Diversity of endophytic bacteria within nodules of the Sphaerophysa salsula in different regions of Loess Plateau in China

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Abstract

A total of 115 endophytic bacteria were isolated from root nodules of the wild legume Sphaerophysa salsula grown in two ecological regions of Loess Plateau in China. The genetic diversity and phylogeny of the strains were revealed by restriction fragment length polymorphism and sequencing of 16S rRNA gene and enterobacterial repetitive intergenic consensus-PCR. Their symbiotic capacity was checked by nodulation tests and analysis of nifH gene sequence. This is the first systematic study on endophytic bacteria associated with S. salsula root nodules. Fifty of the strains found were symbiotic bacteria belonging to eight putative species in the genera Mesorhizobium, Rhizobium and Sinorhizobium, harboring similar nifH genes; Mesorhizobium gobiense was the main group and 65 strains were nonsymbiotic bacteria related to 17 species in the genera Paracoccus, Sphingomonas, Inquilinus, Pseudomonas, Serratia, Mycobacterium, Nocardia, Streptomyces, Pseudonibacter, Brevibacillus, Staphylococcus, Lysinibacillus and Bacillus, which were universally coexistent with symbiotic bacteria in the nodules. Differing from other similar studies, the present study is the first time that symbiotic and nonsymbiotic bacteria have been simultaneously isolated from the same root nodules, offering the possibility to accurately reveal the correlation between these two kinds of bacteria. These results provide valuable information about the interactions among the symbiotic bacteria, nonsymbiotic bacteria and their habitats.

Introduction

The establishment of nitrogen-fixing symbiosis (root or stem nodules) with some soil bacteria, collectively called rhizobia, is a unique feature of the plants of the legume family (Leguminosae or Fabaceae). Traditionally, rhizobia are included in the well-known genera Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium and Allorhizobium of Alphaproteobacteria (Zakhia & de Lajudie, 2001). Because of their economic and ecological importance, the rhizobium–legume symbiosis has been investigated intensively, and the nodule-forming bacteria affiliated to the genera Methylobacterium (Sy et al., 2001), Devosia (Rivas et al., 2002), Blastobacter (Van Berkum & Eardly, 2002), Ochrobactrum (Ngom et al., 2004) and Shinella (Li et al., 2008) in Alphaproteobacteria. Burkholderia (Moulin et al., 2001) and Cupriavidus (Chen et al., 2001) in Betaproteobacteria have also been identified.

In addition to rhizobia, other bacteria have been found inside legume nodules (Philipson & Blair, 1957; Sturz et al., 1997; De Lajudie et al., 1999; Bai et al., 2002, 2003; Tokala et al., 2002; Vandamme et al., 2002; Benhizia et al., 2004; Barrett & Parker, 2006; Zakha et al., 2006; Kan et al., 2007; Li et al., 2008; Ibáñez et al., 2009). These bacteria were not able to induce nodules and did not fix nitrogen symbiotically. However, they live inside nodules for at least a part of their life cycle and do not visibly harm host plants and are thus called endophytic bacteria. The endophytic bacteria in nodules were mainly Bacillus, Pseudomonas and enterobacterial species (Zakhia et al., 2006; Kan et al., 2007). Their
ecological role for the plant is so far unknown, but the nodules have been proposed to form an ecological niche for survival of the bacteria (Muresu et al., 2008). These bacteria possibly entered nodules in association with the infecting rhizobia. Enterobacterial species were once reported to have infection ability and nodulation genes (Benhizia et al., 2004). However, a thorough examination of the system revealed that the cultures were mixed with rhizobia that were mainly in an unculurable state when isolated from nodules (Muresu et al., 2008).

The *Leguminosae* family includes more than 18 000 known species (http://www.ildis.org/), but so far only a small fraction of them have been investigated for nodulation (Sprent, 2001) and even fewer have been the subject of studies on their rhizobia and the nodule endophytes. *Swainsona*, or Austrian peaweed [*Sphaerophysa salsula* (Pall.) DC], is one of the highly drought-tolerant perennial herbaceous legumes widely distributed in the Middle-Asia and northwestern China. This deep-rooted wild legume has potential for vegetation of arid regions and highly alkaline soils, and is used as a folk medicine for the treatment of hypertension in China. It forms nitrogen-fixing root nodules but its bacterial partners are so far unknown.

Previous studies have indicated that due to environmental factors, including pH of the soil (Anyango et al., 1995), soil type (Girvan et al., 2003) and agricultural practices (Dowdle & Bohlool, 1985; Palmer & Young, 2000), rhizobial species or populations can alternate to nodulate the leguminous plants, in addition to the specific affinities among the rhizobia and legumes. Therefore, each legume, like *S. salsula*, grown in certain geographic regions could serve as a model for studying the interactions among the symbiotic bacteria, nonsymbiotic bacteria, host plant and their habitats.

The aims of the present study were to isolate and characterize root nodule-inhabiting bacteria associating with *S. salsula* grown in two ecological regions in China and to investigate whether there is a relationship between the biogeography of the host legume and the composition of nodule bacterial communities.

**Materials and methods**

**Collection of root nodules from *S. salsula***

Root nodules were collected in July 2008 when *S. salsula* was flowering, the growth stage facilitating the plant identification and the effectivity of most of the nodules. The geographical location of the sampling sites was determined by GPS. The plants were photographed, and foliage and flowers were preserved as specimens for further identification. Plants grown at six sampling sites within two ecological regions in Loess Plateau of China were sampled (Fig. 1). Ecoregion I (Gansu province) has a typical temperate and monsoon climate with sandy loam and saline/alkaline soil. Ecoregion II (Ningxia Autonomous Region) has a semi-arid temperate climate with clay soil. Root-attached soils were also collected and stored at 4 °C for subsequent characterization. Physicochemical characteristics such as organic carbon content, total N content, C/N, total K content, total P content and pH of soil samples were analyzed. Detailed information about the sampling sites is available in Supporting Information, Table S1.

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**Fig. 1.** Simplified map of sampling area showing location of Zhangye (Zy), Gaotai (Gt), Guazhoun (Gz) and Minqin (Mq) in Gansu province in a zone of 305 × 665 km (south to north × east to west) and Zhongwei (Zw) and Qingtongxia (Q) in Ningxia Autonomous Region in a zone of 90 × 68 km. Number of acquired bacterial isolates in each site is presented in parentheses.
For nodule collection, the distance between sampling sites was at least 50 km, and at least three subsites, separated by more than 1 km, were included within a sampling site. From each subsite, 10–15 plants separated at least by 30 m between each other were chosen randomly and uprooted. Three or four undamaged, healthy root nodules of similar size were excised from the lateral roots of each plant, brushed free of soil debris and then immediately either desiccated over silica gel in microtubes (1.5 mL) with cotton wool or surface sterilized (see procedure below) and temporarily stored in test tubes with yeast-mannitol agar (YMA) medium (Vincent, 1970).

**Isolation of bacteria from nodules**

Healthy, nonruptured nodules were carefully washed under running water in a fine-mesh plastic holder and surface sterilized by immersion in 95% ethanol for 30 s, then in 5% sodium hypochlorite for 3 min, and finally rinsed eight times in sterile distilled water. The surface-sterilized root nodules were crushed on a sterile plate, and the bacteria were isolated by streaking the nodule juice on YMA plates containing Congo red (Vincent, 1970), potato dextrose agar (PDA) (extract of 200 g potato, 20 g glucose, 18 g agar, 1 L distilled water), King’s B medium (10 g peptone, 10 g glycerol, 1.5 g K2HPO4, 1.5 g MgSO4, 20 g agar, 1 L distilled water; pH 7.2) and nutrient agar (NA) (5.0 g peptone, 1.5 g yeast extract, 1.5 g beef extract, 5.0 g NaCl, 20 g agar, 1 L distilled water; pH 7.2). Isolates were incubated at 28 °C, and the purity of the isolates was guaranteed by repeated streaking of single colonies on the same media and by microscopic examination. Pure cultures were preserved at 4 °C for temporary storage or in 30% v/v glycerol at −80 °C for long-term storage.

To confirm that the surface sterilization process was successful, the surface-sterilized nodules were rolled on YMA plates and aliquots of the sterile distilled water from the final rinse were plated onto YMA plates as controls to detect possible contaminants. Only the isolates from nodules without growth on the control plates were considered nodule-inhabiting bacteria.

**Plant inoculation tests**

*Sphaerophysa salsula* seeds were surface sterilized by the protocol of immersion in 96% sulfuric acid for 20 min, washing 10 times with sterile water to remove all traces of acid, immersion in 75% ethanol for 30 s, followed by stirring in 0.1% HgCl2 for 5 min and rinsing eight times in sterile distilled water. In each of the sterile Petri dishes, 10 surface-sterilized seeds were put separately on moist filter paper for germination at 28 °C. The germinated seeds were inoculated by immersing them for 8 h in a thick suspension (approximately 10⁹ CFU ml⁻¹) of bacterial culture grown overnight, and then were transferred to sterile glass tubes containing nitrogen-free plant nutrient solution (Vincent, 1970) sealed with cotton plugs. All nodulation tests were performed in triplicate and noninoculated plants were included as blank control. Plants were grown in growth chamber with a 16-h daylight photoperiod at 28 °C, a night temperature of 20 °C and 65% relative humidity. Nodule formation was observed after 6–8 weeks of growth.

**PCR-based restriction fragment length polymorphism (RFLP) and sequencing of 16S rRNA genes**

Total DNA was extracted according to the method of Terefe-work et al. (2001) and was used as templates in PCR with primers fD1 and rD1 (Weisburg et al., 1991), which respectively correspond to *Escherichia coli* 16S rRNA gene positions 8–27 and 1524–1540. Procedures described by Van Berkum et al. (1996) were used for PCR amplification of almost complete 16S rRNA gene. The PCR products were digested separately with restriction endonucleases Mspl, HaeI, HinfI and Hhal as recommended by manufacturer TaKaRa. The restriction fragments were separated by electrophoresis in 1.5% w/v agarose gels and photographed under UV light after staining with 0.5 μg ml⁻¹ of ethidium bromide. The RFLP patterns obtained from the digestions with the four restriction endonucleases were combined for comparison and isolates sharing the same RFLP patterns were designated an rRNA type.

With the same primers and procedures used for PCR-RFLP, the 16S rRNA genes of the representative strains for each rRNA type were sequenced using the method of direct sequencing from PCR products (Hurek et al., 1997). The sequences obtained were used to retrieve those of related species from the GenBank database. The sequences were aligned using the programs in the package *CLUSTALX* version 1.83 (Nick et al., 1994; Thompson et al., 1997) and the similarity of each pair of sequences was computed using *DNAMAN* (version 6.0.3.40, Lynnon Corporation). The phylogenetic tree was constructed by the neighbor-joining method and the Jukes–Cantor model, and bootstrapped with 1000 replications of each sequence using *TREECON* version 1.3b (van de Peer & De Wachter, 1994).

The criterion for operational taxonomic units (OTUs) defined by 16S rRNA gene sequence was set at <3% divergence (Vinuesa et al., 2005b). The isolation frequency of each OTU was calculated as \( F = n/N \), where \( n \) is the number of sites where an OTU was isolated and \( N \) is the total number of sampling sites. The richness of an OTU in a sampling site was expressed as \( R = s/S \), where \( s \) is the number of strains in an OTU and \( S \) is the total strain number obtained in the site.
Enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis

ERIC-PCR fingerprinting allowed the differentiation of related strains (De Bruijn, 1992). In the present study, ERIC-PCR was performed using total DNA as template with the primers ERIC1R (5' -ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5' -AAG TAA GTG ACT GGG GTG AGC G-3') for PCR amplification as described by De Bruijn (1992). The amplified DNA fragments were separated by electrophoresis in 1.8% w/v agarose gel and visualized as in the RFLP analysis. To determine the cut-off for ERIC type (pattern), each of the randomly selected isolates Qtx-3, Qtx-11, Zw-22-2 and Gaoshi-1 was incubated in three independent replicates, which were used separately as templates in PCR. Similarities among the PCR patterns of the three repetitions were calculated and the average of the four isolates was used as the cut-off. Strains sharing similarities of PCR patterns greater than the cut-off were designated as the same ERIC type (pattern). All PCR patterns were used in clustering analysis with Dice coefficient and the method of UPGMA in NTSYS pc2.1 software package (Vauterin & Vauterin, 1992). Statistical analysis of the ERIC-PCR DNA fingerprints was performed with the spss program (SPSS, version 12.0 for Windows). Computer-assisted analysis of the fingerprints was performed using GELCOMPAR II (version 1.5; Applied Maths, Kortrijk, Belgium). ERIC groups were defined by taking the 16S rRNA RFLP type as reference.

Amplification and sequencing of nifH gene

The nifH gene encodes dinitrogenase reductase, which is essential for nitrogen fixation in the diazotrophic organisms (Raymond et al., 2004). Using the primers 34F [5'-AAA GG(C/T) GG(A/T) ATC GG(C/T) AA(A/G) TCC ACCAC-3'] and 491R [5'-TTG TT(G/C) GC(G/C) GC(G/C) GC(A/G) TAC AT(G/C) GCC ATC AT-3'] and the procedures of Rosch et al. (2002), the nifH fragments were used for amplification. The PCR products were electrophoresed in 1% w/v agarose gel and the bands corresponding to the expected size (460 bp) were cut, purified and sequenced directly (van Berkum et al., 1996). Sequences were assembled and aligned, and a phylogenetic tree was constructed as described for the 16S rRNA gene analysis.

Effects of endophytes on S. salsula plants

The isolate (nonsymbiotic bacterium) alone and the mixture of each isolate with Mesorhizobium gobiense Zw-19 (1:1) were inoculated on the surface-sterilized S. salsula seeds. Negative controls without inoculation and positive controls inoculated with M. gobiense Zw-19 were included for comparison. The surface sterilization, germination, inoculation and incubation of the plants were performed as described previously (Vincent, 1970). All the pot experiments were performed in triplicate and plants were harvested after 55 days of inoculation when well-developed nodules could be detected. Various parameters such as plant fresh weight, chlorophyll content, shoot length, nodule number per plant and nodule weight per plant were determined.

Statistical analysis

The correlations of the diversity (RFLP types) and the geographic origins (sampling sites) were examined with correspondence analysis (CA), using the spss 12.0 package (Data Theory Scaling System Group, Faculty of Social and Behavioral Sciences, Leiden University, the Netherlands). The data in inoculation tests were statistically analyzed to estimate the effects of endophytic bacteria on the nodulation and growth of the S. salsula plants with the model of post hoc multiple comparisons, one-way ANOVA program in the spss 12.0 package.

Results

Isolation of bacteria from nodules

In the course of isolation, the surface-sterilization treatment was generally efficient, as in most cases no bacterial colonies were observed on the control plates. Most of the nodules yielded a single colony type, but two or more colony types were recovered from some of the nodules. In all, 115 bacterial colonies were recovered, 66 from Ecoregion I and 49 from Ecoregion II (Table 1, Fig. 1). The isolates defined as symbiotic bacteria (rhizobia) in the subsequent analyses were only obtained from medium YMA. The isolates belonging to nonsymbiotic bacteria were from one to three other medium, such as NA medium, King's B medium and PDA. Detailed information of the genera in each culture is shown in Table S2.

PCR-based RFLP analysis and 16S rRNA gene sequencing

Twenty-five different rRNA types (RFLP patterns) were obtained from the isolates (Table 1). The sequences of 16S rRNA genes acquired from isolates representing the RFLP types have been deposited in GenBank under accession numbers GU129566–GU129569 (Fig. 2). By BLAST searching (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic analysis, 50 isolates within the RFLP types 1–8 were identified as Rhizobium, Sinorhizobium or Mesorhizobium (Table 1, Fig. 2). The 65 endophytes found had a high level of genetic diversity and belonged to three phyla: Proteobacteria, Actinobacteria and Firmicutes. Within phylum Proteobacteria, four isolates belonged to the class Alphaproteobacteria (Paracoccus, Sphingomonas, Inquilinus) and 11 to the class Gammaproteobacteria (Pseudomonas and Serratia). The six isolates of phylum Actinobacteria were related to
Table 1. Isolates obtained from *Sphaerophysa salsula* root nodules and relevant information

<table>
<thead>
<tr>
<th>Isolates*</th>
<th>RFLP types</th>
<th>ERIC types</th>
<th>Nod, nifH²</th>
<th>Ecoregions¹</th>
<th>Most related bacteria (16S rRNA gene similarity)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qtx-3</td>
<td>1</td>
<td>1</td>
<td>+, –</td>
<td>II</td>
<td><em>Rhizobium giardinii</em> (97%)</td>
</tr>
<tr>
<td>Qtx-14</td>
<td>1</td>
<td>2</td>
<td>+, –</td>
<td>II</td>
<td><em>Rhizobium leguminosarum</em> (100%)</td>
</tr>
<tr>
<td>Qtx-142</td>
<td>1</td>
<td>3</td>
<td>+, –</td>
<td>II</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Mq-141</td>
<td>1</td>
<td>4</td>
<td>+, –</td>
<td>I</td>
<td><em>Paracoccus halophilus</em> (96%)</td>
</tr>
<tr>
<td>Mq-142</td>
<td>1</td>
<td>5</td>
<td>+, –</td>
<td>I</td>
<td><em>Sphingomonas pruni</em> (98%)</td>
</tr>
<tr>
<td>Qtx-111, Qtx-112, Qtx-113, Qtx-141, Qtx-191, Qtx-192, Zy-31, Zy-32, Zy-3-3</td>
<td>2</td>
<td>7</td>
<td>+, +</td>
<td>I, II</td>
<td><em>Rhizobium galegae</em> (99%)</td>
</tr>
<tr>
<td>Qtx-10-1, Qtx-1</td>
<td>3</td>
<td>6</td>
<td>+, +</td>
<td>I</td>
<td><em>Rhizobium leguminosarum</em> (100%)</td>
</tr>
<tr>
<td>Gz-202, Mq-3</td>
<td>4</td>
<td>9</td>
<td>+, +</td>
<td>I</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Gz-341, Zw-23</td>
<td>4</td>
<td>10</td>
<td>+, +</td>
<td>I, II</td>
<td><em>Mesorhizobium amorphae</em> (99%)</td>
</tr>
<tr>
<td>Qtx-5, Qtx-102, Zw-19, Zw-91</td>
<td>4</td>
<td>11</td>
<td>+, +</td>
<td>II</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Gt-32, Gt-271, Gz-4, Zw-2, Zw-4, Zw-2-1</td>
<td>4</td>
<td>12</td>
<td>+, +</td>
<td>I, II</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Zw-8</td>
<td>4</td>
<td>13</td>
<td>+, +</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Mq-18</td>
<td>4</td>
<td>14</td>
<td>+, +</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Zy-10</td>
<td>4</td>
<td>15</td>
<td>+, +</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Mq-13</td>
<td>4</td>
<td>16</td>
<td>+, +</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Qtx-161</td>
<td>4</td>
<td>17</td>
<td>+, +</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Zw-81</td>
<td>4</td>
<td>18</td>
<td>+, +</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Zw-191</td>
<td>4</td>
<td>19</td>
<td>+, +</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Qtx-11</td>
<td>4</td>
<td>20</td>
<td>+, +</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Zw-9</td>
<td>4</td>
<td>22</td>
<td>+, +</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Qtx-17</td>
<td>4</td>
<td>23</td>
<td>+, +</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Gz-34, Gt-11, Gt-92, Qtx-15, Qtx-21, Zw-111</td>
<td>5</td>
<td>8</td>
<td>+, +</td>
<td>I, II</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Zy-2</td>
<td>6</td>
<td>24</td>
<td>+, +</td>
<td>I</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Qtx-24</td>
<td>7</td>
<td>21</td>
<td>+, +</td>
<td>I</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Qtx-8-1, Qtx-82</td>
<td>8</td>
<td>25</td>
<td>+, –</td>
<td>II</td>
<td><em>Mesorhizobium gobiense</em> (100%)</td>
</tr>
<tr>
<td>Zy-3, Zw-11</td>
<td>9</td>
<td>30</td>
<td>–, –</td>
<td>I, II</td>
<td><em>Paracoccus halophilus</em> (96%)</td>
</tr>
<tr>
<td>Guashi-1</td>
<td>10</td>
<td>29</td>
<td>–, –</td>
<td>I</td>
<td><em>Sphingomonas pruni</em> (98%)</td>
</tr>
<tr>
<td>Mq-10</td>
<td>11</td>
<td>51</td>
<td>–, –</td>
<td>I</td>
<td><em>Inquilinus limbos</em> (99%)</td>
</tr>
<tr>
<td>Zy-2-1</td>
<td>12</td>
<td>31</td>
<td>–, –</td>
<td>I</td>
<td><em>Pseudomonas fluorescens</em> (99%)</td>
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<tr>
<td>Zy-22</td>
<td>12</td>
<td>32</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Zy-23</td>
<td>12</td>
<td>33</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Gz-20</td>
<td>12</td>
<td>34</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Qtx-252</td>
<td>12</td>
<td>35</td>
<td>N, N</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Zw-22, Zw-221, Zw-82, Qtx-30</td>
<td>13</td>
<td>26</td>
<td>–, –</td>
<td>II</td>
<td><em>Serratia plymuthica</em> (99%)</td>
</tr>
<tr>
<td>Gt-101</td>
<td>13</td>
<td>27</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Gz-171</td>
<td>13</td>
<td>28</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Qtx-25</td>
<td>14</td>
<td>38</td>
<td>N, N</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Mq-21, Qtx-19</td>
<td>14</td>
<td>39</td>
<td>–, –</td>
<td>I, II</td>
<td><em>Mycobacterium sacrum</em> (99%)</td>
</tr>
<tr>
<td>Gt-25</td>
<td>15</td>
<td>37</td>
<td>–, –</td>
<td>I</td>
<td><em>Nocardia uniformis</em> (100%)</td>
</tr>
<tr>
<td>Gt-20, Gt-10</td>
<td>16</td>
<td>40</td>
<td>–, –</td>
<td>I</td>
<td><em>Streptomyces bottropensis</em> (100%)</td>
</tr>
<tr>
<td>Gt-1</td>
<td>17</td>
<td>41</td>
<td>–, –</td>
<td>I</td>
<td><em>Paenibacillus amylyticus</em> (100%)</td>
</tr>
<tr>
<td>Mq-31</td>
<td>17</td>
<td>42</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Gt-11</td>
<td>17</td>
<td>43</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Mq-17</td>
<td>18</td>
<td>44</td>
<td>–, –</td>
<td>I</td>
<td><em>Brevibacillus borstelensis</em> (100%)</td>
</tr>
<tr>
<td>Zw-13-3</td>
<td>19</td>
<td>45</td>
<td>–, –</td>
<td>I</td>
<td><em>Lysinibacillus fusiformis</em> (100%)</td>
</tr>
<tr>
<td>Gt-8, Mq-101</td>
<td>20</td>
<td>46</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Gt-51, Gt-191, Gz-1, Gz-22, Zw-13</td>
<td>20</td>
<td>47</td>
<td>N, N</td>
<td>I, II</td>
<td></td>
</tr>
<tr>
<td>Qtx-11, Qtx-12</td>
<td>20</td>
<td>55</td>
<td>–, –</td>
<td>I, II</td>
<td><em>Bacillus simplex</em> (100%)</td>
</tr>
<tr>
<td>Gz-2</td>
<td>20</td>
<td>56</td>
<td>N, N</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Gt-19</td>
<td>20</td>
<td>57</td>
<td>N, N</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Gz-201</td>
<td>21</td>
<td>48</td>
<td>N, N</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Gt-6, Gt-12, Gt-13, Gt-21, Gt-23, Gt-31, Gt-41, Gt-42, Gt-49, Gt-52, Gt-61, Gt-62, Gt-71, Gt-72, Gz-6, Gz-41, Mq-19, Mq-143, Mq-144, Zw-132, Zw-2, Zw-2-1</td>
<td>21</td>
<td>50</td>
<td>–, –</td>
<td>I, II</td>
<td><em>Bacillus safensis</em> (100%)</td>
</tr>
</tbody>
</table>
Mycobacterium, Nocardia and Streptomyces, all belonging to the order Actinomycetales. The 44 isolates grouped in the phylum Firmicutes were identified as Paenibacillus, Brevibacillus, Staphylococcus, Lysinibacillus and Bacillus. Five species of Bacillus were found, making it the most ubiquitous nodule endophyte, accounting for 58.5% (38 isolates) of all the endophytes within nodules of S. salsula, of which Bacillus simplex and Bacillus safensis were the predominant groups. The species Serratia plymuthica, Lysinibacillus fusiformis and Staphylococcus saprophyticus have never been recorded previously as nodule endophytes. Furthermore, Qtx-3 (related to Rhizobium giardinii) and Zy-3 (Paracoccus) had sequence similarities of < 97% to the most related species and could be novel species (Table 1, Fig. 2).

**ERIC-PCR analysis**

ERIC-PCR resulted in specific PCR bands, from 10 to 15 for each strain and with fragments sized from 0.2 to 2.0 kb. In the analysis of the cut-off value for ERIC types, the PCR patterns of three independent cultures for the same strain showed similarities ranging from 86.8% (for strains Qtx-3) to 100% (for Qtx-11) with an average of 94.0 ± 3.9%. We therefore took 94.0% similarity as the cut-off for ERIC types in the cluster analysis (Fig. S1). Among the 115 isolates, 55 ERIC types were identified (Table 1). In general, single unique ERIC types were defined in the RFLP types which harbored one or two isolates, as well as in RFLP types 2 and 5 (corresponding to Rhizobium galegae and Mesorhizobium amorphae) with respectively nine and six isolates. The other main RFLP types harboring three isolates or more showed polymorphism in ERIC-PCR patterns.

When checking the main ERIC types, it was commonly found that strains isolated from different sites in the two provinces shared the same ERIC types, including ERIC types 7 (R. galegae, nine strains), 8 (M. amorphae, six strains), 9–12 (M. gobiense, with two, two, four and six strains, respectively), 26 (S. plymuthica, four strains), 30 (Paracoccus halophilus, two strains), 39 (Mycobacterium sacrum, two strains), 47 (B. simplex, five strains) and 59 (B. safensis, 21 strains).

**Nodulation tests and sequence analysis of nifH genes**

In this analysis, effective pink nodules were found on the S. salsula plants inoculated with the 50 isolates in Mesorhizobium, Rhizobium and Sinorhizobium, and the nodulated plants looked healthy (with dark green leaves). In contrast, no nodule was induced by the 17 isolates representing other bacterial genera (Table 1, root growth), or by the noninoculated controls, despite strain Paracoccus sp. Zy-3 causing a black-coloured covering of the root.

In the nifH gene amplification, PCR fragments of nifH corresponding to the expected size of 460 bp were produced from only six RFLP types (representing rDNA types 2–7), respectively related to R. galegae, Rhizobium leguminosarum bv. vicieae, M. amorphae, M. gobiense, Mesorhizobium tian Shanense and Mesorhizobium mediterraneum. No PCR product was obtained from the representative strains of R. giardinii, Sinorhizobium meliloti and the other bacteria. The divergence between their nifH sequences (accession numbers GU201840–GU201872) was very limited and they were found in the same phylogenetic clade (Fig. 3).

**Isolation frequency and richness of the OTUs**

Among the symbiotic bacteria, M. gobiense was the most ubiquitous and predominant symbiont; it was isolated from all six sampling sites and contained 20 isolates (Table 1). Mesorhizobium amorphae was the second most common symbiotic bacterium, found in four sites and consisting of six isolates. Rhizobium giardinii and R. galegae were found in two sites with five and nine isolates, respectively. The
remaining putative species relating to *R. leguminosarum*, *M. tianshanense*, *M. mediterraneum* and *S. meliloti* were found in one site and harbored one or two isolates.

Among the other bacteria, *Bacillus* species were the ubiquitous nonsymbiotic nodule endophytes, found in five of the six sampling sites, and were predominant with 38 isolates. *Serratia* and *Pseudomonas* were also common, respectively found in four and three sites, but only six and five isolates were contained in each. *Mycobacterium* and *Paenibacillus* were isolated from two sites and the remaining OTUs were isolated from one site only.

Including both symbiotic and nonsymbiotic bacteria, diverse bacteria were isolated from the root nodules of *S. salsula* grown in the Loess Plateau of China. In each site, five (in the case of Zhangye) to 12 (in the case of Qingtongxia) putative species (OTUs) were found. There were 10 OTUs isolated from nodules in Gaotai, eight in Guazhou and in Minqin and seven in Zhongwei (Table 1).

**Fig. 2.** Phylogram of 16S rRNA gene showing relationships of bacteria (in bold) associated with *Sphaerophysa salsula* root nodules. The tree was generated with the neighbor-joining method. Bootstrap values (1000 replicates) above 70% are indicated at the node. Scale bar indicates 1% substitution of nucleotide.
number and average nodule weight of the plant (Table S3). Nitrogen-free culture conditions also could increase nodule length. Their coinoculation with such as chlorophyll content, fresh weight of plant and shoot promoting activity with respect to various plant parameters representative endophytic isolates showed plant growth-promoting activity with respect to various plant parameters.

Under pot culture conditions, inoculation of 15 of the 17 Effects of endophytes on S. salsula plants

Distribution of bacterial genotypes across sites

In the CA, three bacterial groups were defined according to their geographic origin (Fig. 4), demonstrating a clear correlation between the RFLP types and the sampling sites. RFLP types 4, 15, 16, 23, 24 and 25 (corresponding to M. gobiense, Nocardia, Streptomyces, Bacillus cereus, Bacillus pumilus and Staphylococcus) and Gt (Gaotai, Ecoregion I) sampling site formed group I. RFLP types 2, 6, 9, 10, 11, 17, 18, 20, 21 and 22 (corresponding to R. galegae, M. tianshanense, Paracoccus, Sphingomonas, Inquilinus, Paenibacillus, Brevibacillus, B. simplex, B. safensis and Bacillus licheniformis) and three sampling sites Gz (Guazhou), M (Minqin) and Zw (Zhongwei) formed the third group. In general, the majority of genotypes (64.0%) were found to be unique to one site, except for nine genotypes that included samples from different regions (Table 1). A high Pearson’s correlation coefficient (0.838) was observed, suggesting that the level of similarity between fingerprints reflected the genetic relatedness of closely related bacteria.

Effects of endophytes on S. salsula plants

Under pot culture conditions, inoculation of 15 of the 17 representative endophytic isolates showed plant growth-promoting activity with respect to various plant parameters such as chlorophyll content, fresh weight of plant and shoot length. Their coinoculation with M. gobiense Zw-19 under nitrogen-free culture conditions also could increase nodule number and average nodule weight of the plant (Table S3). Among them, B. pumilus Qtx-10 was an excellent plant growth-promoting bacterium, which enhanced the seeding fresh weight by 87.5% and the shoot length by 89.4%.

Discussion

In the present study, RFLP and 16S rRNA gene sequencing were used to define the bacterial OTUs or putative species, as in other studies (Li et al., 2009; Ruiz-Díez et al., 2009). ERIC-
PCR was used to reveal the genetic diversity within bacterial species (de Bruijn, 1992). Twenty-five OTUs (RFLP types) were defined in the bacterial isolates from root nodules of *S. salsula* (Table 1). Combined with the results of noduleation tests and *nifH* gene amplification, 50 isolates were identified as symbiotic bacteria within eight OTUs belonging to *Rhizobium*, *Sinorhizobium* or *Mesorhizobium*. The remaining 65 isolates were nonsymbiotic bacteria (Table 1, Fig. 2).

*Mesorhizobium gobiense* was the main microsymbiont of *S. salsula*, followed by *R. galegae*, *M. amorphae* and *R. giardinii*. The remaining five rhizobial OTUs only contained one or two isolates each. Kuklinsky-Sobral *et al.* (2004) reported that *nifH* gene was not able to be amplified from some symbiotic bacteria, which might be due to the variability of this gene (Zehr *et al.*, 2003). This could explain the failure of amplification of *nifH* gene from isolates of rRNA type 1 (related to *R. giardinii*) and type 8 (related to *S. meliloti*) in the present study. These results imply that the host *S. salsula* might host prefer the chromosome background of *M. gobiense* as its microsymbiont, with *R. galegae*, *R. giardinii* and *M. amorphae* as its secondary preference; the remaining rhizobia were not competitive in forming noduleation of this plant in the studied area.

The high similarities of *nifH* gene sequences among the different rhizobial species (Fig. 3) demonstrated that (1) lateral transfer of symbiotic genes might have occurred among the *Mesorhizobium* and *Rhizobium* species, similar to the previously reported cases between different symbiotic bacteria (Barcellos *et al.*, 2007; Han *et al.*, 2008, 2009) or between symbiotic and nonsymbiotic endophytes (Li *et al.*, 2008); and (2) *S. salsula* strongly selected the symbiotic background of its symbiotic bacteria.

The existence of nonsymbiotic endophytic bacteria in leguminous root nodules is a universal phenomenon (De Lajudie *et al.*, 1999; Wang *et al.*, 2006). Using a direct PCR protocol, Ben Romdhane *et al.* (2005) found that 25.8% of the bacteria in *Acacia tortilis* nodules were not rhizobia. Some of the nodule endophytes, such as *Agrobacterium* strains, had a positive (Liu *et al.*, 2010) or negative (Mrabet *et al.*, 2006) impact on noduleation, and they also could have an effect on the growth of host plants (Wang *et al.*, 2006). Although *Agrobacterium* is the most studied nodule endophyte, it was not isolated in the present study, confirming that this bacterium is not a predominant nodule endophyte (Zakhia *et al.*, 2006). The finding of *Bacillus*, *Pseudomonas* and enterobacterial species as common nodule endophytes also confirmed previous reports (Zakhia *et al.*, 2006; Kan *et al.*, 2007).

In general, the nonsymbiotic endophytes coinhabit naturally with symbiotic bacteria, for example *Bacillus subtilis* and *Bacillus thuringiensis* in soybean nodules together with *Bradyrhizobium japonicum* (Bai *et al.*, 2003), and *Agrobacterium* together with *S. meliloti* in nodules of some woody legumes (Liu *et al.*, 2010). In the present study, most of the root nodules of *S. salsula* prevalingly harbored rhizobia, and some of them were also colonized internally by nonsymbiotic endophytes (Fig. 5); for example *B. pumilus* Qtx-10 cohabited with *R. leguminosarum* Qtx-10-1 and *M. gobiense* Qtx-102, *P. halophilus* Zy-3 with *R. galegae* Zy-31 and Zy-32. However, in some nodules, only endophytic bacteria were isolated, for example Gaoshi-1 (*B. cereus*) and Gaoshi-7 (*S. saprophyticus*) (Fig. 5). In this case, it is possible that the growth of symbiotic bacteria in the same nodule was suppressed by the endophytic bacteria during the isolation because the endophytic bacteria grew faster or produced antibiotics. Another reason might be related to the viable but nonculturable state of rhizobia within nodules (Alexander *et al.*, 1999; Toffanin *et al.*, 2000; Muresu *et al.*, 2008). We are considering, for future studies, direct PCR analysis of nodule endophytes to examine the existence of the nonculturable symbiotic bacteria within nodules of *S. salsula*, to provide a better insight into the diversity of natural legume–endophyte associations.

According to the inoculation tests, most of these endophytic bacteria had no negative effects on the host plants, in comparison with the noninoculated controls (Table S3). However, the interactions between symbiotic and nonsymbiotic endophytes are still unclear and deserve further study.

Previously, biogeographical distribution has been shown in the symbiotic bacteria associated with faba bean (Tian *et al.*, 2007), soybean (Man *et al.*, 2008; Han *et al.*, 2009), *Caragana* spp. (Lu *et al.*, 2009) and some agroforestry legumes (Bala *et al.*, 2003), as well as endophytes (Dalmastri *et al.*, 1999; Li *et al.*, 2008). In the present study, biogeographical distribution was also detected for the symbiotic and nonsymbiotic bacteria of *S. salsula*, as revealed by CA (Fig. 4). The biogeography of rhizobia has been explained by the abiotic conditions (temperature, soil pH, rainfall, etc.) and biotic factors (genotypes of host plants and their distribution), as reported in previous studies (Moulin *et al.*, 2001; Vinuesa *et al.*, 2005a; Diouf *et al.*, 2007), which were also confirmed in the present study, as all the bacteria originating from Gaotai formed a cluster in CA that was distinctive from the other three sites in the same ecological region (Fig. 4); this could be related to the soil of this region having the highest C, N and P concentrations but lowest K (Table S1).

The distribution of some isolates within the same ERIC types in different sites of both ecological regions (Fig. 5) implies that soil characteristics might be more important than climate factors as determinants of bacterial biogeography, as reported previously (Lu *et al.*, 2009). As plant-associated bacteria, the community composition and abundance of symbiotic and nonsymbiotic bacteria of the leguminous root nodules are also affected by the genomic background of their host plants (Yang *et al.*, 2001). However, there is no information available about the genetic variations of this wild plant at this moment.
In conclusion, this is the first systematic study on root nodule endophytes associated with S. salsula grown in two ecological regions of Loess Plateau in China. The present study differed from other similar studies in that both the symbiotic and nonsymbiotic bacteria were isolated simultaneously from the same root nodules. Our results revealed that S. salsula were nodulated with symbiotic bacteria belonging to Mesorhizobium, Rhizobium and Sinorhizobium, mainly M. gobiense; and nonsymbiotic endophytes cohabited universally with the microsymbionts in the nodules with bacteria related to Paracoccus, Sphingomonas, Inquilinus, Pseudomonas, Serratia, Mycobacterium, Nocardia, Streptomyces, Paenibacillus, Brevibacillus, Staphylococcus, Lysinibacillus and Bacillus. The species S. plymuthica, L. fusiformis and S. saprophyticus have never previously been recorded as root nodule endophytes. These results demonstrated that this plant is associated with diverse symbiotic and nonsymbiotic bacteria in its nodules, grown in the Loess Plateau of China. The nifH genes amplified from the Rhizobium and Mesorhizobium isolates showed close relationships despite their chromosomal backgrounds, implying that horizontal gene transfer might have occurred and the host legume might have a strong selection for the symbiotic gene background of rhizobia. The correlation between bacterial genotypes and their geographical origin implies the existence of biogeographical distribution in the nodule endophytes of this plant.

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References

**References**


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. UPGMA dendrogram resulting from ERIC-PCR patterns showing the diversity of 115 isolates obtained from root nodules of the *Sphaerophysa salsula*.

Table S1. Physicochemical characteristics of the studied soils.

Table S2. Medium used for the isolation of bacteria from *Sphaerophysa salsula* root nodules.

Table S3. Results of comparative growth profile of inoculation tests.

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