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Soil Biology & Biochemistry 40 (2008) 238–246

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China

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Received 7 May 2007; received in revised form 29 July 2007; accepted 22 August 2007
Available online 21 September 2007

Abstract

A total of 98 non-symbiotic endophytic bacterial strains isolated from soybean root nodules were classified into eight rDNA types in ARDRA analysis and 21 BOX types in BOX-PCR. The phylogenetic analysis of 16S rDNA identified these strains as *Pantoea, Serratia, Acinetobacter*, *Bacillus*, *Agrobacterium*, and *Burkholderia.* Limited genetic diversity was revealed among these bacteria since most of the strains (85.7%) were found in three very similar rDNA types corresponding to *Pantoea agglomerans*, and many strains shared the same BOX-PCR patterns. The inoculation of nodule endophytes had no significant effects on the growth and nodulation of soybean, but most of the strains produced indoleacetic acid (IAA), could solubilize mineral phosphate, and could fix nitrogen, implying that they are a valuable pool for discovering plant growth promoting bacteria. Our results demonstrated that the nodule endophytes were common in soybean and their diversity was affected by the plant's character and the soil conditions. The 99% similarities found in the *nifH* genes of *Bradyrhizobium japonicum* and of the endophytic *Bacillus* strains strongly indicated that horizontal transfer of symbiotic genes happened between the symbiotic bacteria and the endophytes.

Keywords: Diversity; Endophyte; Root nodule; Soybean; *nifH*; Horizontal transfer; PGPM; Phylogeny; Enterobacteria; *Bacillus*

1. Introduction

The root nodules of leguminous plants are symbiotic organs induced by root nodule bacteria called rhizobia. Inside these root nodules the rhizobia are protected and can obtain carbon from the plant and can supply ammonia to the plant by fixing gaseous nitrogen. As well as the rhizobia, some non-symbiotic bacteria have also been isolated from the root nodules of a wide range of legumes (de Lajudie et al., 1999; Gao et al., 2001; Kan et al., 2007; Zakhia et al., 2006). These non-symbiotic bacteria were endophytes living inside nodules and did not cause visible damage to the plants. These nodule endophytic bacteria have been studied poorly compared with the endophytes living in other plant tissues. The most studied nodule endophytes are *Agrobacterium tumefaciens* strains (de Lajudie et al., 1999; Gao et al., 2001; Mrabet et al., 2006), while diverse bacteria, including *Bacillus* and *Pseudomonas* (Zakhia et al., 2006) and enterobacteria (Kan et al., 2007) were also isolated from nodules. It has been argued that the endophytes only coexist with symbiotic bacteria in nodules and they do not induce nodules (Wang et al., 2006b).

It has been reported that the endophytic bacteria may have two main effects. They may increase the ability of plants to absorb nutrients from the soil by increasing root development and by assisting in solubilizing phosphorus (Kuklinsky-Sobral et al., 2004). They may also control soil-borne pathogens. The inoculation of endophytic bacteria has shown a positive effect on plant growth in contaminated soil (Taghavi et al., 2005). The nodule endophytic *Agrobacterium* strains might specifically reduce the nodulation of *Rhizobium gallicum* in the common bean (Mrabet

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0038-0717/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2007.08.014

et al., 2006), but they did not affect nodulation of *Sinorhizobium meliloti* with alfalfa (Wang et al., 2006b). Also, the nodule endophytic bacteria might evolve into symbiotic bacteria by acquiring symbiotic genes from the rhizobia by lateral gene transfer inside the nodules, as reported in rhizobia (Trinick et al., 1989) and in endophytic bacteria (Taghavi et al., 2005). In order to understand the plant–bacteria interactions, it is essential to study the diversity of nodule endophytic bacteria and their impacts on and interactions with rhizobia and host plants.

Soybean (*Glycine max* (L.) Merrill) is one of the most important legume crops originated in China. It has been cultivated for more than 5000 years. This plant forms root nodules with different symbiotic bacteria (Chen et al., 2005). Also, diverse endophytic bacteria have been isolated from roots and stems of soybean (Kuklinsky-Sobral et al., 2004). In a survey of soybean rhizobia performed in our laboratory, many non-symbiotic bacteria were isolated from root nodules. We were interested in studying these nodule endophytic bacteria because no information was available about the nodule endophytic bacteria of soybean. The study of these non-symbiotic endophytes could offer information on the bacterial communities associated with root nodules and on the interactions among the symbiotic bacteria, endophytes, host plants, and environmental factors. The aims of our work were to analyze the diversity of endophytic bacteria isolated from root nodules of soybean and to identify the potential of these bacteria for promoting plant growth.

2. Materials and methods

2.1. Isolation of nodule endophytic bacteria

Root nodules were sampled from soybean plants grown in fields at Beian (126°31′E, 48°17′N), Yian (125°18′E, 47°54′N), Yilan (129°35′E, 46°18′N) and Muling (130°33′E, 44°56′N) of Heilongjiang province, in the northeast of China, the original center of cultivated soybean. The nodules were collected in July. Three or four nodules per plant were randomly selected for isolation of rhizobia by a standard procedure and YMA medium (Vincent, 1970) and the non-symbiotic nodule endophytes were obtained as by-products. To test the surface-sterilization process, aliquots of the sterile distilled water used in the final rinse were plated onto YMA medium and the plates were incubated at 28 °C for 4 d (Kuklinsky-Sobral et al., 2004). All strains were incubated at 28 °C. The nodule formation was checked for each isolate by inoculating soybean seedlings as described (Vincent, 1970). The nodule-forming strains were used in another study and the non-symbiotic bacteria were further characterized in this work.

2.2. ARDRA and sequencing of 16S rDNA

Total DNA was extracted from each strain with the method of Terefework et al. (2001) and was used as templates to amplify the 16S rDNA with primers fD1 and rD1 (Weisburg et al., 1991) and PCR procedure of van Berkum et al. (1996). Restriction endonucleases *Msp*I, *Hinf*I, *Alu*I, and *Hae*III recommended by Laguerre et al. (1994) were used separately to digest PCR products. The restriction fragments were separated by electrophoresis in 2.5% (w/v) agarose gels supplied with 0.5 μg ml^{-1} of ethidium bromide (Laguerre et al., 1994). The profiles were photographed under UV light and the restriction patterns of four endonucleases were combined and clustered using the method of unweighted pair grouping with mathematic average (UPGMA) in the Gelcompar II 3.5 software package. Strains sharing identical RFLP patterns were defined as an rDNA type.

The 16S rDNAs amplified with the same primers and procedures from several representative strains were sequenced directly (van Berkum et al., 1996) and the acquired sequences were compared with those of related species found in the GenBank database. All sequences were aligned using MEGA 3.1 software (Kumar et al., 2004). The phylogenetic tree was reconstructed using the Jukes–Cantor distances and the neighbor-joining method, and was bootstrapped using 1000 replicates for each sequence. The similarity criterion for operational taxonomic units (OTUs) defined by 16S rDNA sequence divergence is less than 3% (Vinuesa et al., 2005). The isolation frequency of each OTU was calculated as $F = n/N$, where n is the number of sites where an OTU was isolated; 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 the total strain number obtained in the site.

2.3. BOX-PCR

In order to reveal the genetic diversity of endophytic bacteria, BOX-PCR was performed using the DNA as templates, the BOXA1R primer (5′- CTA CGG CAA GGC GAC GCT GAC G- 3′) and the procedure of Nick et al. (1999). The amplified DNA fragments were separated by electrophoresis in 1.5% (w/v) agarose gel. The visualization and pattern analysis were the same as in ARDRA.

2.4. Effects of nodule endophytic bacteria on soybean plants

The isolate alone and the mixture of each isolate with *Bradyrhizobium japonicum* B15 (1:1) were inoculated on the surface of sterilized soybean seeds. Blank controls without inoculation and nodulation control inoculated with *B. japonicum* B15 were included for comparison. The surface sterilization, germination, inoculation, and incubation of the plants were performed as described elsewhere (Vincent, 1970). Number of nodules, fresh weight, and height of seedlings were recorded after 1 month of growth of the plants. All of these data were statistically analyzed t o estimate the effects of endophytic bacteria on the