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Rhizobia inhabiting nodules and rhizosphere soils of alfalfa: A strong selection of facultative microsymbionts

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is an important leguminous forage crop, which is hypothesized to select compatible rhizobial partners from rhizosphere to form nitrogen-fixing nodules. Here we investigated the diversity of rhizobia from both nodules and rhizosphere of alfalfa from farmlands and natural ecosystems along the Yarlung Zangbo River valley in the Tibetan Plateau. 581 nodule isolates were characterized using BOX-A1R PCR fingerprinting, sequences of *rpoB* and nodulation gene *nodC*. They belong to four *rpoB* haplotypes of *Sinorhizobium meliloti* (*rpoB*-I, 473 isolates; *rpoB*-II, 95; *rpoB*-III, 1; *rpoB*-IV, 10), *Sinorhizobium medicae* (1 isolate) and *Rhizobium* sp. (1 isolate). Re-inoculation experiments of the 32 representative strains suggested a great variation in their symbiotic performance on alfalfa. High-throughput sequencing of *rpoB* for 34 rhizosphere samples uncovered that the predominant *rpoB*-I in nodules also dominated among fourteen *rpoB* haplotypes of *S. meliloti* in rhizosphere soils. In addition to *S. meliloti*, more than 40 rhizobial species documented as microsymbionts of other legumes were also identified. Although a very low level of nucleotide diversity for *S. meliloti* was found among sampling sites, multivariate statistical analysis demonstrated a significant differentiation of total rhizobial community between farmlands and natural ecosystems. Moreover, edaphic conditions, especially pH and nitrogen content, were revealed as important deterministic factors shaping the observed beta-diversity of rhizobial community. Taken together, these results demonstrated that the rhizospheric pool of rhizobial germplasms was significantly affected by land tillage compared to that of natural ecosystems, while a predominant population of *S. meliloti* was selected by alfalfa.

1. Introduction

Biological nitrogen fixation by prokaryotes is an essential component of nitrogen cycle and makes substantial contributions to the pool of soil nitrogen, which is fundamental to crop productivity. The most efficient way of biological nitrogen fixation has been found in legume nodules, which rhizobia colonize intracellularly and in which they obtain essential nutrients from the plant host and reduce atmospheric dinitrogen, to the benefit of the host (Dixon and Kahn, 2004; Udvardi and Poole, 2013). In practice, diverse legumes can be cultivated not only to meet the demand of food and forage as crops but also to restore damaged lands as green manure and pioneering plants (Jensen and Hauggaard-Nielsen, 2003; Rajwar et al., 2013; Russelle et al., 2001). For example, *Medicago sativa* (alfalfa) is one of the most important forage crops around the world, not only due to its high productivity and palatability, but also for its ecological ability in preventing soil erosion and elevating soil fertility. However, the amount of symbiotically fixed nitrogen by the alfalfa-rhizobium system can range from zero to 210 kg of N ha^{-1} $year^{-1}$, showing both strain- and cultivar-dependent variations (Jia et al., 2013; Provorov and Tikhonovich, 2003; Russelle et al., 2001).

The efficiency of rhizobium-legume symbiosis is modulated by the specificity and effectiveness of natural populations of rhizobia (Broughton and Perret, 1999; Downie and Walker, 1999; Peix et al., 2014). Rhizobia associated with alfalfa have been intensively studied. *S. meliloti* is the main rhizobial species, which establishes nitrogen-fixing symbiosis with alfalfa around the world, though *S. medicae* has been documented as another rare microsymbiont of alfalfa (Carelli

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et al., 2000; Donnarumma et al., 2014; Roumiantseva et al., 2002; Silva et al., 2007; Zribi et al., 2005). Legumes including alfalfa are well known for their specific selection of rhizobial strains with superior competitiveness for nodule occupancy. This greatly challenges our effort to improve symbiotic nitrogen fixation by introducing commercial inocula into soils harboring such indigenous rhizobia of superior competitiveness (Brockwell and Bottomley, 1995; Peoples et al., 2012; Thilakarathna and Raizada, 2017). Therefore, to reveal the germplasms' pool of indigenous rhizobia in rhizosphere soils is critical for further inoculation practices.

With the revolutionary advances in molecular phylogenetic approaches, sequencing techniques and computational methodologies in recent years (Caporaso et al., 2010; Kozich et al., 2013), many studies had investigated the microbial community and the related edaphic conditions shaping its variations among a wide range of plant rhizosphere soils by adopting the 16S rRNA gene high-throughput sequencing technology (Bulgarelli et al., 2015; Mendes et al., 2014; Ofek-Lalzar et al., 2014; Peiffer et al., 2013). However, the highly conserved 16S rRNA gene is not informative regarding our understanding of the important ecological differentiation in closely related organisms within a genus or a species (Nemergut et al., 2013), and multiple copies (e.g. from one to eleven copies in *Proteobacteria*) of this gene may lead to misinterpretation of the biogeographical patterns (Case et al., 2007). Fortunately, taxonomists have used housekeeping genes such as *recA* and *rpoB* among others to achieve the intraspecific resolution for the purpose of species identification (Miranda-Sanchez et al., 2015; Zhang et al., 2012). In contrast to *recA* and many other housekeeping genes of phylogeny incongruent to the species tree, the *rpoB* gene has been demonstrated to be a good taxonomic and phylogenetic marker proved by DNA-DNA hybridization and phylogenomic analyses (Adekambi et al., 2008, 2009; Tian et al., 2012; Zhang et al., 2012). Recently, *rpoB* was further proposed to be a robust marker for intraspecific level bacterial diversity analysis focusing on specific taxonomic groups in pyrosequencing studies (Vos et al., 2012; Zhang et al., 2017).

In this study, we aimed to uncover the pool of rhizobial species inhabiting alfalfa rhizosphere and nodulating alfalfa across sampling sites in both farmland and natural ecosystem, and investigate the potential factors shaping the beta diversity of rhizobia. To this end, we surveyed 18 agricultural alfalfa lands (farmland) and 16 non-agricultural lands where alfalfa plants were found (natural ecosystem), in the Tibetan Plateau of China. Alfalfa nodule isolates were characterized using repetitive extragenic palindromic PCR (BOX-A1R-PCR), *rpoB* and *nodC* PCR sequencing. Rhizobial diversity in alfalfa rhizosphere soils was investigated by *rpoB* Illumina sequencing at the intraspecific level. The factors shaping the biogeographical patterns of identified rhizobial species were studied and discussed in the context of lifecycles of rhizobia with typical characteristics of facultative microsymbionts.

2. Materials and methods

2.1. Sampling, DNA extraction, soil physicochemical properties characterization

Alfalfa nodules and rhizosphere soils were collected from farmlands and natural ecosystems along the Yarlung Zangbo River valley, which is the major growing region of alfalfa in the Tibetan Plateau. Briefly 3–10 plants were examined for the presence of root nodules per sampling site. The roots of alfalfa were shaken to remove the loose soil and the remaining attached soil were collected and considered to be the rhizosphere soil. Rhizosphere soils and all nodules associated with three plants were collected, and soils were mixed as a single sample for each sampling site. Nodules were stored over silica gel in closed drying tubes until their isolation in the laboratory. The rhizosphere soil samples were stored in ice bags, then transported to laboratory and preserved at −80 °C before DNA extraction. All the nodule and soil samples were collected in 2014 during the vegetative growing season when the alfalfa started blooming. Total DNA of the rhizosphere soil samples from farmlands (18) and natural ecosystems (16) were extracted using the FastDNA SPIN Kit (MP biomedicals, Cleveland, OH, USA), and the quality and quantity of DNA were checked with a NanoDrop device (ND-1000, Thermo Fisher, USA) and kept at −20 °C before use. Moreover, total nitrogen, available phosphorus, organic matter, available potassium, pH, salt content, and ion concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, Cl^{-}, K^{+}, HCO_3^{-} and SO_4^{2-} for air-dried soil samples were determined at the Plant Nutrient and Resource Research Institution, Beijing Academy of Agriculture and Forestry Sciences (Table S1), using methods described previously (Lu, 2000). Briefly, total N was determined by the Kjeldhl method; available phosphorus was measured by Mo-Sb colorimetric method; organic matter was determined by potassium dichromate oxidation and subsequent titration with ferrous sulfate; available potassium was extracted with ammonium acetate and determined using a flame photometer; pH was determined using an acidometer; salt content was measured by gravimetry of the evaporation residue; levels of Cl^{-} and HCO_3^{-} were determined by silver nitrate titration and potentiometric titration, respectively; concentrations of Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, and SO_4^{2-} were measured using inductively coupled plasma atomic emission spectrometry; electrical conductivity (EC) was determined in deionized water (1:5 w/v). Climate data of the sampling sites were acquired from DIVA-GIS (http://www.diva-gis.org) (Table S2).

2.2. DNA fingerprinting, rpoB and nodC sequencing of nodule isolates

Nodules stored in closed drying tubes were soaked for half an hour in sterile water before being surface sterilized, using 95% ethyl alcohol for 30 s and 1% (w/v) calcium hypochlorite for 3 min, and then rinsed five times in sterile water. The resultant nodules were crushed, spread onto TY solid medium and incubated at 28 °C for 24 h (Vincent, 1970). Nodule isolates were purified for two times and characterized using DNA fingerprinting methods BOX-A1R-PCR with PCR reaction and amplification procedures described earlier (Versalovic et al., 1994). Representative strains (based on electrophoresis patterns of the BOX-A1R-PCR products) were selected by Gelcompar II software (Vauterin and Vauterin, 1992) and identified by *rpoB* and *nodC* sequencing. The *rpoB* primers (*rpoB*-F: 5′-GCT NTC GCA GTT CAT GGAC-3′ and *rpoB*-R: 5′-GCA GGT RTT CTG GTT BGAR-3′) were designed using the software Primer 5.0 based on an alignment of homologous regions in genomes of *S. meliloti* and *S. medicae* (Galibert et al., 2001; Reeve et al., 2010). The amplified fragment of 759 bp starts from 1560 bp to 2319 bp positions of *rpoB* in *S. meliloti*. *The nodC* genes were amplified using *nodC*rev1160 (5′- CGY GAC ARC CAR TCG CTR TTG-3′) (Sarita et al., 2005) and a modified version of *nodC*for540 (5′-TGA TYG AYA TGG ART AYT GGP CR-3′). PCR reactions were carried out in a 25-μL mixture solution with 1 μL of template DNA, 1 μL of each primer, 10 μL of Taq-mix plus 12 μL of sterile water, and amplification consisted of an initial denaturing step at 95 °C for 5 min and 30 cycles of amplification (94 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min plus a final extension of 72 °C for 10 min). The PCR products were then purified and sequenced.

2.3. Plant assays

Alfalfa seeds were surface sterilized in 3% (vol/vol) NaClO solution, allowed to germinate, and inoculated with 1 ml of rhizobial culture at OD_{600} of 0.2. Plants were grown in vermiculite moistened with low-N nutrient solution (Vincent, 1970). At 60 days post inoculation, leaf chlorophyll content was determined using a SPAD-502 meter (Konica Minolta). The height of seedlings and number of nodules were recorded. Plant shoots were dried at 65 °C for five days and shoot dry weight per plant was then determined. Uninoculated plants were used as the negative control. Thirty-two representative rhizobial strains were selected based on sampling origin, and sequence types of *rpoB* and *nodC*. Reference strains of *S. meliloti* (USDA 1002^{T}, 1021) and *S. medicae* (USDA

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