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Full Length Research Paper

Antagonistic bioactivity of an endophytic bacterium isolated from *Epimedium brevicornu* Maxim

Rong-lin He[1], Guo-ping Wang[2], Xiao-Hong Liu[1], Chu-long Zhang[1]* and Fu-cheng Lin[1]*

[1]State Key Laboratory for Rice Biology, Institute of Biotechnology, Zhejiang University, Kaixuan Road 268, Hangzhou 310029, China.
[2]Dayang Chemical CO. Ltd., Chaoyang Road 22, Dayang town, Jiande, 311616, China.

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Endophytic bacteria are one of the most potential biological control agents in plant disease protection. The aim of this work was to evaluate the antimicrobial activities of a strain of endophytic bacterium which was isolated from *Epimedium brevicornu* Maxim. The dual tests revealed that this endophytic bacterium strain displayed a wide-spectrum antimicrobial activity against 14 fugal phytopathogens and one bacterial phytopathogen; it especially strongly inhibited *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*, *Botrytis cinerea* and *Botrytis fabae*. Identification of this strain based on morphology, physiological and chemical characteristics and 16S rRNA gene sequence analysis demonstrated that it belonged to the genus *Phyllobacterium*.

Key words: Endophytic bacteria, antimicrobial bioactivity, 16S rRNA gene.

INTRODUCTION

Endophytes, by definition, are microorgnasims (mostly fungi and bacteria) which inhabit in healthy living plant tissues for all or part of their life cycle without causing apparent harmful symptoms to the host (Sturz et al., 2000; Wellington and Marcela, 2004). In resent years, much research has focused on the bioactivities of endophytic fungi and endophytic bacteria (Strobel, 2003). Because of living in a relatively steady environment – within plant tissues, endophytes may be much more bioactive than rhizosphere microbes or any other microbes which were isolated from plant surface or soil are (Dowler and Waiver, 1974; Andrews, 1992). As a member of Endophytes, endophytic bacteria have also received more attention on their antagonistic bioactivities including biological control of plant diseases, plant growth stimulation, nitrogen-fixing and so on (Harris et al., 1994; Chen et al., 1995; Graner et al., 2003; Cui et al., 2003; Qiao et al., 2006).

Nowadays, it is known to all that excess use of fungicides leads to severe large-scale pollution. With increasing environmental awareness, developing biological strategies using endophytic bacteria are new alternatives to solving the contamination problems. The biocontrol mechanisms of endophytic bacteria include the production of second metabolites, nutrient and ecological niche competition, and the induction of systemic acquired resistance of the host (Sturz et al., 2000; Kong and Ding, 2001). In fact, some species of *Pseudomonas* and *Bacillus* species have already been successfully used as biological control agents (Braun-Kiewnick et al., 2000; Cartwright et al., 1995; Kudryashava et al., 2005 Shoda, 2000). Therefore, searching for new endophytic bacteria is a way of controling plant diseases by biocontrol methods.

In this paper, an isolate of endophytic bacterium from *Epimedium brevicomum* Maxim was screened for its antimicrobial activity *in vitro*. In the dual culture analysis, it showed a broad-spectrum antimicrobial activity against 14 stains of fungal as well as one stain of bacterial phytopathogen; it especially strongly inhibited the growth of *Alternaria alternata* and *Sclerotinia sclerotiorum*. Finally, the endophytic bacterium strain EBBLQ01 was identified through morphology, physiological and chemical characteristics and homology of 16S rRNA gene sequence by NCBI program BLAST and phylogenetic tree analysis.

MATERIAL AND METHODS

Test phytopathogens and media

16 strains of fungal and 3 strains of bacterial phytopathogens for an-

*Corresponding author. E-mail: clzhang@zju.edu.cn, fuchenglin@zju.edu.cn, Tel: +86 571 8697 1185.

timicrobial activities assays were used in this study. The tested isolates; *Alternaria alternata, Aspergillosis, Botrytis cinerea, Rhizoctonia solani, Sclerotium rolfsii, Pythium ultimum, Fusarium oxysporum* f.sp *cucumber, Colletotrichum orbiculare*, *Verticillium dahliae*, *S. sclerotiorum*, *Pestalotia diospyri*, *Paecilomyces Lilaciaus, Pyricularia oryzae*, *Botrytis fabae*, *Didymella bryoniae, Chryseobacterium* sp., *Pseudomonas solanacearum* and *Pseudomonas cachrymans* 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.

Isolation of the endophytic bacterium strain

The healthy plant materials of *E. brevicomum* Maxim were collected from Longquan, Zhejiang province, China in May 2008. Then the plant samples were placed in a plastic bag within 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 three rinses in sterilized distilled water. The inner part of the root 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 25°C for 3 - 7 days. After incubation, the endophytic bacterial colony appeared and showed antimicrobial activity against other isolates of endophytic fungi which had been isolated at the same time. The bacterial colony was picked out, streaked on NA and incubated at 28°C for 2 days to get the pure culture. After purification, the bacterial isolate (named as EBBLQ01) was cultivated in 5 ml of NA liquid medium with constant shaking at 28°C for 2 days. The culture was suspended in 20% glycerol solution and stored at -80°C.

Antimicrobial activity assay and scanning electron microscopy

The inoculum of EBBLQ01 was prepared from cultures incubated in NA liquid medium at 28°C for 3 - 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 removed to a new centrifuge tube and filter sterilized. Both the bacterial inoculum and supernatant were used for the dual tests.

For the antifungal assay, a plug of mycelium of each fungus (5 mm diameter) was plated at the centre of the Petri dish containing 25 ml PDA and 50 µl liquid endophytic bacterial cultures or supernatant aliquots were put into the holes (5 mm diameter) 3 cm away from the centre. All the tested plates were incubated at 25°C for 5 - 7 days and the inhibition effects were evaluated by measuring the diameters of the inhibition zones.

For the antibacterial assay, the bacterial pathogens were cultivated in Erlenmeyer flask with 100 ml liquid NA medium at 28°C for 2 - 3 days. After being diluted 1000 times, the bacterial cultures were spread on the Petri dishes containing 25 ml NA. The following procedures were carried out as described previously. All the tested plates were incubated at 28°C for 24 h days and then the diameters of the inhibition zones were observed. All the experiments above were repeated at least 3 times with 3 replications and sterilized distilled water was used as CK. For scan electron microscopy (SEM), the samples observations were performed as described everywhere (Liu et al., 2007).

Characterization of the endophytic bacterium strain EBBLQ01

The physiological and biochemical tests were carried out as described for preliminary characterization (Knösel, 1984). For further identification, 16S rDNA sequence analysis was conducted. Genomic DNA of EBBLQ01 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 boiled water for 5 min, and centrifuged at 12000 rpm for 5 min. The supernatant was removed 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'-AAGGAGGTGATCCAGCCGCA-3'). The amplification conditions 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).

PCR product was checked by gel electrophoresis in 1.0% agarose gel stained with ethidium bromide (EB). The resulting PCR product was purified by DNA gel extraction kit (Axygen, USA) and the purified PCR product was used directly for sequencing. Alignments between the sequences were performed by using CLUSTAL W software (Thompson et al., 1997) and a phylogenetic tree was made using PAUP version 3.1.1 (Swofford, 1993).

RESULTS

Antimicrobial tests and scanning electron microscopy observation

The bacterial strain EBBLQ01 was isolated from *E. brevicornu* Maxim. It was resistant to streptomycin and ampicillin and depressed the growth of the endophytic fungi. In the dual tests, EBBLQ01 produced inhibition zones against 14 strains of phytopathogenetic fungi and one strain of phytopathogenetic bacterium. The inhibition zones against *A. alternata*, *S. sclerotiorum*, *V. dahliae*, *B. fabae*, *B. cinerea* and *Rhizopus* sp. were larger than 20 mm. We also found that the bioactivities of ferment aliquots were weaker than that of the liquid endophytic bacterial cultures (Table 1).

After analysis of antagonistic activity, *S. sclerotiorum* which was intensively inhibited by EBBLQ01 was picked out as the model of studying the mechanism of its antimicrobial activity. In the dual test plate, the inhibition zones were obvious and the diameters were 23 mm (Figure 1A). In the SEM photographs of confront culture (Figure 2A), it could be seen that the hyphae of *S. sclerotiorum* first swelled then ruptured, and the cytoplasm started to extravagate outside. In contrast, the hyphae were straight and thrived in normal growth conditions (Figure 2B).

Identification of the endophytic bacterium EBBLQ01

The colony of EBBLQ01 on LB was yellowish, smooth and opaque. The thallus was rod-shaped and motiled by means of a polar flagellum (Figure 1B). EBBLQ01 was characterrized as Gram-negative, catalase-positive, methyl red reaction (MR) positive, Voges-Proskauer (V-P) reaction positive, oxidative metabolism of glucose positive and was capable of producing H_2S. It hydrolyzed starch

Table 1. Antimicrobial activities of EBBLQ01 against phytopathogens.

Phytopathogen	Inhibition zone diameters (mm)	
	Fermentation aliquot	Bacterial culture
Alternaria alternata	16	26
Botrytis cinerea	12	22
Botrytis fabae	13	23
Colletotrichum orbiculare	11	19
Didymella bryoniae	9	16
Fusarium oxysporum f.sp *cucumerium*	9	12
Pestalotia diospyri	11	16
Pythium ultimum	/	/
Rhizoctonia solani	11	17
Paecilomyces Lilaciaus	8	13
Rhizopus sp.	13	22
Sclerotinia sclerotiorum	14	23
Sclerotium rolfsii	/	/
Verticillium dahliae	14	23
Penicillum digitatum	13	19
Aspergillosis sp.	11	16
Chryseobacterium sp.	12	17
Pseudomonas lachrymans	-	-
Pseudomonas solanacearum	-	-

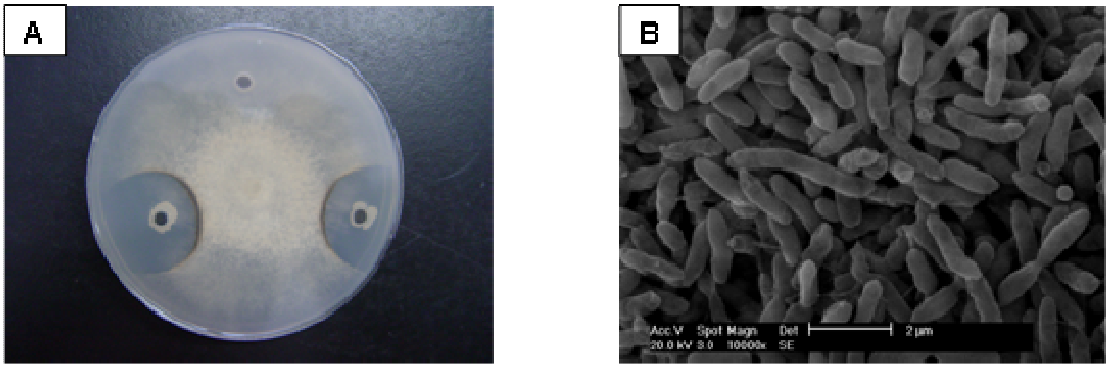

Figure 1. (A) Inhibition effect of EBBLQ01 against *Sclerotinia sclerotiorum*. (B) Thallus of EBBLQ01. They are arrayed in pairs, 1.1 µm × 2.2 µm in size.

and Tween 20, and could grow on basal medium, yeast-mannitol agar, peptone agar, glucose-yeast extract agar and nutrient agar. Growth in the presence of NaCl concentrations was up to 5% and the optimal temperature of its growing was 30 - 37℃.

For further identification of EBBLQ01, 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 bacterium strain showed 99% identity to that of *Phyllobacterium myrsinacearum*. It was indicated that this strain was phylogenetically related to members of the genus *Phyllobacterium*. The resulting phylogenetic tree (Figure 3) showed that EBBLQ01 and *P. myrsinacearum* clustered together within one subclade with a bootstrap support of 96%. Taken together this suggested that strain EBBLQ01 belonged to different species.

DISCUSSION

Previous studies demonstrated that some species of *Phyllobacterium* sp. are resistant to many kinds of antibiotics (Mahdhi et al., 2007). In the present study, one

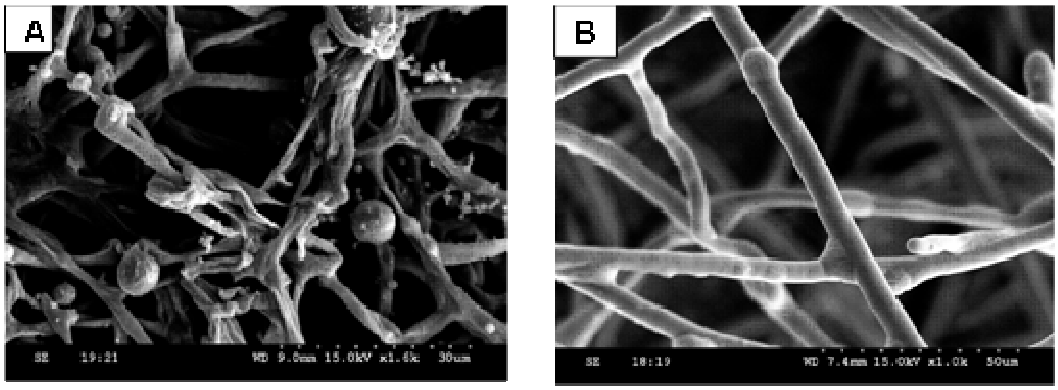

Figure 2. Hyphae of *Sclerotinia sclerotiorum* (A) hyphae in dual test plate was wrinkle and the cytoplasm was concentrated. (B) Normal hyphae.

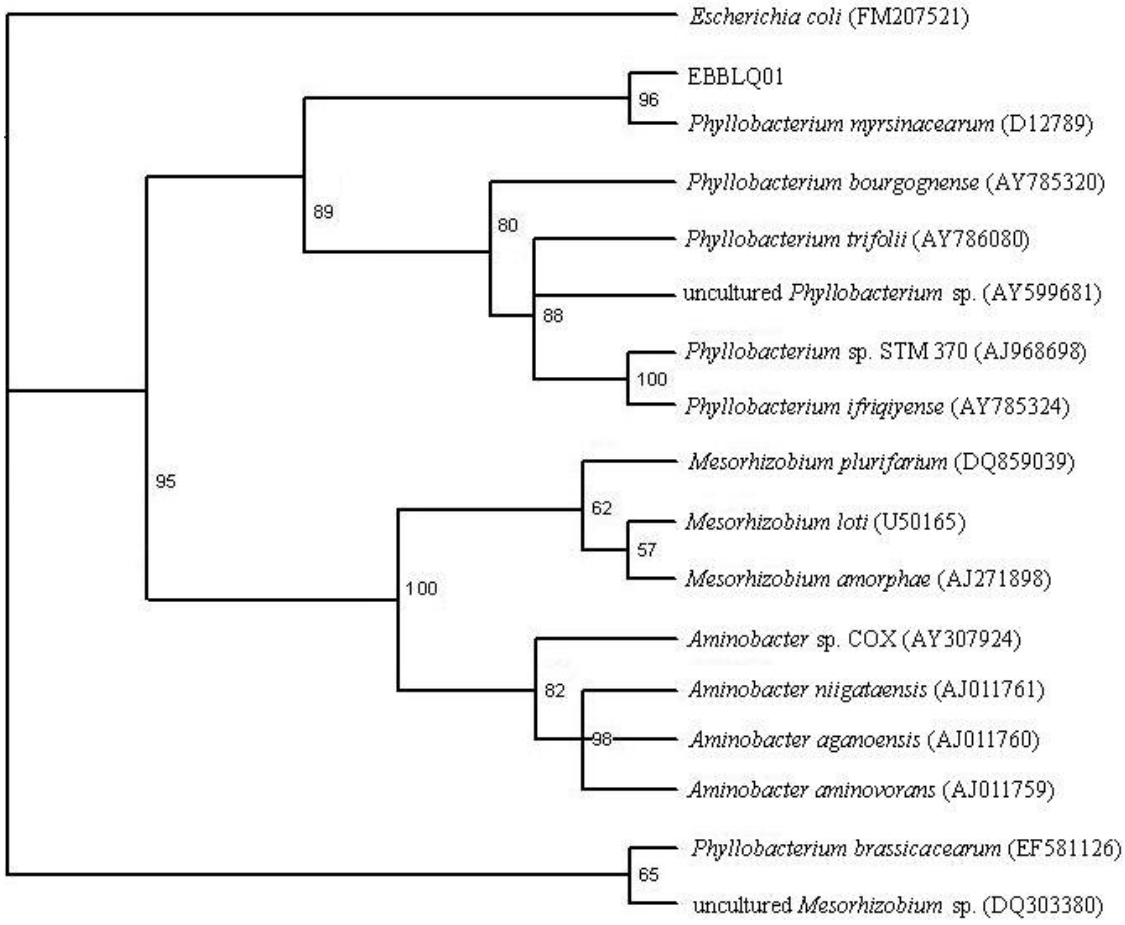

Figure 3. Phylogenetic relationships of the 16S rRNA gene from EBBLQ01 (GenBank No. FJ178785) and representative related strains from GenBank. *Escherichia coli* (FM207521) was used as outgroup. Percentages above the branches were the frequencies which had been given in 1000 bootstrap replications.

isolate of endophytic bacterium EBBLQ01 was resistant to streptomycin and ampicillin which was consistent with the former research.

On the basis of physiological and chemical tests and 16S rRNA gene sequence analysis, strain EBBLQ01 was designed as *Phyllobacterium* sp. Because we only performed preliminary physiological and biochemical characteristics tests and sequenced only 16S rRNA gene,

it was difficult to determine precisely which species it belonged to (Angel et al., 2005; Jurado et al., 2005).

In the past, the genus *Phyllobacterium* had always been studied for its capability of forming nodules to fix N_2 (Adriana et al., 2001; Rasolomampianina et al., 2005), while little attention was paid on its antimicrobial abilities. However, the antifungal bioactivities spectrum of *Phyllobacterium* FTP 3 isolated from sugar beet has been reported (Bart et al., 1990). Our study revealed that the *Phyllobacterium* sp. isolated from the roots of *E. brevicornu* Maxim have a wide antagonistic spectrum against a lot of common fungal and bacterial phytopathogens. Except *P. ultimum*, *S. rolfsii*, *P. cachrymans* and *P. solanacearum*, all the diameter of inhibition zones of the tested pathogens were larger than 10 mm and the inhibition zones of *A. alternata*, *S. sclerotiorum* and *V. dahliae* were up to 26, 23 and 23 mm, respectively. Therefore it could be a candidate biological control agent for these three fungal pathogens or may control different plant diseases.

When the antimicrobial activities of inoculum cultures and fermentation aliquots were compared, it was clear that the bioactivities of the inoculum cultures were much stronger than that of the aliquots. It is probable that the bioactive substance secretes outside very slowly and thus there were less bioactive substance in the fermentation aliquots than that in inoculum cultures. SEM analysis also showed that the antagonistic mechanism of EBBLQ01 is due to the production of secondary metabolites rather than competing with phypathogens.

Conclusion

In conclusion, the results of this study indicated that EBBLQ01 is a broad-spectrum antagonistic bacterium which provides us with new insights in the biological control of plant disease. For further studies of EBBLQ01, we will research on its colonization on plant roots which is good for growth stimulation and disease prevention and its bioactive components.

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