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Full Length Research Paper

Antagonistic bioactivity of endophytic strains isolated from *Salvia miltiorrhiza*

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The antibiotic-producing potential of endophytic populations from medical plant of *Salvia miltiorrhiza* was examined. A total of 63 isolates was screened against five fungal and three bacterial species for the production of antimicrobial compounds. It showed that more isolates was antagonistic to fungi than to bacteria. One bacterial isolate named DX30 showed broad-spectrum of antagonistic activity against all the target organisms in plate assays. Morphology, physiological, chemical characteristics and 16S rRNA gene sequence analysis of strain DX30 demonstrated that it belonged to the genus *Bacillus*. The strain DX30 had maximum activity at temperature of 25°C and pH of 6.0 respectively and remained stable activity at temperature between 30 and 100°C and pH between 6 and 12. Strain DX30 seems promising in the biological control.

Key words: Endophytes, antimicrobial bioactivity, 16S rRNA gene, *Salvia miltiorrhiza.*

INTRODUCTION

Endophytes are organisms inhabiting in plant tissues whether they are neutral, beneficial or detrimental to hosts (Sturz et al., 2000). In recent years, many researches have focused on the bioactivities of endophytes (Strobel, 2003; Cui et al., 2003). In endosphere, endophytes are in protected environment that gives them competitive advantage over organisms of the rhizosphere and phyllosphere. Because of living in a relatively steady environment, endophytes may be much more bioactive (Dowler and Waiver, 1974; Andrews, 1992). The future use of biological–chemical combinations of endophytes in combination with commercial pesticides applied to seed or seedling could lead to synergistic effects on one or multiple disease causing agents. Moreover, the biological agent could provide continuing effective control into the crops production cycle (Shoda, 2000).

Nowadays, it is well known that excess use of fungicides leads to severe large-scale pollution. With increasing awareness of environmental protection, biological strategies using endophytic bacteria are new promising alternatives to solving the contamination problems. Therefore, biological pesticides, especially endophytic bacteria, is an effective and environmental tool in controlling plant diseases instead of using chemical pesticide (Kong et al., 2001).

Salvia miltiorrhiza is a famous Chinese medicinal plant, its root stalk is used for the treatment of angina, coronary heart disease, etc (Zhang and Ma, 1997). During the cultivation, some diseases such as ceitocybe bescens were found, which affects the quality of the root product. Use of chemical pesticide will not only contaminate the environment, but also pollute residue on the roots (Fu et al., 2008).

The aim of this study was to isolate antagonistic strains from tissues of *S. miltiorrhiza* and characterize bio-activities. In our study, strains with ideal antifungal activity were identified. Among them, an isolate named Dx30 was chosen for further study.

MATERIALS AND METHODS

Tested pathogens and media

The tested pathogens for antagonism are *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Candida albicans*, *Rhizotonia solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum* and *Curvularia lunata*, which were all stored in our laboratory. Potato dectrose agar (PDA) was used for antagonistic tests and fungal phytopathogens maintaining. Nutrient agar (NA) was used for bacteria culture.

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Isolation of the endophytic bacterium strains

The healthy plant material of *S. miltiorrhiza* was collected from the test field in Yangling, Shaanxi Province, China. Then the plant samples were placed in a plastic bag stored in an ice box and transported to the laboratory as soon as possible. Samples were flushed with running water to remove the soil. The stems and roots were cut into 10 × 10 mm pieces and treated with 75% ethanol for 1 min, 5.2% NaClO for 3 min, and then rinsed three times with sterilized distilled water. The inner part of the tissues were all placed on water agar (WA) medium (containing 60 µg/ml streptomycin and 100 µg/ml ampicillin) and the plates were incubated at 28°C for 3 to 7 days. The colonies were picked out and streaked on NA and PDA to get the pure culture. After purification, the isolates were cultivated in 5 ml of NA or PDA liquid medium with constant shaking at 28°C for 2 to 4 days. The culture was suspended in 20% glycerol solution and stored at -80°C.

Antimicrobial activity assay

The inoculum of endophytes were prepared from cultures incubated in NA or PDA liquid medium at 28°C for 3 to 5 days with constant shaking. 20 ml of the fresh liquid culture was centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was transferred to a new centrifuge tube and filter sterilized. The supernatant was used for the tests.

For the antibacterial assay, the tested pathogens (including *C. albicans*) were cultivated in Erlenmeyer flask with 100 ml liquid NA (or PDA) medium at 28°C for 2 to 3 days. The cultures were spread on the Petri dishes containing 25 ml NA (or PDA). The following procedures were carried out as described previously.

All the tested plates were incubated at 28°C for 24 h (for bacteria and *C. albicans*) or 72 h (for fungi) and then the diameters of the inhibition zones were observed. All the experiments above were repeated 3 times with 3 replications and distilled water was used as CK.

Characterization of the endophytic bacterium strain DX30

The physiological and biochemical tests for strain DX30 were carried out for preliminary characteristics (Knösel, 1984). 16S rDNA sequence analysis was also conducted for further identification. Genomic DNA of DX30 was extracted as follows: a single colony of the bacterial strain grown on NA for 2 days was picked out with a sterilized toothpick and suspended in an Eppendorf tube with 50 µl TE buffer. The tube was heated in boiling water for 5 min, and centrifuged at 12000 rpm for 5 min. The supernatant was transfered to a new Eppendorf tube and used as DNA template. The 16S rRNA gene sequence was amplified by primers BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3'), BSR1541/20 (5'-AAGGA GGTGATCCAGCCGCA-3'). The amplification was performed as the following program: initial denaturation (5 min at 94°C), 35 cycles of denaturation (60 s at 94°C), annealing (30 s at 55°C) and extension (90 s at 72°C), and final extension (10 min at 72°C). Polymerase chain reaction (PCR) product was checked by gel electrophoresis in 1.0% agarose gel stained with ethidium bromide (EB). The PCR product was purified by DNA gel extraction kit (Axygen, USA) and the purified PCR product was used directly for sequencing. The 16S rRNA gene sequence of the strain was aligned by Clustal_X version 1.8 (Thompson et al., 1997) with the 16S rRNA gene sequences of recognized species of the genus *Bacillus* obtained from the GenBank/EMBL/DDBJ database. A phylogenetic tree was constructed using neighbour-joining (Saitou and Nei, 1987) method in the TREECON software package version 1.3b (Van de Peer and De Wachter, 1994). The genetic distance matrices were estimated by the Kimura two-parameter model (Kimura, 1980). The topology of the tree was evaluated by bootstrap analysis based on 100 replicates (Felsenstein, 1985).

Physiological characterization

Methods and media used for physiological test were as described by Luedemann and Brodsky (1964) and Luedemann (1971). All the cultures were incubated at 28°C for 10 days. The assay for enzymatic activity was performed according to Hopwood (1957).

Morphological characterization

The morphology of the spores was determined by light microscopy. A small peripheral portion of well-grown mature part of the colony was picked using a sterile inoculation loop. The sample was then transferred to a glass slide and observed under Nikon photo micrographic unit at the magnification of 1000X.

The stability of bioactivity under different pH and temperature

Antagonistic activity under different temperature, pH, and UV were evaluated. The supernatant were prewarmed to temperature gradient from 30 to 100°C in water bath at an interval of 10°C, and then cooled to room temperature. The pH-dependence was determined by adjusting pH ranged from 2 to 12 at an interval of 2. To determine the effect of UV on bioactivity, the supernatant was exposed to UV for 30 min.

The primitive purification of active compounds and GC-MS chromatograph

The broth of DX30 was centrifuged and the insoluble mycelium remains were removed by centrifugation. The aqueous solution was then extracted with ethyl acetate and the extract applied to a column of silica gel, eluted with a gradient of ethyl acetate - methanol from 100:1 to 10:1. The active fraction was concentrated to dryness. The fraction was analyzed using GC-MS (Agilent 7890A/5975C).

RESULTS

Antimicrobial tests

Altogether, 63 isolates were separated from tissues of *S. miltiorrhiza,* of which 32 were fungus and 31 were bacteria. 26 isolates out from 63 showed bioactivity (Table 1). Strain DX30 showed the widest antagonistic activity against almost all the test targets, especially active to phytopathogen, such as *Rhizotonia solani*, and it was chosen for further study. After analysis of antagonistic activity, *Bacillus subtilis* that was intensively inhibited by DX30 was picked out as the target of further study (Table 1).

The stability of bioactivity under different pH and temperature

The results show that the fermentation broth of Dx30

Table 1. Inhibition zone diameters (mm) of isolates against tested pathogens.

Isolate	Tested pathogen							
	1	2	3	4	5	6	7	8
Dz2	—	—	—	18	—	11	—	—
Dz4	—	—	12	—	26	12	—	—
Dz7	11	—	11	12	37	—	—	—
Dz9	—	11	11	—	23	—	—	14
Dz10	—	—	—	18	34	13	—	12
Dz14	—	20	—	23	—	9	13	8
Dz20	—	—	—	11	20	11	—	8
Dz26	—	—	—	11	24	—	—	—
Dz28	—	—	11	11	22	—	—	—
Dz30	—	—	—	12	25	—	—	—
Dx1	13	—	15	—	25	—	—	15
Dx2	24	—	—	14	18	—	—	—
Dx4	12	—	—	11	23	12	14	-
Dx6	12	15	17	13	—	—	—	20
Dx8	17	20	18	—	26	13	—	—
Dx9	15	—	15	17	27	—	15	12
Dx10	—	20	—	—	22	11	10	8
Dx11	19	—	17	—	—	—	—	—
Dx14	—	22	—	—	26	11	—	17
Dx18	—	17	—	—	29	—	—	9
Dx19	23	—	—	13	26	—	11	9
Dx20	—	—	12	—	—	12	13	12
Dx23	—	—	—	18	19	16	—	—
Dx24	21	—	23	—	21	11	9	11
Dx28	12	—	—	20	18	18	17	8
Dx30	12	17	19	17	29	13	19	13

—, negtive, 1. *Escherichia coli*, 2. *Staphylococcus aureus*, 3. *Bacillus subtilis*, 4. *Candida albicans*, 5. *Rhizotonia solani*, 6. *Fusarium oxysporum*, 7. *Sclerotinia sclerotiorum*, 8. *Curvularia lunata.*

exhibited some degree of stability to heat treatment. As temperature rose, the bioactivity decreased slowly, but the diameter of the inhibition zone remained above 13 mm even at 100°C, which was 31.6% smaller than the original supernatant (Figure 1). Within pH of 6 to12, the activity was consistent, but the antibacterial activity decreased in acidic environment (pH <6) (Figure 2). When treated under UV, the activity decreased apparently, which showed the ultraviolet light can damage the structure of the active substance.

Identification of the endophytic bacterium DX30

The colony of DX30 on NA was yellowish, smooth and opaque. The thallus was rod-shaped. DX30 was characterized as Gram-negative, catalase-positive, methyl red reaction (MR) positive and was capable of producing H_2S. It hydrolyzed starch and liquefied isinglass. When it grows in solution of 5% (v/v) NaCl and the optimal growing temperature was 30 to 37°C.

For further identification of DX30, we amplified the 16S rRNA gene sequence and compared the sequence with sequences from GenBank using BLAST program (Altschul et al., 1990). The 16S rRNA gene sequence of the strain showed 99.443% identity to that of *Bacillus methylotrophicus*. It was indicated that this strain was phylogenetically related to members of the genus *Bacillus*. The phylogenetic tree (Figure 3) showed that DX30 and *B. methylotrophicus* clustered together within one subclade with a bootstrap support of 96%. The two experimental results suggested that strain DX30 should belong to *B. methylotrophicus*.

GC-MS chromatograph of active fraction

The LC-MS analysis indicated that 3-Oxo-4-phenyl-butyronitrile and some organic acids exist in the active fraction. However, further investigations are required to

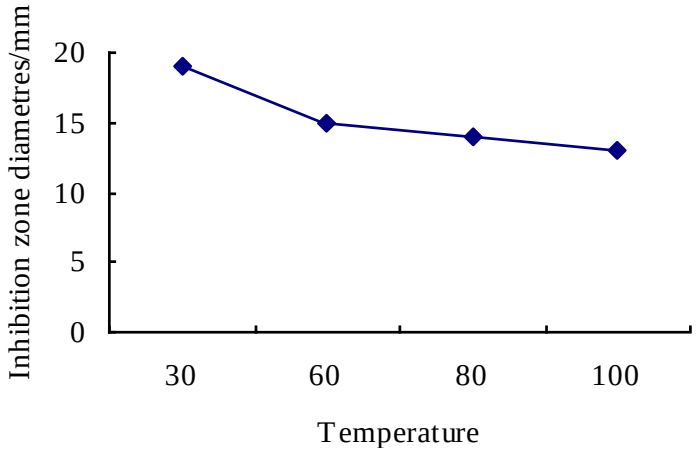

Figure 1. Influence of temperature on activeness.

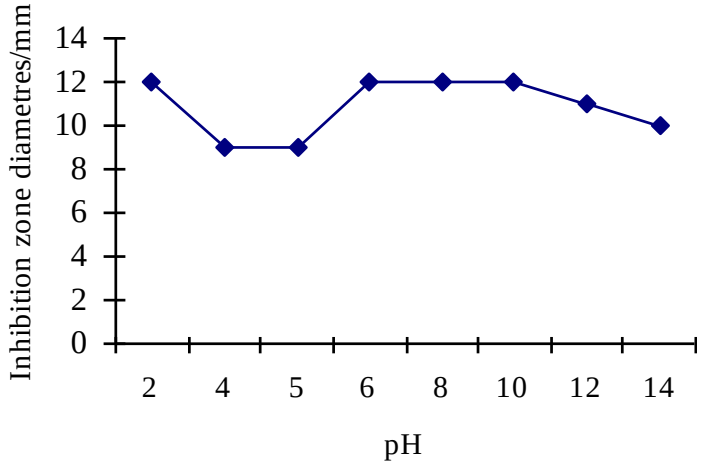

Figure 2. Influence of pH to activeness.

confirm whether there are other activecompounds or not.

DISCUSSION

In the course of screening for novel antimicrobial substances (antibiotics) from endophytes, antibiotics-producing cultures were screened from the tissue of *S. miltiorrhiza*. In recent years, some medicinal plants had been investigated for endophytes (Huang et al., 2008; Guo et al., 2008; Jalgaonwala et al., 2010), but only a few were focused on ndophytic actinomyce -tes *isolatedSalvia miltiorrhiza* (Zhao et al., 2011). Our study demonstrates that many endophytic bacteria also had broad-spectrum antimicrobial activity. As stated earlier, endophytes can enter the inner part of plant and help to prevent the plant from pathogenic infection (Barka et al., 2002; Bargabus et al., 2002) and also produce important bioactive compounds with high commercial value. The presence of relatively large population of antagonistic endophytes in the tissue of *S. miltiorrhiza* indicates that it is a suitable ecosystem that is suitable for the isolation of endophytes. One isolate of endophytic bacterium DX30 inhibited the growth of all tested indicator organisms, making it a good candidate for further studies. DX30 was identified using bacteriological and molecular methods. The morphological, physiological and biochemical characters and 16S rDNA sequence analysis (Figure 3) of the antagonistic strain agreed with those of *Bacillus*. The results based on both mophology demonstrated that the antagonist was a member of *Bacillus*. Because we only performed preliminary physiological and biochemical tests and sequenced only 16S rRNA gene, it was difficult to determine precisely which species it belonged to, further analysis is needed.

Conclusion

In conclusion, the results of this study indicated that Dx30

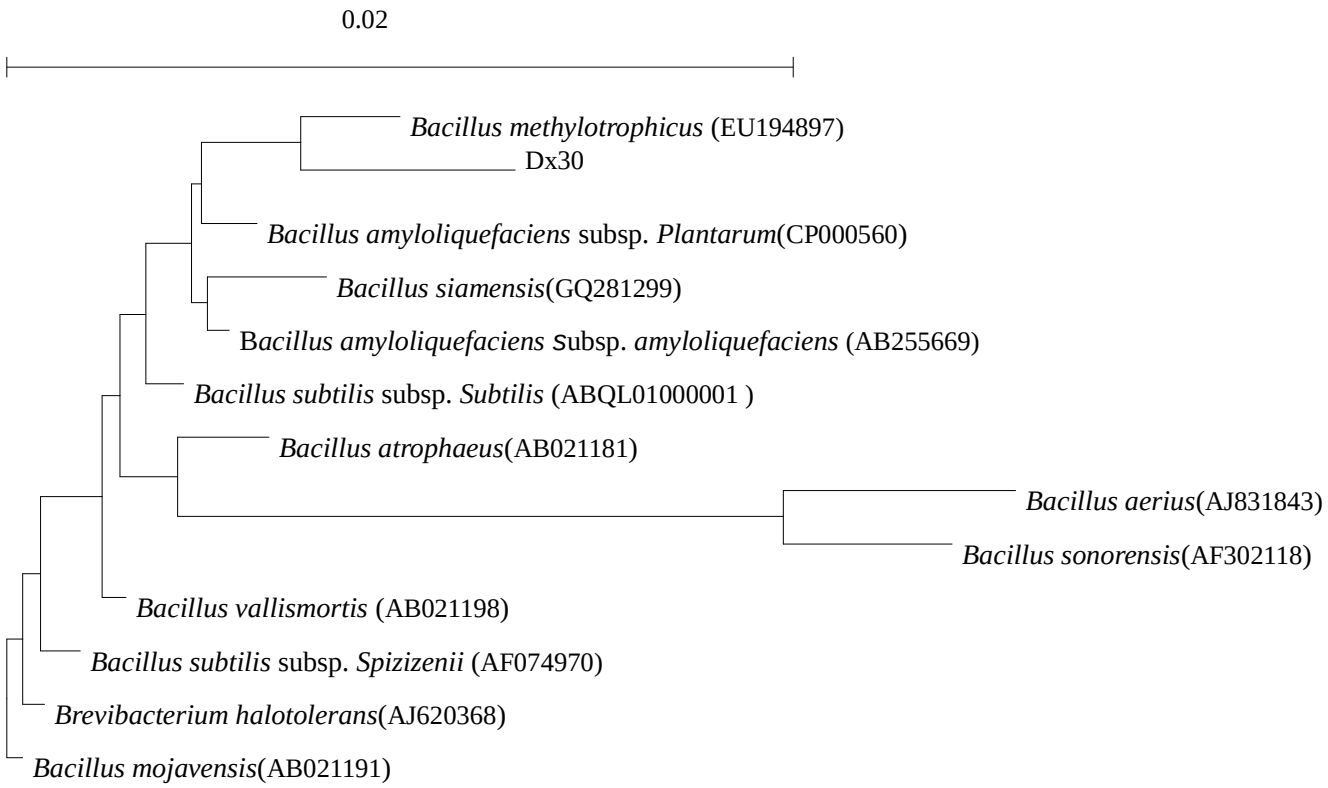

Figure 3. Phylogenetic N-J tree of DX30.

is a broad-spectrum antagonistic bacterium which provides us with new insights into the biological control of plant disease. We will further investigate its colonization on plant roots and DX30 bioactive components, which could be good for the growth stimulation and disease prevention.

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