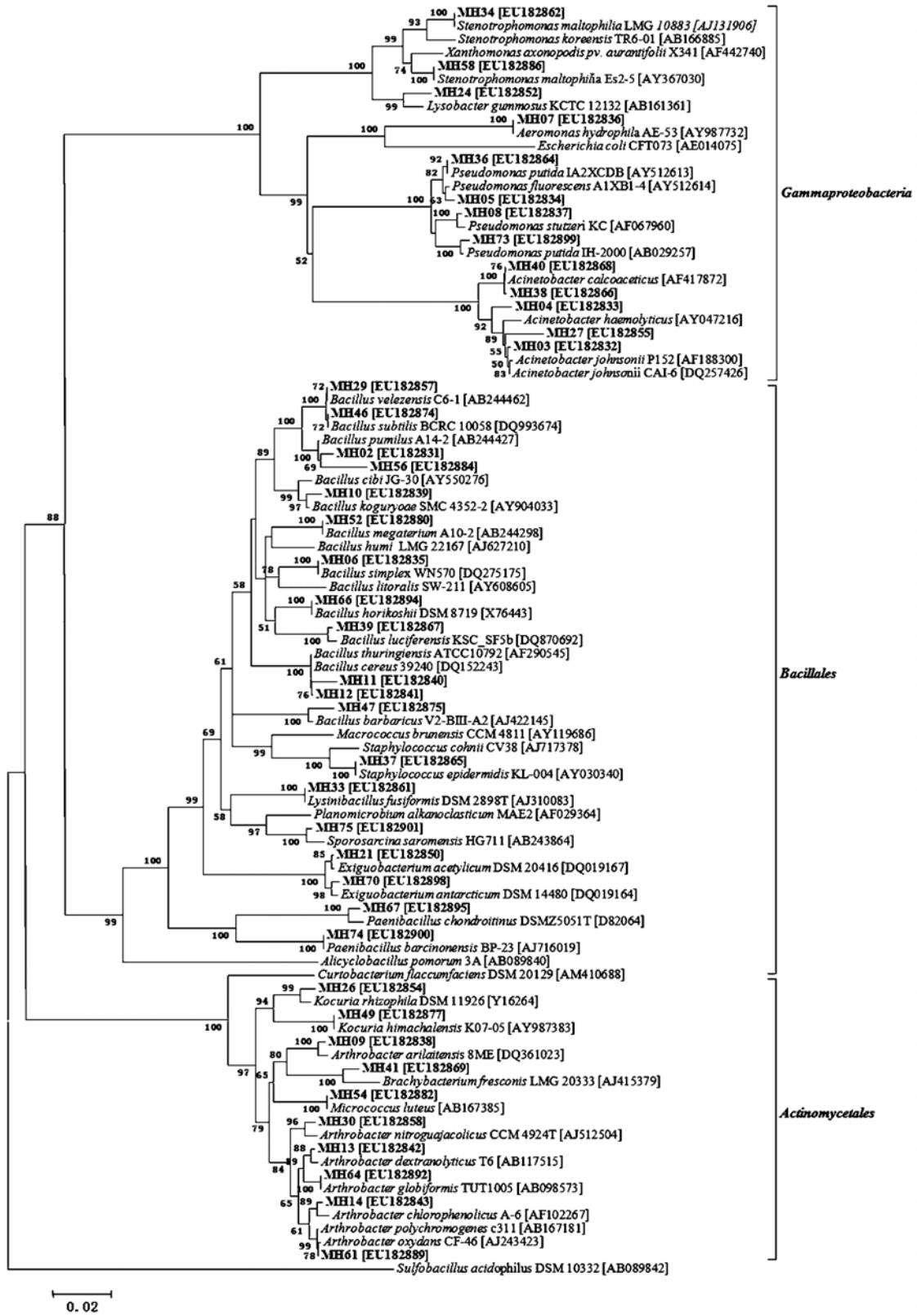

Fig. 1. 16S rDNA-based dendrograms showing phylogenetic relationships of fungistatic bacteria (shown in bold) to closely related sequences from public database. Bootstrap values (n=1000 replicates) of ≥50% are reported as percentages. The scale bar represents the number of changes per nucleotide position. *Sulfobacillus acidophilus* (AB089842) are used as outgroups. Accession numbers are given at the end of each sequence.

T2, and B). Their sequences matched to five genera of *gammaproteobacteria* (97∼100% similarities): *Acinetobacter* (32 isolates, 3 types), *Aeromonas* (6, 1), *Lysobacter* (11, 1), *Pseudomonas* (139, 3), *Stenotrophomonas* (22, 1). Among these groups, the types I1 (44 isolates), I2 (49 isolates) and I3 (46 isolates) were the most frequently encountered. They were found phylogenetically associated with the species *Pseudomonas stutzeri*, *P. putida*, and *P. fluorescens*, respectively.

The group of *Actinomycetales* included 89 isolates represented by 10 ARDRA types (R1-R6, O1, O2, W2, and M). They were phylogenetically associated with nine genera of *Actinomycetales* (97∼100% similarities): *Arthrobacter* (75 isolates, 6 types), *Brachybacterium* (3, 1), *Kocuria* (7, 2) and *Micrococcus* (4, 1). Within this group, types R4 (24 isolates) and R5 (23 isolates) were the most frequently encountered. They were found phylogenetically associated with the species *Arthrobacter nitroguajacolicus* and *A. oxydans*, respectively.

Discussion

Soil microorganisms are a causal agent of soil fungistasis. The most popular hypotheses to explain the microbial cause of fungistasis, were the nutrient-deprivation (Lockwood, 1964, 1977) and the germination inhibitor hypothesis (Romine and Baker, 1973). The latter hypothesis stated that fungistasis could be caused by antifungal compounds produced by soil microorganisms. Microbial antifungal compounds suppressed fungi by (1) interfering with conidial germination and germ tube elongation (Silo-Suh *et al*., 1994; Thrane *et al.*, 1997); (2) inhibition resulting from unusual hyphal swelling (Lim *et al.*, 1991); (3) lysis and complete degradation of the hyphal tip (Lim *et al*., 1991). In previous studies, several volatile fungistatic compounds from soil have been reported, including ethylene, ammonia, methyl vinyl ketone (Lockwood, 1977; Herington *et al*, 1987), trimethylamine, 3-methyl-2-pentanone, dimethyl disulfide, methyl pyrazine, 5-dimethylpyrazine, benzaldehyde, N,N-dimethyloctylamine and nonadecane (Xu *et al*., 2004). In addition, volatile compounds of bacterial origins (acetamide, benzaldehyde, benzothiazole, 1-butanamine, methanamine, phenylacetaldehyde, and 1-decene) were proposed to play important roles in soil fungistasis (Zou *et al*., 2007).

The results of Liebman and Epstein (1992) indicated that water-soluble and possibly nonvolatile fungistatic compounds occurred in a variety of soils. Recently, we purified a fungistatic compound of the iturin A group from *Bacillus* sp. strain H6, a strain representing the dominant colony type isolated from fungistatic soils (Li *et al*., 2007). This non-volatile inhibitor showed strong inhibitory activity against nematophagous fungi by inducing unusual swelling of conidia and germ tubes, thereby preventing the fungi from proliferating.

In this study, 24.67% of 2,100 randomly selected bacteria produced non-volatile fungistatic inhibitors. Among them, members of the *Bacillales* contributed the most. Species of *Bacillus* have been repeatedly reported to produce antifungal compounds and been implicated to function as antagonists of plant pathogens (Ryder *et al*., 1999; Bhaskar *et al*., 2005). This group of bacteria exhibited fungistatic activity mediated by producing volatiles (Zou *et al*., 2007) as well as non-volatile inhibitors (Li *et al*., 2007; this study). De Boer *et al*. (2003) concluded that the presence and antifungal activity of *Pseudomonadaceae* could be essential in soil fungistasis. In this investigation, 139 isolates (represented by ARDRA types of I1, I2, I3) of *Pseudomonas* were the most frequently encountered and phylogenetically associated with the species *Pseudomonas stutzeri*, *P. putida*, and *P. fluorescens*, respectively. Additionally, 75 isolates (represented by 6 ARDRA types) of the genus *Arthrobacter* were found to suppress fungal growth with non-volatiles substances (this study), although members of this genera have been indicated to involve in soil fungistasis by producing volatiles of 1-butanamine, benzothiazole, benzaldehyde (Zou *et al*., 2007). This study revealed that besides volatile inhibitors, non-volatile fungistatic compounds including their phylogenetically diverse producer strains are important components of the soil fungistasis effect. Species of the genus *Streptomyces* are known as major antibiotic producers but not be found in this investigation. This maybe due to the medium BPSA used is unsuitable for this group.

Conclusively, results from this study indicate that the spore germination of fungi will be suppressed severely by non-volatile inhibitors from many kinds of soil bacteria. However, the molecular mechanism of fungistatic compounds inhibiting germination is unknown. This is one of the vital issues for us to understand the phenomenon of soil fungistasis.

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