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Community Analysis of Endophytic Bacteria from the Seeds of the Medicinal Plant *Panax notoginseng*

Shuai Liu[1,†], Dinghua Li[1,†], Xiuming Cui[1], Limei Chen[1] & Hongjuan Nian[1]

[1] Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming Key Laboratory of Sustainable Development and Utilization of Famous-Region Drug, Key Laboratory of *Panax notoginseng* Resources Sustainable Development and Utilization of State Administration of Traditional Chinese Medicine, Kunming, China

Correspondence: Hongjuan Nian, Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, China. Tel: 86-871-6592-0213. E-mail: hjnian@163.com

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† *Shuai Liu and Dinghua Li contributed equally to this work.*

Abstract

Panax notoginseng is a traditional Chinese medicine. The roots of *P. notoginseng* can be used for treatment of diseases and raw materials in Chinese medicinal products. High yield and quality roots require cultivation in shade and humid conditions for 3 years. The long period cultivation makes *P. notoginseng* vulnerable to infect by pathogens. So control diseases are vital for the high yield and quality of *P. notoginseng*. The seed is the carrier systems of many probiotics and pathogens. To explore the indigenous bacterial community diversity, the endophytic bacteria from the seeds of the medicinal plant *P. notoginseng* were isolated and identified using traditional cultivation methods in combination with molecular technique. A total of 137 endophytic bacteria strains were isolated. The 16S rDNA of these strains was amplified and subjected to amplified ribosomal DNA restriction analysis (ARDRA) with restriction enzyme *Hae*III. All the isolated strains were grouped into 9 OTUs (Operational Taxonomic Units) on the basis of the similarity of the ARDRA band profiles. Each representative strain of 9 OTUs was selected for sequencing. γ-proteobacteria was the most dominant group among the isolates (98.5%), containing eight genera. *Pseudomonas* was the most dominant genus (58 of 135 isolates), whose isolates occurred in the seeds collected from all three places. The second dominant genus was *Enterobacter* (20.7%), followed by uncultured bacterium (14.8%) and *Stenotrophomonas* (10.4%). Among the six areas sampled, endophytic bacteria in the seeds collected from Panlong of Yanshan exhibited species diversity and contained the most isolates. These results suggest an abundant diversity of bacterial community within the seeds of *P. notoginseng*. These data provide insights into monitoring the seed health and disease outbreak during seeding.

Keywords: ARDRA, endophytic bacteria, diversity, OTUs, *Panax notoginseng*

1. Introduction

Endophytic bacteria ubiquitously inhabit a majority of plant species (Lodewyck et al., 2002). These organisms can be isolated from surface-disinfected plant tissues, including seeds, roots, stems and leaves and are not harmful to the hosts (Hallmann et al., 1998). Endophytic bacteria may promote plant growth and suppress plant diseases (Feng et al., 2006). Several studies have reviewed endophytic bacteria community structures and their potential biological functions (Senthilkumar et al., 2011; Sturz et al., 2000).

Panax notoginseng is a traditional Chinese medicine plant specifically grown in the Wenshan region of Yunnan Province. The root of *P. notoginseng* is often used to treat cardiovascular diseases, inflammation, different body pains, trauma, and internal and external bleeding due to injury (Sun et al., 2005). *P. notoginseng* is a 3-year-old plant. High quality roots require 3-year cultivation in the shade and humid conditions. The long period growth makes *P. notoginseng* vulnerable to be infected by pathogens. The root-rot disease is the most destructive one (Sun et al., 2004), which was caused by soil-borne fungal pathogens (including *Alternaria panax*, *Alternaria tenuis*, *Cylindrocarpon destructans*, *Cylindrocarpon didynum*, *Fusarium solani*, *Fusarium oxysporum*,

Phytophthora cactorum, *Phoma herbarum* and *Rhizoctonia solani*), bacterial pathogens (*Pseudomonas* sp. and *Ralstonia* sp.), and parasitic nematodes (such as *Ditylenchus* sp., *Rhabditis elegans* and *Meloidogyne* spp.) (Miao et al., 2006). Control diseases are vital for the high yield and quality of *P. notoginseng*.

The seeds of plants are the carrier systems of many probiotics and pathogens that play an important role in formation of rhizosphere microbial communities (Patkowska, 2001). However, studies concerning the interaction of the microbial community and plant seed genotype are lacking. Therefore, a better study of endophytic bacteria may help increase the current understanding of their function and potential role for the development of a more sustainable system for crop production. Few studies concerning endophytic bacterial community structures and their biological functions in *Panax* spp. have been reported, with the exception of reports on the endophytic bacterial community (Cho et al., 2007; Vendan et al., 2010). Ma et al. (2013) isolated 1000 endophytic bacterial strains from the root, stems, petioles, leaves and seeds of *P. notoginseng*, of which 104 strains exhibited antagonistic properties against at least one of three major pathogens (*F. oxysporum*, *Ralstonia* sp. and *Meloidogyne hapla*) related to the root-rot disease of *P. notoginseng*. Therefore, knowledge concerning the endophytic bacterial structures and species is vital for the growth of *Panax* plants, the monitoring of seed health and the control of seedling disease. Thus, the aims of the present study were to characterize the endophytic bacteria present in the seeds of the medicinal plant *P. notoginseng*, to obtain a better understanding of endophytic bacterial community structures and diversities and to identify potential biological control candidates against pathogens leading to seedling and root diseases.

2. Materials and Methods

2.1 Microorganisms and Culture Conditions

The bacterial strains were cultured on Luria-Bertani (LB) solid medium (peptone 10 g, yeast extract 5 g, NaCl 10 g, agar powder 15 g, and distilled water 1000 mL, pH 7.2) or in LB liquid broth at 37 °C.

2.2 Isolation and Purification of Endophytic Bacteria

The seeds were collected from 1-3-year-old healthy *P. notoginseng* plants, cultivated in Yanshan County, Maguan County, Guangnan County, Xichou County, Wenshan County and Qiubei County of the Wenshan region, Yunnan Province, China.

The samples were disinfected according to Li et al. (2010), with some modifications. The seeds were rinsed three times with sterile water, and subsequently, the moisture was absorbed using filter paper. The seeds were sterilized with 70% ethanol for 3 min, immersed in 2.6% sodium hypochlorite solution for 5 min, soaked in 70% ethanol for 30 s, and subsequently washed three times with sterile water. The water samples of the last rinse were inoculated onto LB agar plates as a negative control. Simultaneously, the surface sterilized seeds were pressed onto an LB agar plate to test the sterilization efficiency. The seeds that were not detected as contaminated by cultivable microorganisms were considered successfully surface disinfected and were subsequently used for the isolation of endophytic bacteria (Schulz et al., 1993).

The surface sterilized seeds were divided into three groups. Five seeds in each group were homogenized with 2 mL of sterile water. The homogenates were diluted 1000-fold, and 100-μL dilutions were then spread onto LB agar plates. Each sample was replicated three times. The plates were cultured at 37 °C for 72 h. The bacterial colonies with obvious morphological differences were purified. Colonies without distinct morphological differences were randomly selected.

2.3 DNA Extraction from Bacteria

The purified strains were inoculated in LB liquid medium and shaken at 200 rpm for 12 h at 37 °C. The cultures were centrifuged and subsequently collected. Genomic DNA was extracted using the Genomic DNA Purification Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer's instructions.

2.4 Amplification of the Bacterial 16S rRNA Gene

The genomic DNA was used as the PCR template. A pair of primers, 799f and 1492r [13], were used to amplify the 16S rDNA. In total, a 20-μL PCR reaction mixture contained 1 μL of genomic DNA, 1 μL of each primer (10 μM), and 1 μL 2 × Es Taq MasterMix (Es Taq DNA Polymerase, 2 × Es Taq PCR buffer, 3 mM $MgCl_2$, and 400 μM dNTP) (ComWin Biotech, Beijing, China). After initial denaturing at 94 °C for 3 min, thermal cycling proceeded with, denaturing at 94 °C for 30 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 45 s. At the end of 30 cycles, a final extension step was performed at 72 °C for 10 min.

2.5 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The specificity of the PCR product was examined on an agarose gel. The specific PCR product was used for digestion. The PCR product was completely digested with restriction enzyme *Hae*III (NEB, Beijing, China), an enzyme that identifies four bases. The reaction mixtures contained 2 μL of 10 X incubation buffer, 2 μL of *Hae*III (10 U/μL) and 16 μL of the PCR product. The digestion was performed according to the manufacturer's instructions. Reaction mixtures were incubated overnight at 37 °C. The total volume of each restriction-digested product was separated on a 1% (w/v) agarose gel and subsequently photographed. According to ARDRA patterns, the strains were grouped into Operational Taxonomic Units (OTUs) according to Sessitsch et al. (2002).

2.6 Sequencing and Phylogenetic Analysis

The PCR products of 16S rDNA from each ARDRA pattern were selected for sequencing at Sangon Biotech Co., Ltd (Shanghai, China). All obtained sequences were compared with the sequences in the GenBank database using the BLASTN search program. The most similar sequences were further aligned using Clustal W software (Tompson et al., 1994). All reference sequences were obtained from the National Center for Biotechnology Information (NCBI). Phylogenetic trees were constructed using the neighbor-joining method with two-parameter MEGA software (version6.0) (Saitou & Nei, 1987). Statistical significance levels of interior nodes were determined by bootstrap analysis (1,000 data resamplings) (Felsenstein, 1985).

2.7 Nucleotide Sequence Accession Numbers

The nucleotide sequences of the 16S rRNA gene for each analyzed strain have been deposited in GenBank and have been assigned accession numbers (Table 1).

3. Results

3.1 Isolation of Endophytic Bacteria

The surface sterilized seeds were divided into groups and then homogenized with sterile water. The homogenates were diluted and spread on LB agar plates. The plates were cultured at 37 °C for 72 h. Colonies with obvious morphological differences were purified. Colonies without morphological differences were randomly selected. A total of 137 colonies were isolated and purified.

3.2 16S rRNA Amplification and ARDRA Analysis

A single colony was inoculated in LB broth and cultured at 37 °C for 12 h. The culture sample was used for DNA extraction. A pair of PCR primers (799f and 1492r) was used to amplify the 16S rRNA gene of the 137 isolates. Genomic DNA was used for the templates. The fragment size of 16S rDNA is approximately 700bp (Figure 1). The specific PCR product was completely digested with the restriction enzyme *Hae*III. Based on ARDRA patterns, the isolated bacterial strains were grouped into different OTUs (Table 1). An OTU was defined as a group of clones with an identical banding patterns obtained from digestion. According to the ARDRA patterns (Figure 2), 137 strains were grouped into 9 OTUs.

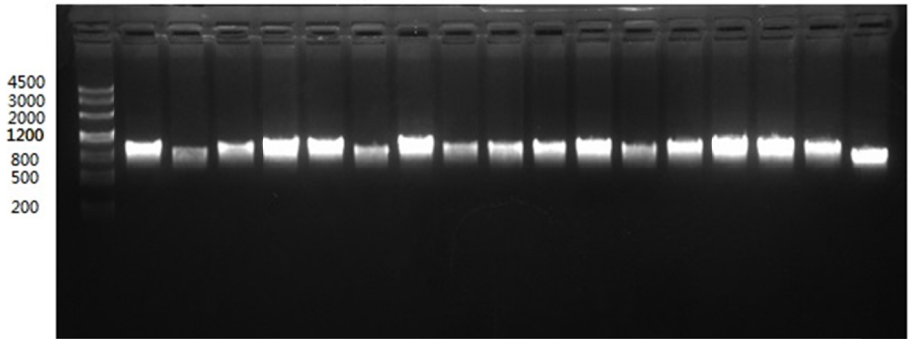

Figure 1. PCR amplification of the 16S rRNA gene

Note. The primers 799f and 1492r were used. Genomic DNA of endophytic bacteria was used as a template, and the annealing temperature of the PCR was 50 °C. The predicted product was approximately 700 bp.

Table 1. Number of phylotypes per OTU and their *Hae*III patterns

OTU Identification	Phylotypes ID	Amplified product (bp)	*Hae*III pattern
γ-proteobacter			
Closest to *Stenotrophomonas* sp.	WSDS21	689	335,204,150
	PL122	693	336,206,151
	PL232	700	335,211,154
	PL11	700	335,212,153
Closest to *Pseudomonas* sp.	WSDS121	699	320,179,119,81
	SLY22	703	321,179,122,81
	SLY13	694	318,178,118,80
	PL12	701	322,178,120,81
	PL133	698	321,180,118,79
Closest to Uncultured bacterium	YNZJ312	703	331,197,148,27
	YNZJ323	699	328,201,141,29
	SLY321	694	327,198,141,28
	DYPE121	704	332,196,145,31
Closest to *Pectobacterium carotovorum* subsp.	SLY33	699	446,244,9
Closest to *Yokenella regensburgei* strain	MGJ121	682	357,201,79,33,12
Closest to *Citrobacter* sp.	PL212	703	501,118,84
Closest to *Kluyvera ascorbata* strain	MGJ136	700	357,217,126
Closest to *Enterobacter* sp.	PL231	703	301,212,179,11
	SLY32	703	302,211,179,11
Firmicutes			
Closest to *Paenibacillus* sp.	PL222	700	441,177,52,30

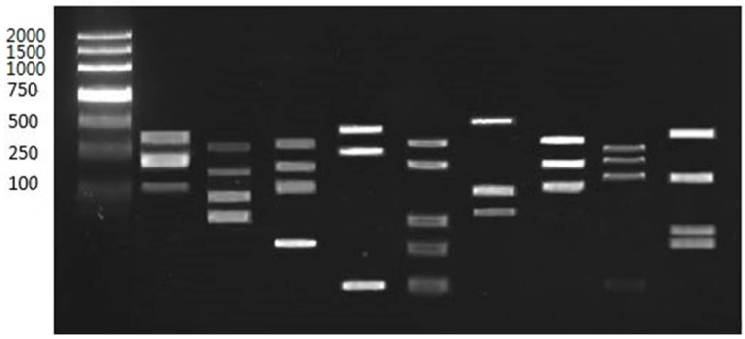

Figure 2. ARDRA analysis patterns of the 16S rDNA.

Note. The specific PCR products were completely digested using the restriction enzyme *Hae* III. The digested products were separated by electrophoresis. Strains with the same digestion patterns were grouped into OTUs.

3.3 Diversity and Distribution Analysis of Endophytic Bacteria

Representative isolates of specific groups were selected for sequencing and compared with the sequences in GenBank using the BLASTN search program. All isolates showed high similarities (≥ 99%) with their closest related species. The details of representative strains are listed in Table 2. Sequence analysis revealed the bacterial diversity of *P. notoginseng* seeds. These strains contain 2 Gram-positive species in one genus and 135 Gram-negative species in eight genera, indicating the complexity of endophytic population present in the seeds of *P. notoginseng* plants. The relationship between the isolates and the reference species is shown in the phylogenetic tree (Figure 3). γ-proteobacteria was the most dominant group among the isolates (135 of 137 isolates) and comprised eight genera, with *Pseudomonas* being dominant (58 of 135 isolates). The second dominant genus was *Enterobacter* (20.4%), followed by uncultured bacterium (14.6%) and *Stenotrophomonas* (10.2%).

Table 2. Similarity of the16S rDNA sequences of partial endophytic bacterial strains from *P. notoginseng* seeds

Group	Isolates (accession number)	No. of isolates	Closest relative (accession number)[a]	Similarity (%)
γ-proteobacteria	WSDS21 (KX688530)	3	*Stenotrophomonas maltophilia* strain G10b (KC136828.1)	99
	PL11 (KX688526)	4	*Stenotrophomonas* sp. REp-tet_144 (JX899643.1)	99
	PL232 (KX688525)	5	Uncultured *Stenotrophomonas* sp. (LC002923.1)	99
	PL122 (KX688520)	2	*Stenotrophomonas maltophilia* strain ZJB-14120 (KM655831.1)	99
	WSDS121 (KX688529)	3	*Pseudomonas* sp. Tibet-YD5003-3 (KF805078.1)	99
	SLY22 (KX688532)	4	*Pseudomonas* sp. BS29 (KR063209.1)	99
	SLY13 (KX688533)	1	*Pseudomonas* sp. +Y33 (JX113247.1)	99
	PL12 (KX688521)	26	*Pseudomonas beteli* strain RRLJ SMAR (DQ299947.1)	99
	PL133 (KX688522)	24	*Pseudomonas* sp. PW49 (KT726998.1)	99
	YNZJ312 (KX688537)	8	Uncultured bacterium clone Y1-5 (JF766465.1)	99
	YNZJ323 (KX688538)	9	Uncultured bacterium clone M1_209_H3 (JN683972.1)	99
	SLY321 (KX688535)	2	Uncultured bacterium clone 3Y-35 (EU786145.1)	99
	DYPE121 (KX688536)	1	Uncultured bacterium clone c93 (KC954365.1)	99
	SLY33 (KX688534)	1	*Pectobacterium carotovorum* subsp. carotovorum strain RN24 (KC790284.1)	99
	MGJ121 (KX688527)	2	*Yokenella regensburgei* strain NvH01 (KJ397957.1)	100
	PL212 (KX688523)	7	*Citrobacter* sp. F41 (FJ405282.1)	99
	MGJ136 (KX688528)	5	*Kluyvera ascorbata* strain IHB B 7177 (KJ767338.1)	99
	PL231 (KX688524)	24	*Enterobacter* sp. HT-Z52-B2 (KJ516915.1)	100
	SLY32 (KX688531)	4	*Enterobacter aerogenes* strain IEY (GQ165811.1)	100
Firmicutes	PL222 (KX688519)	2	*Paenibacillus* sp. MC5-3 (FJ932657.1)	99

Note. [a] Closest relative species and its accession number in the 16S rDNA sequence database.

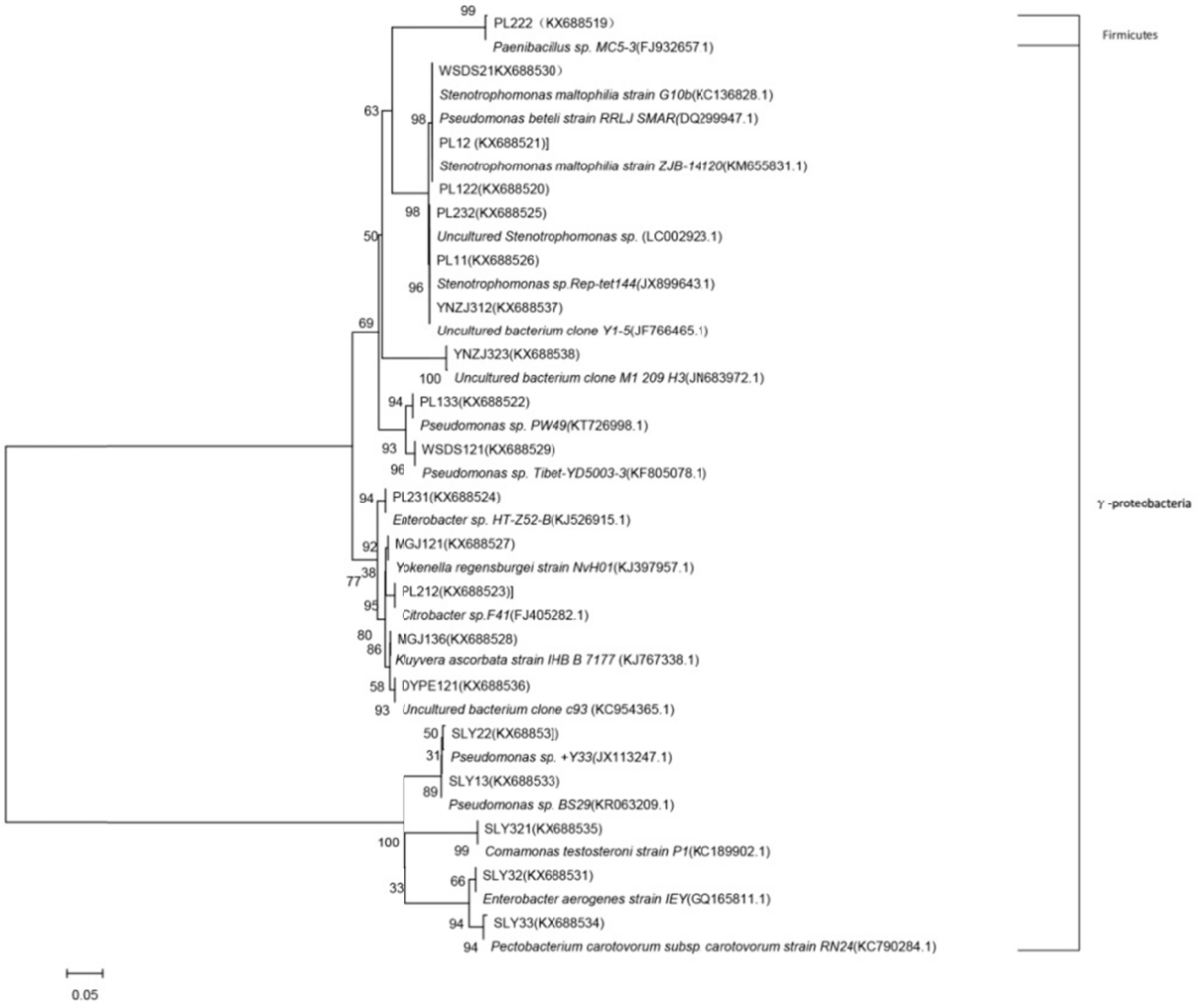

Figure 3. Phylogenetic tree based on partial 16S rDNA sequences of the endophytic bacteria of the *P. notoginseng* seeds using the neighbor-joining method

Note. The numbers in round brackets indicate the accession number in GenBank. The numbers at branch points indicate the bootstrap threshold (samples drawn 1000 times). Scale bar, 0.05 substitutions per base position.

The diversity of the endophytic bacteria of *P. notoginseng* plants was assessed using the seeds collected from the following six growing areas of the Wenshan region: Panlong of Yanshan (PL), Wenshandongshan of Wenshan (WSDS), Zhujie of Guangnan (ZJ), Hanqing of Maguan (MG), Shuanglongying of Qiubei (SLY), and Xichou (DYPE). The *Pseudomonas* genus showed a predominant existence and wide distribution in seeds collected from three places, which included Panlong of Yanshan, Wenshandongshan of Wenshan and Shuanglongying of Qiubei. Both the second and the third dominant genera, *Enterobacter* and *Stenotrophomonas*, were isolated from Panlong of Yanshan and Shuanglongying of Qiubei. The most endophytic bacteria isolates (94 of 137 isolates) originated from Panlong of Yanshan. With the exception of Panlong, the highest number of species (5 of 9 species) occurred in the seeds collected from Shuanglongying of Qiubei.

4. Discussion

Molecular approaches based on 16S rRNA gene analysis have been successfully used for bacterial community analysis. The ARDRA technique has been widely applied in genetic diversity studies of rhizobacteria (Dellagnezze et al., 2016; Mehri et al., 2011; Nievas et al., 2012; Santoro et al., 2016; Sanyal et al., 2016). In the present study, ARDRA analysis was applied to group the endophytic bacterial strains into different OTUs. The sequencing of 16S rDNA proved that each representative strain from each OTU represents a genus. The results suggest that this method is accurate and effective for the isolation and identification of the endophytic bacteria

from *P. notoginseng* seeds, provides a preliminary screening before sequencing and avoids several rounds of sequencing.

Endophytes have been considered as abundant sources for probiotics. Ratnaweera et al. (2013) isolated eight endophytic fungi from *Opuntia dillenii*, seven of which exhibited antimicrobial activity. The most biologically active species is *Fusarium* sp., and the second most active is *Aspergillus niger*. Chadha et al. (2015) suggested that plant endophytic fungi can protect plants against various pathogen and pests and help plants survive under harsh biological or abiotic stresses. Tantirungkij et al. (2015) reported that rice leaves harbor several new yeast strains. Khan et al. (2015) isolated two fungal strains, *Fusarium tricinctum* RSF-4L and *Alternaria alternata* RSF-6L, which promote the growth of the host by phytohormone secretion. Ma et al. (2013) isolated endophytic bacteria from *P. notoginseng*, and 104 strains showed antagonism against *Fusarium oxysporum*, *Ralstonia* sp. and *Meloidogyne hapla*, which are three major pathogens associated with the root-rot disease complex of *P. notoginseng*. Shahzad et al. (2016) isolated an endophytic *Bacillus amyloliquefaciens* with the potential to produce gibberellins (GAs) and that plays a role in improving host-plant physiology.

In the present study, the bacterial endophytes isolated from the seeds of *P. notoginseng* belonged to four bacterial groups, including γ-proteobacteria and *Firmicutes*, *Enterobacter*, uncultured bacteria and *Firmicutes*. These results suggest that *P. notoginseng* seeds carry abundant microbial resources. Among the isolated bacteria, the dominant bacteria was *Pseudomonas* sp., followed by *Enterobacter* sp., uncultured bacteria clones, and *Stenotrophomonas* sp. To our knowledge, this report is the first comprehensive study on the isolation of endophytic bacteria from *P. notoginseng* seeds.

Strains isolated from the seeds may have biological activity against pathogens or perhaps are pathogens leading to diseases that occur during seed germination, seedling growth or plant development. Some *Pseudomonas* strains serve as plant growth-promoting rhizobacteria (PGPR) or biological control agents against plant pathogens (de Bruijn et al., 2007; Raaijmakers et al. 2010). *Pseudomonas* sp. is also one of bacterial pathogens that cause *P. notoginseng* root-rot disease (Miao et al., 2006). In the present study, the dominant endophytic bacteria were *Pseudomonas* sp. Whether the subsequent harboring of these *Pseudomonas* sp. strains leads to disease or plays a beneficial role on plants requires further research. *Stenotrophomonas maltophilia* has been reported to promote plant growth due to its production of phytohormones (Park et al., 2005; Naz et al., 2009) and to be a biological control agent due to its production of antibacterial compounds and secretion of fungicidal metabolites (Messiha et al., 2007; Taghavi et al., 2009). *Pectobacterium carotovorum* is a plant pathogen with a diverse host range and that causes bacterial soft rot (Mansfield et al., 2012). *Enterobacter aerogenes* is a nosocomial and pathogenic bacterium that causes opportunistic human infections. Whether these pathogens cause diseases on *P. notoginseng* remains unknown. Therefore, the isolation of these endophytic bacterial strains not only helps to further current understanding of the outbreak of seedling or plant diseases, but also provides good candidates for biological control against soil-borne root diseases.

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