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Isolation, characterization, and antimicrobial activity of endophytic bacteria from *Polygonum cuspidatum*

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Polygonum cuspidatum* Sieb. et Zucc. is a traditional Chinese medicinal plant that produces polydatin, a glycosylated derivative of resveratrol. Thus far, 244 prokaryotic endophytes have been isolated and identified from *P. cuspidatum* using 16s rDNA sequence. The results show that the endophytic bacteria in *P. cuspidatum* belong to five orders, namely, Actinomycetales, Bacillales, Rhizobiales, Pseudomonadales, and Enterobacteriales. At the genus level, 244 strains were identified, including *Lysinibacillus, Paenibacillus, Pseudomonas, Bacillus, Kocuria, Streptomyces, Providencia, Rhizobium, Leucobacter, Brachybacterium,* and *Mycobacterium.* These bacteria were classified into 36 groups based on their 16s rDNA sequence. The endophytic bacteria isolated from *P. cuspidatum* show inhibitory activity against pathogenic fungi and bacteria. Among them, 13 isolates showed inhibitory activity against *Gibberella fujikuroi*, 18 isolates against *Aspergillus niger*, 6 isolates against *Aspergillus fumigatus,* 4 isolates against *Klebsiella pneumoniae,* 12 isolates against *Staphylococcus aureus*, and 6 isolates against *Bacillus subtilis*. However, none of the bacteria inhibited *Escherichia coli.

Key words: *Polygonum cuspidatum*, endophytic bacteria, phylogenetic analysis, antimicrobial activity.

INTRODUCTION

Plant-associated microorganisms fulfill important functions for plant growth and health. On the one hand, plants protect themselves by producing some compounds called secondary metabolites against pathogenic microbes. Some endophytic microorganisms produce antibiotics or growth-stimulating factor to benefit plant. Plant endophytes are microorganisms that live in the internal organs or cell gaps of healthy plants. Many factors, such as soil conditions and phytopathogen populations, influence the population structures of endophytic bacteria (Asraful Islam et al., 2010). Trivedi et al. (2010) found that infection of citrus plants by "*Ca.* Liberibacter asiaticus" has a profound effect on the structure and composition of the bacterial community associated with citrus roots.

Endophytic bacteria have been isolated from the interior of the stems and roots of many plants, such as ginseng, cotton, sweet corn, canola, wheat, and others (Cho et al., 2007; Justin et al., 2003).

Many endophytes have antimicrobial activity. Seo et al. (2010) found that some endophytic bacteria isolated from young radishes can be used as biocontrol agents against human and plant pathogens. Castillo et al. (2002) found that the endophytic *Streptomyces* sp. NRRL 30562

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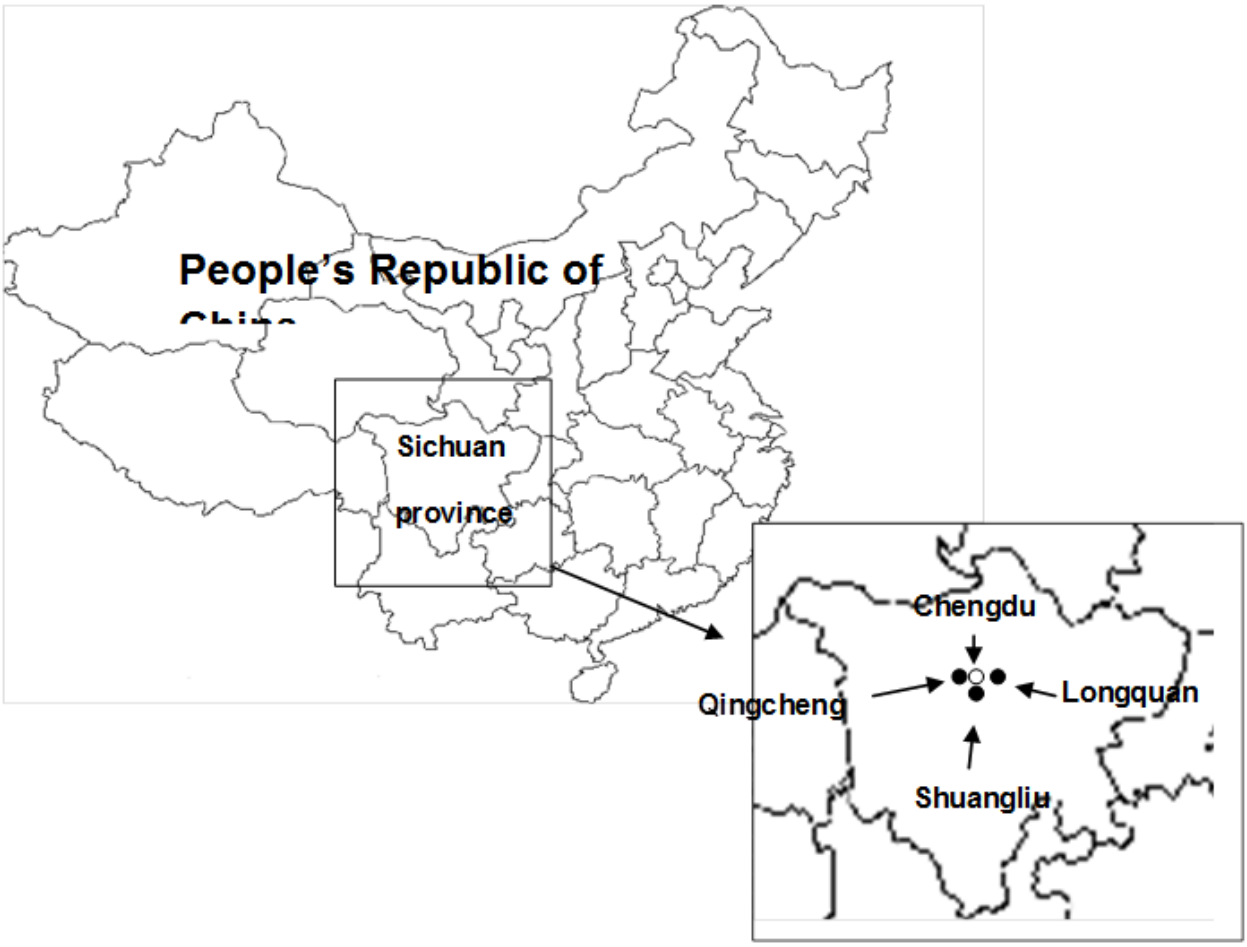

Figure 1. Collection sites in Sichuan, China.

obtained from snakevine produces novel peptide antibiotics that possess wide-spectrum activity against many pathogenic fungi and bacteria. Endophytic bacteria are considered potential resource of antimicrobial agents for biocontrol and pharmaceutical use. In recent years, endophytes have become a hot research topic. *Polygonum cuspidatum* Sieb. et Zucc., a traditional Chinese medicinal plant known in the UK as Japanese knotweed, is mainly distributed in the southern regions of China and is harvested in spring and autumn. In China, *P. cuspidatum* roots are dried and they have been used for centuries as a traditional herbal remedy for many diseases, including heart disease and stroke (Kimura et al., 1985). The active ingredients of this medicine are believed to be *trans*-resveratrol and glucoside (Soleas et al., 1997). The aims of the present study were as follows: (1) to examine the population structures of endophytic bacteria in *P. cuspidatum* from Shuangliu, Longquan, and Qingcheng in Sichuan Province, China; and (2) to investigate the inhibitory activity of the endophytic bacteria in *P. cuspidatum* against pathogenic microorganisms such as *Gibberella fujikuroi, Aspergillus niger, Aspergillus fumigatus, Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli,* and *Bacillus subtilis* for their utility as biological control agents or pharmaceutical use. Endophytic bacteria with antifungal and antibacterial activity are reported in this paper.

MATERIALS AND METHODS

Sample collection

Samples of *P. cuspidatum* root were collected from pot-grown plants in Qingcheng, Shuangliu, and Longquan, around Chengdu, the capital of Sichuan Province (Figure 1), from April 2010 to May 2010. Immediately after collection, the plants were washed with tap water and processed for the isolation of endophytic bacteria.

Media preparation and growth conditions of microorganism

The bacterial medium used in the isolation of endophytic bacteria contained the following: tryptone, 20 g/L; yeast extract, 5 g/L; trace salt (1 mL/L: $FeSO_4 \cdot 7H_2O$, 0.01 g/L; $MnCl_2 \cdot 4H_2O$, 0.01 g/L; $ZnSO_4 \cdot 7H_2O$, 0.01 g/L); glucose, 5 g/L; agar, 15 g/L; at pH 7.3. Nystatin (0.1 g/L) was added to the media to prevent fungal contamination. Luria–Bertani agar medium [LB; tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L; agar, 15 g/L] was used in the

purification of endophytic bacteria. Tap water-yeast extract agar modified medium [TWYE (Crawford et al., 1993); yeast extract, 0.25 g/L; K_2HPO_4, 0.5 g/L; agar, 15 g/L] was prepared for the isolation of endophytic actinobacteria. Actidione (0.1 g/L) and penicillin (0.1 g/L) were added to the media to prevent the growth of fungi and bacteria. ISP-3 medium [malt extract, 20 g/L; trace salt (1 mL/L; $FeSO_4 \cdot 7H_2O$, 0.01 g/L; $MnCl_2 \cdot 4H_2O$, 0.01 g/L; $ZnSO_4 \cdot 7H_2O$, 0.01 g/L); and agar, 15 g/L; at pH 7.2] was used in the purification of endophytic bacteria. LB medium [tryptone, 10 g/L; yeast extract, 5 g/L; and NaCl, 10 g/L; at pH 7.0], which is used to culture the endophytic bacteria on a small scale, was used for extracting bacterial metabolites. Actinomycetes culture medium [glucose, 10 g/L; dextrin, 25 g/L; oat meal, 20 g/L; PMM, 10 g/L; fish meal, 5 g/L; B-molasses, 5 g/L; Ebios, 2 g/L; and $CaCO_3$, 3 g/L] was used for the small scale multiplication of endophytic actinobacteria. Microorganisms were cultivated at 28°C for 2 d (for bacteria) or 7 d (for actinobacteria).

Isolation of endophytic bacteria

Endophytic bacteria were isolated from *P. cuspidatum* roots from three different locations in Sichuan: Qingcheng, Shuangliu, and Longquan. The roots of *P. cuspidatum* were surface sterilized with 99% ethanol for 60 s followed by 3.125% sodium hypochlorite for 6 min, washed in 99% ethanol for 30 s, and finally rinsed in sterile water. The surface-sterilized roots were then aseptically sectioned into 1 cm fragments, distributed onto the isolation media, and incubated at 28°C for 2 to 15 days. The only bacterial colonies that developed in the media were separately transferred to fresh media to obtain a pure culture.

DNA extraction, PCR, Sequencing

After subculturing the bacterial strains on LB agar medium for 2 d, fresh mycelia were inoculated in a 100 mL Erlenmeyer flask containing 25 mL of liquid LB medium and cultured in a shaking incubator in darkness at 28°C and 220 rpm for 2 d. The bacterial cells were obtained by centrifugation, suspended in 500 µL of lysozyme solution, and incubated at 37°C for 60 min. Subsequently, 250 µL of 2% SDS was added and the mixture was agitated for 1 min. Then, 250 µL of neutral phenol chloroform was added and the mixture was agitated for 5 min, and then centrifugated at 1500 *g* for 5 min. Then, 80 µL of 3 M sodium acetate (pH 4.8) and 800 µL of isopropyl alcohol were added to the supernatant liquid, mixed gently, and then centrifugated at 1500 *g* for 2 min. Finally, the supernatant liquid was poured off and the DNA pellets were washed with ice-cold 70% ethanol. The DNA pellets were vacuum dried and dissolved in 50 µL of TE (pH 8.0).

Each bacterial DNA was amplified with the universal primers: 887F: 5′-CGGAGAGTTTGATCCTGG-3′; and 878R: 5′-TACGGCTACCTTGTTAGCGAC-3′. Amplification was performed in a 25 µL reaction system that contained 2 µL of template DNA, 2.5 µL of 10 mmol/L of each primer, 0.25 µL of 1 U/µL Taq polymerase, 0.75 µL/L of 2.5 mmol/L dNTP, 2.5 µL of 10× Buffer, 2.5 µL of $MgCl_2$, and 14 µL of distilled water. The PCR was carried out according to the following protocol: initial denaturation 94°C for 3 min; denaturation 94°C for 45 s; annealing 50°C for 45 s, extension 72°C for 1 min. From each PCR reaction, 5 µL was obtained and the PCR products were examined through agarose gel electrophoresis (0.8% w/v) using ethidium bromide staining. The anticipated product, approximately 750 bp, was isolated from the amplified mixture after agarose gel electrophoresis using the SanPrep column DNA gel extraction kit, and was sequenced by Sangon Biotech (Shanghai) Co., Ltd. The partial sequences of the 16S rDNA of the isolated strains were submitted to GenBank and the accession numbers of the sequences are listed in Table 1.

Phylogenetic analysis

The sequence-based identification and phylogenetic analysis were based on data on sequences obtained from BLAST searches using the EzTaxon server 2.1 (Chun et al., 2007). Sequences were aligned using BioEdit (Hall 1999). The overhanging ends were removed from both ends to ensure that all sequences were of the same length. The tree was constructed with the MEGA 4.1 software package using the neighbor-joining method. Bootstrap tests were performed using 1000 replicates (Tamura et al., 2007).

Antimicrobial assay

The inhibitory activity of *P. cuspidatum* endophytic bacteria on the growth of *G. fujikuroi, A. niger, A. fumigatus, K. pneumoniae, S. aureus, E. coli,* and *B. subtilis* was determined through the filter paper method. Before final concentration of $2\text{-}5\times10^7$ cells mL^{-1} was regulated, microbe suspension (for bacteria) or spores suspension (for fungi) was mingled with LB or PDA medium respectively. Paper disc were immersed in bacterial fermentation liquor and placed on the surface of assay plates . After incubation at 37°C (for bacteria) or at 28°C (for fungi) for 24~48 h, the inhibition zones around each disc was measured in diameter Φ. The antimicrobial activity was qualitatively evaluated. according to the diameter of clear zone of growth inhibition.

For the minimal inhibitory concentration (MIC) determination, the indicator bacteria cultured on slants were washed with sterile water. Then, the bacterial or bacterial spore cultures were diluted to 10^7 CFU/mL and stored at 4°C. Before use, the cultures were mixed with appropriate amounts of medium (the final approximate bacterial concentration was 10^5 CFU/mL and the final approximate fungal concentration was 10^4 CFU/mL). The samples were separately dissolved and the resulting mixtures were diluted twofold, and were introduced into the 96-cell plates. The culture medium containing only the indicator bacteria, without the sample was used as the negative control, and the culture medium was used as the blank control. Both the bacterial and fungal cultures were cultured at 28°C for 24 h. The absence of bacterial growths and the MIC was determined by visual observation. To obtain statistically significant results, test of inhibitory activity to indicator strains were carried out three times, the data of the diameter were the average of three assays.

RESULTS

Isolation and phylogenetic placement of endophytic bacteria from *P. cuspidatum* roots

The diversity of endophytic bacteria in *P. cuspidatum* was assessed using root samples collected from three different locations: Shuangliu, Qingcheng, and Longquan (Figure 1). In total, 244 culturable endophytes were recovered from the interior of *P. cuspidatum* roots (Table 1). Among them, 80 strains were obtained from Shuangliu, 82 strains from Longquan, and the remainder, from Qingcheng.

The results of the phylogenetic analysis of *P. cuspidatum* endophytic bacteria isolated from Shuangliu are shown in Figure 2A. Based on the 16S rDNA sequence analysis, three orders were identified, namely, Bacillales, Actinomycetales, and Pseudomonadales, and 80 endophytic bacteria were classified into 6 genera, including 11 distinct species (Table 1): *Lysinibacillus*

Table 1. Similarity values of 16S rDNA sequence retrieved from the endophytic bacteria from the *Polygonum cuspidatum Sieb. et Zucc.* roots (Shuangliu, Longquan, Qingcheng).

Isolates (accession number)	No. of isolates	Order	Nearest relative (accession number)	Similarity (%)
Shuangliu				
CB1(JN120807)	38	Bacillales	*Lysinibacillus sphaericus* C3-41(CP000817)	99.876
CB2(JN120808)	16	Bacillales	*Paenibacillus pabuli* JCM 9074(T)(AB073191)	99.586
CB3(JN120809)	1	Pseudomonadales	*Pseudomonas nitroreducens* DSM 14399(T)(AM088474)	99.721
CB4(JN120810)	2	Bacillales	*Bacillus safensis* FO-036b(T)(AF234854)	99.860
CB5(JN120811)	4	Bacillales	*Bacillus cereus* ATCC 14579(T)(AE016877)	99.857
CB6(JN120812)	9	Bacillales	*Bacillus thuringiensis* ATCC 10792(T)(ACNF01000156)	99.857
CB7(JN120813)	5	Bacillales	*Paenibacillus alvei* DSM 29(T)(AJ320491)	99.145
CB8(JN120814)	2	Bacillales	*Bacillus simplex* NBRC 15720(T)(AB363738)	100.000
CB9(JN120815)	1	Actinomycetales	*Kocuria rosea* DSM 20447(T)(X87756)	99.711
CB10(JN120816)	1	Actinomycetales	*Streptomyces umbrinus* NBRC 13091(T)(AB184305)	98.563
CB11(JN120817)	1	Actinomycetales	*Streptomyces olivochromogenes* NBRC 3178(T)(AB184737)	99.747
Longquan				
CB12(JN120818)	11	Bacillales	*Lysinibacillus sphaericus* C3-41(CP000817)	99.876
CB13(JN120819)	4	Enterobacteriales	*Providencia rettgeri* DSM 4542(T)(AM040492)	99.708
CB14(JN120820)	6	Rhizobiales	*Rhizobium radiobacter* ATCC 19358(T)(AJ389904)	99.854
CB15(JN120821)	16	Bacillales	*Bacillus thuringiensis* ATCC 10792(T)(ACNF01000156)	99.857
CB16(JN120822)	1	Bacillales	*Bacillus atrophaeus* JCM 9070(T)(AB021181)	99.861
CB17(JN120823)	31	Actinomycetales	*Leucobacter aridicollis* CIP 108388(T)(AJ781047)	98.138
CB18(JN120824)	1	Pseudomonadales	*Pseudomonas nitroreducens* DSM 14399(T)(AM088474)	99.721
CB19(JN120825)	2	Bacillales	*Bacillus cereus* ATCC 14579(T)(AE016877)	99.857
CB20(JN120826)	1	Actinomycetales	*Brachybacterium faecium* DSM 4810(T)(CP001643)	97.718
CB21(JN120827)	1	Actinomycetales	*Mycobacterium frederiksbergense* DSM 44346(T)(AJ276274)	99.566
CB22(JN120828)	3	Actinomycetales	*Streptomyces atroolivaceus* LMG 19306(T)(AJ781320)	99.447
CB23(JN120829)	2	Actinomycetales	*Streptomyces griseoplanus* AS 4.1868(T)(AY999894)	100.000
CB24(JN120830)	1	Actinomycetales	*Streptomyces ciscaucasicus* NBRC 12872(T)(AB184208)	99.649
CB25(JN120831)	2	Actinomycetales	*Streptomyces ederensis* NBRC 15410(T)(AB184658)	99.593
Qingcheng				
CB26(JN120832)	2	Bacillales	*Paenibacillus pabuli* JCM 9074(T)(AB073191)	99.586
CB27(JN120833)	2	Enterobacteriales	*Providencia rettgeri* DSM 4542(T)(AM040492)	99.708
CB28(JN120834)	1	Bacillales	*Bacillus atrophaeus* JCM 9070(T)(AB021181)	99.861
CB29(JN120835)	25	Bacillales	*Bacillus thuringiensis* ATCC 10792(T)(ACNF01000156)	99.857
CB30(JN120836)	31	Bacillales	*Bacillus amyloliquefaciens subsp. plantarum* FZB42(T)(CP000560)	99.862
CB31(JN120837)	4	Bacillales		100.000
CB32(JN120838)	4	Bacillales	*Bacillus aryabhattai* B8W22(T)(EF114313)	98.996
CB33(JN120839)	10	Rhizobiales	*Paenibacillus alvei* DSM 29(T)(AJ320491)	100.000
CB34(JN120840)	1	Actinomycetales	*Rhizobium radiobacter* ATCC 19358(T)(AJ389904)	98.153
CB35(JN120841)	1	Actinomycetales	*Leucobacter aridicollis* CIP 108388(T)(AJ781047)	100.000
CB36(JN120842)	1	Actinomycetales	*Streptomyces intermedius* NBRC 13049(T)(AB184277) *Streptomyces murinus* NBRC 12799(T)(AB184155)	100.000

sphaericus (CB1, 38 isolates), *Paenibacillus pabuli* (CB2, 16 isolates), *Pseudomonas nitroreducens* (CB3, 1 isolate), *Bacillus safensis* (CB4, 2 isolates), *Bacillus cereus* (CB5, 4 isolates), *Bacillus thuringiensis* (CB6, 9 isolates), *Paenibacillus alvei* (CB7, 5 isolates), *Bacillus simplex* (CB8, 2 isolates), *Kocuria rosea* (CB9, 1 isolate), *Streptomyces umbrinus* (CB10, 1 isolate), and *Streptomyces olivochromogenes* (CB11, 1 isolate). The 16S rDNA sequences of these bacteria were 98%–100% similar to those found in databases.

Figure 2B shows the phylogenetic analysis of *P. cuspidatum* endophytic bacteria from Longquan. Five orders were identified: Actinomycetales, Bacillales, Rhizobiales, Pseudomonadales, and Enterobacteriales. The 82 isolates from Longquan represented 10 genus and 14 species (Table 1); the species are as follows:

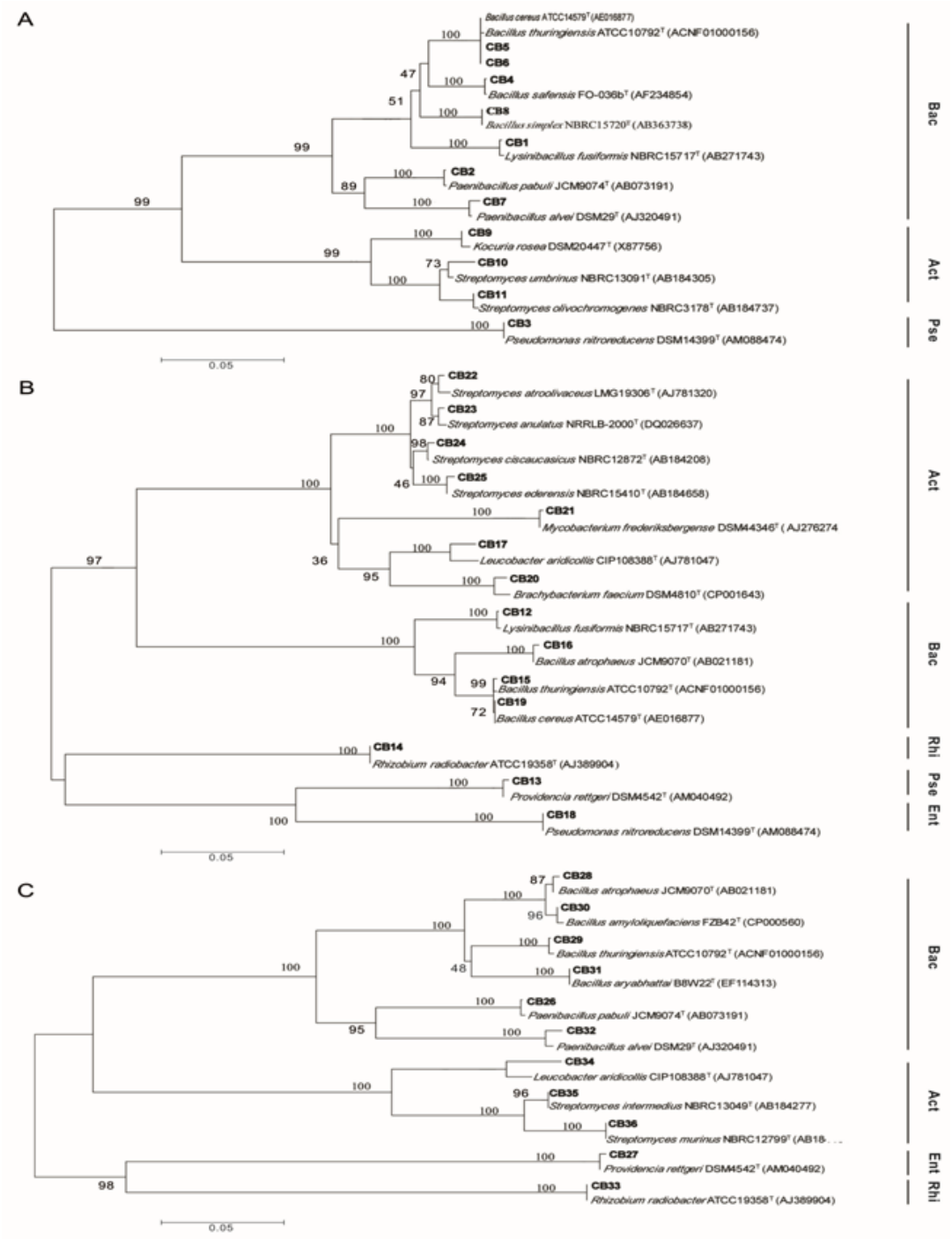

Figure 2. Phylogenetic analysis of 16S rDNA sequences of the endophytic bacteria of *P. cuspidatum* root collected from Shuangliu (A), Longquan (B), and Qingcheng (C). The numbers above each node are confidence levels (%) generated from bootstrap analysis using 1000 replicates. The scale bar has fixed nucleotide substations per sequence position. Actinomycetales (Act); Bacillales (Bac); Rhizobiales (Rhi); Pseudomonadales (Pse); Enterobacteriales (Ent).

Lysinibacillus sphaericus (CB12, 11 isolates), *Providencia rettgeri* (CB13, 4 isolates), *Rhizobium radiobacter* (CB14, 6 isolates), *B. thuringiensis* (CB15, 16 isolates), *Bacillus atrophaeus* (CB16, 1 isolate), *Leucobacter aridicollis* (CB17, 31 isolates), *P. nitroreducens* (CB18, 1 isolate), *B. cereus* (CB19, 2 isolates), *Brachybacterium faecium* (CB20, 1 isolate), *Mycobacterium frederiksbergense* (CB21, 1 isolate), *Streptomyces atroolivaceus* (CB22, 3 isolates), *Streptomyces griseoplanus* (CB23, 2 isolates), *Streptomyces ciscaucasicus* (CB24, 1 isolate), and

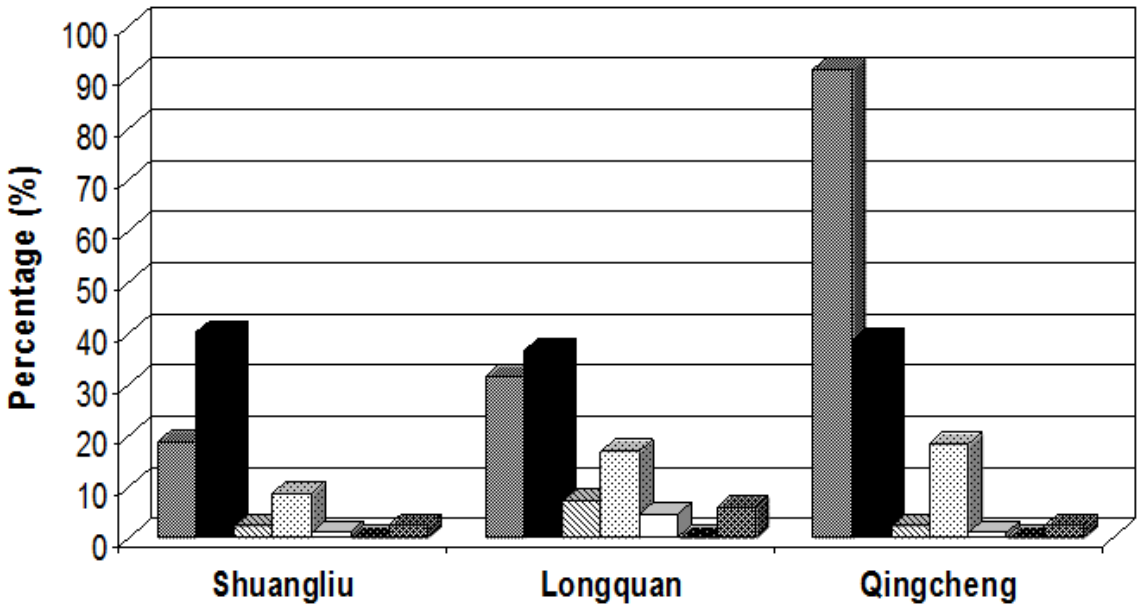

Figure 3. Distribution of the antimicrobial activity of isolates from the three sample sites: Shuangliu, Longquan, and Qingcheng. Percentage of microbe in each of the three sample sites is shown. *Gibberella fujikuroi* (▩); *Aspergillus niger* (■); *Aspergillus fumigatus* (▨); *Staphylococcus aureus* (▩); *Klebsiella pneumoniae* (□); *Escherichia coli* (▩); *Bacillus subtilis* (▩).

Streptomyces ederensis (CB25, 2 isolates). The 16S rDNA sequences of these 82 isolates were 98–100% similar to the sequences in databases, except CB20, which was 97.718% similar to its corresponding sequence.

Figure 3C shows the phylogenetic analysis of *P. cuspidatum* endophytic bacteria from Qingcheng. Four orders were identified: Bacillales, Actinomycetales, Rhizobiales, and Enterobacteriales. These endophytes were classified into 6 genus and 11 species (Table 1); the species are as follows: *Paenibacillus pabuli* (CB26, 2 isolates), *Providencia rettgeri* (CB27, 2 isolates), *B. atrophaeus* (CB28, 1 isolate), *B. thuringiensis* (CB29, 25 isolates), *B. amyloliquefaciens* subsp. *plantarum* (CB30, 31 isolates), *B. aryabhattai* (CB31, 4 isolates), *Paenibacillus alvei* (CB32, 4 isolates), *R. radiobacter* (CB33, 10 isolates), *Leucobacter aridicollis* (CB34, 1 isolate), *Streptomyces intermedius* (CB35, 1 isolate), and *Streptomyces murinus* (CB36, 1 isolate). The 16s rDNA sequences of these strains were 98%–100% similar to those found in databases.

The strains under the order Bacillales were broadly distributed in the roots of *P. cuspidatum* collected from the three sites. Of the 244 strains collected, 71% (173/244) belonged to this order. Bacteria most closely related to *B. thuringiensis* ATCC 10792 were found in all three sites. The strains *B. cereus*, *L. sphaericus*, *P. pabuli*, *P. alvei*, and *B. atrophaeus* were the predominant species; they were found in two sample sites and were isolated more than once in each site. Actinomycetes are an important group in the root of *P. cuspidatum*. Streptomycetes was the predominant group of endophytic actinobacteria found. *Streptomyces* strains were found in samples from all three sites.

Antimicrobial activity of endophytic bacteria from *P. cuspidatum*

Selected endophytic bacteria from the three sites were studied to determine their *in vitro* inhibitory activity against pathogenic fungi (*G. fujikuroi, A. niger,* and *A. fumigatus*) and pathogenic bacteria (*K. pneumoniae, S. aureus, E. coli,* and *B. subtilis*) (Table 2). Five isolates (CB22, CB23, CB25, CB35, and CB36) appeared to have a broad spectrum of antifungal and antibacterial activity *in vitro.* The isolates were all *Streptomyces* sp. CB36 (*S. murinus*) in particular, exhibited a notable antifungal activity against all pathogenic fungi tested. CB23, isolated from Longquan, not only had a strong activity against pathogenic fungi (*A. niger* and *A. fumigatus*), but also against pathogenic bacteria (*K. pneumoniae* and *B. subtilis*). CB10 (*S. umbrinus*) and CB22 (*S. atroolivaceus*) showed activity against pathogenic *S. aureus.* In this study, some *Bacillus* strains showed significant antifungal activities, such as *B. thuringiensis* (CB6, CB15, and CB29), which showed inhibitory activity against *G. fujikuroi*, and *B. cereus* (CB5 and CB19) and *B. atrophaeus* (CB16 and CB28), which showed inhibitory activity against *A. niger. Rhizobium* sp. (CB14 and CB33)

Table 2. Inhibitory activity against the microbe by Polygonum cuspidatum endophytic bacteria.

Isolates	*Gibberella fujikuroi*	*Aspergillus niger*	*Aspergillus fumigatus*	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Escherichia coli*	*Bacillus subtilis*
Shuangliu							
CB1	-	-	-	-	-	-	-
CB2	-	+	-	-	-	-	-
CB3	-	-	-	-	-	-	-
CB4	-	+	+	-	-	-	-
CB5	-	++	-	-	-	-	+
CB6	++	+	-	-	-	-	-
CB7	+	-	-	-	+	-	-
CB8	-	-	-	-	-	-	-
CB9	+	-	-	-	+	-	-
CB10	-	-	-	-	+++	-	-
CB11	-	+	-	+	-	-	-
Longquan							
CB12	-	-	-	-	-	-	-
CB13	-	+	-	-	-	-	-
CB14	+	-	-	-	+	-	-
CB15	++	+	-	-	-	-	-
CB16	-	++	+	-	+	-	-
CB17	-	-	-	-	-	-	-
CB18	-	-	-	-	-	-	-
CB19	-	++	-	-	-	-	+
CB20	+	-	-	-	-	-	-
CB21	-	-	-	-	+	-	+
CB22	+	+	+	-	++	-	-
CB23	-	++	+	+++	-	-	+++
CB24	-	-	-	-	+	-	-
CB25	-	+	-	+	+	-	-
Qingcheng							
CB26	-	+	-	-	-	-	-
CB27	-	+	-	-	-	-	-
CB28	-	++	+	-	+	-	-
CB29	++	+	-	-	-	-	-
CB30	+	-	-	-	-	-	-
CB31	+	-	-	-	-	-	-
CB32	+	-	-	-	+	-	-
CB33	+	-	-	-	+	-	-
CB34	-	-	-	-	-	-	-
CB35	-	++	-	+	-	-	+
CB36	+	+++	++++	-	-	-	+

The antimicrobial activity was estimated by measuring the diameter of the clear zone (including paper disks, 8 mm diameter) of growth inhibition. Symbols: -, indicates no antimicrobial activity; +, indicates the clear zone 9~11 mm; ++, indicates the clear zone 11~13 mm; +++, indicates the clear zone 13~15 mm; +++，indicates the clear zone >15 mm.

and *Paenibacillus* sp. (CB7 and CB32) showed weak inhibitory activity against *G. fujikuroi* And *Pseudomonas* sp. (CB3 and CB18), and *Lysinibacillus* sp. (CB1 and CB12) did not show any inhibitory activity against the pathogenic microorganisms tested in this study. None of the *P. cuspidatum* endophytic bacteria showed inhibitory activity against *E. coli* (Figure 3).

As shown in Table 2, the antimicrobial activity of CB36

against *A. fumigatus*, CB10 against *Staphylococcus aureus*, and CB23 against *K. pneumoniae* was high. Hence, we chose to determine the minimal inhibitory concentration (MIC) of these three strains as follows: CB10 was 542 µg/mL, CB 23 was 407 µg/mL, and CB36 was 272 µg/mL respectively.

DISCUSSION

Endophytic bacteria are found in virtually every plant on earth (Ryan et al., 2008). Knowledge on the diversity of endophytic bacteria is important for both ecological and biotechnological studies. Cho et al. (2007) have identified 13 different bacterial genera from 63 isolates from the interior of ginseng root. Similarly, Dias et al. (2009) have isolated 20 different endophytic bacterial genera from the meristematic tissues of three varieties of strawberry. Justin et al. (2003) isolated 49 actinobacteria from surface-sterilized wheat roots. Thus, endophytic communities are clearly distinct in different plant species and the diversity of the communities may vary significantly. Also, endophytic communities from different locations are different. In this study, 244 culturable endophytic bacteria associated with *P. cuspidatum* were isolated and identified. Interestingly, the predominance of Bacillales strains over the other bacterial endoflora of *P. cuspidatum* was observed in the present study. The most prevalent endophytic bacterial groups isolated from *P. cuspidatum* roots from Shuangliu and Qingchen belong to Bacillales. On the other hand, Actinomycetales was the dominant group in the plants from Longquan, because of the great number of CB17, which was most related to *Leucobacter aridicollis* (Table 1). The strains related to *B. thuringiensis* (similarity 99.8%) were the most frequently found in all locations. Furthermore, the other strains, which were related to *L. sphaericus*, *P. pabuli, B. cereus, P. alvei, P. rettgeri, B. atrophaeus*, and *P. nitroreducens*, were isolated from at least two sites. This suggests that the population structures of endophytic bacteria in the root tissues of *P. cuspidatum* are similar despite the large distance between collection sites. The results in this study indicate different ecological characteristics from those presented in previous reports, which imply that the distribution of endophytic bacteria mainly depend on environmental conditions such as temperature, humidity, UV irradiation, and nutrients in the apoplast, but not on the hosts (Durgude et al., 2009; Baker et al., 2010). The higher frequency of Bacillales in plants compared with the other groups observed may indicate that they have formed a beneficial association with plants.

The organisms that reside in the living tissues of host plants form a variety of relationships ranging from symbiotic to pathogenic (Chen et al., 2011). Endophytes may contribute to their host plants by producing a plethora of substances that provide protection, and ultimately have survival value, to the plants (Soca-chafre et al., 2011). Ultimately, these compounds, once isolated and characterized, may also have potential uses in modern medicine, agriculture, and in various industries. Miller et al. (1998) found that *Pseudomonas viridiflava*, a plant-associated bacterium, produces ecomycins, which have significant bioactivity against a wide range of human and plant pathogenic fungi. We have demonstrated that some endophytic bacteria from *P. cuspidatum* inhibit other microorganisms (Figure 3). Most of the crude extracts from 36 endophytes showed different degrees of inhibitory activity against the test organisms (Table 2). Up to 10 strains showed high antifungal activity; they belonged to *Bacillus* (CB5, CB6, CB15, CB16, CB19, CB28, and CB29) and *Streptomyces* (CB23, CB35, CB36). The antifungal activity of *Bacillus* has been reported in previous studies. *Bacillus* has been found to produce antifungal factors such as antifungal hydrolytic enzymes (Chang et al., 2007), spore-specific lipopeptides (Yao et al., 2003), and fengysin (Lin et al., 1999). Furthermore, *Bacillus* strains are stable in soil as spores, and this property is advantageous for their use as biocontrol agents. However, the antifungal activity of *Streptomyces* mainly depends on their secondary metabolites (Shimizu et al., 2000; Taechowisan et al., 2005). The endophytic bacteria from *P. cuspidatum* from Longquan showed apparently high inhibitory activity against *G. fujikuroi* (90%). Less than half of all the strains from the three sample sites were found to exude growth inhibitory substances towards *A. niger* when tested *in vitro* (Figure 3). In our study, *Streptomyces* sp. CB36 showed broad-spectrum antifungal activity and the strongest antifungal activity against *Aspergillus*. *Streptomyces* strains CB10, CB22, and CB23 showed strong antibacterial activity, whereas the other endophytes showed weak or no antibacterial activity. Many antibacterial agents have been reported and identified from *Streptomyces* by previous research (Raja et al., 2011). The results of these studies indicate that strains of endophytic *Streptomyces* play important roles in the antimicrobial mechanism of plants and that they are significant resources for novel antimicrobial agents.

Microbe–plant interactions are far from being fully understood. Nevertheless, more evidence shows plant-associated microorganisms provide substantial benefits to agriculture, industry, and the environment. In brief, this study determined that there are regional differences between microbial communities inside the roots of *P. cuspidatum.* Most of the bacteria we examined had antimicrobial activity. These results show the potential use of endophytic bacteria for biocontrol to protect plants from fungal or bacterial diseases. Further studies are needed to separate and extract the active substances from endophytic bacteria.

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