Systematic and Applied Microbiology 33 (2010) 269–274

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Nodulation in black locust by the Gammaproteobacteria *Pseudomonas* sp. and the Betaproteobacteria *Burkholderia* sp.

Ayami Shiraishi*, Norihisa Matsushita, Taizo Hougetsu

Laboratory of Forest Botany, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 1138657, Japan

ARTICLE INFO

Article history:
Received 12 September 2009

Keywords:
Black locust
Rhizobium
Symbiosis
Pseudomonas sp.
Burkholderia sp.
Horizontal gene transfer

ABSTRACT

Nodulation abilities of bacteria in the subclasses Gammaproteobacteria and Betaproteobacteria on black locust (*Robinia pseudoacacia*) were tested. *Pseudomonas* sp., *Burkholderia* sp., *Klebsiella* sp., and *Paenibacillus* sp. were isolated from surface-sterilized black locust nodules, but their nodulation ability is unknown. The aims of this study were to determine if these bacteria are symbiotic. The species and genera of the strains were determined by RFLP analysis and DNA sequencing of 16S rRNA gene. Inoculation tests and histological studies revealed that *Pseudomonas* sp. and *Burkholderia* sp. formed nodules on black locust and also developed differentiated nodule tissue. Furthermore, a phylogenetic analysis of *nodA* and a BLASTN analysis of the *nodC*, *nifH*, and *nifHD* genes revealed that these symbiotic genes of *Pseudomonas* sp. and *Burkholderia* sp. have high similarities with those of rhizobial species, indicating that the strains acquired the symbiotic genes from rhizobial species in the soil. Therefore, in an actual rhizosphere, bacterial diversity of nodulating legumes may be broader than expected in the Alpha-, Beta-, and Gammaproteobacteria subclasses. The results indicate the importance of horizontal gene transfer for establishing symbiotic interactions in the rhizosphere.

Introduction

In the last 20 years, the number of known rhizobial species has been increasing due to an increase in the number of taxonomic studies and molecular techniques. The rhizobial species belong to two subclasses: Alphaproteobacteria, which include *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* [5,20], and Betaproteobacteria, which include *Burkholderia*, *Cupriavidus*, and *Ralstonia* [2,9,12,16,20]. In 2001, Moulin et al. [9] first reported the symbiotic nodulation ability of Betaproteobacteira, *Burkholderia* species. Their symbiotic *nod* genes have been acquired through horizontal gene transfer. In contrast, leguminous plants have "guest" bacteria, which have no symbiotic or nodulation ability, in their nodules that are formed by rhizobia [7].

In a former study, we determined the spatial distribution and genetic diversity of rhizobia that have a symbiotic relationship with black locust (paper submitted). In that study, several bacterial species not known as rhizobia were isolated from black locust nodules. It has been reported that black locust has a symbiosis with various species of *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* [10,15,22]; therefore, the bacterial strains isolated in the former study might have some nodulation or symbiotic ability in the black locust, as some of them were isolated frequently.

The aims of this study were to test the symbiotic ability of these bacteria, to see the presence or absence of symbiotic genes, and to provide insights into interactions among rhizobia and other soil bacteria in an actual rhizosphere.

Materials and methods

Collection and isolation of bacteria

Collection sites

In November 2005, roots of black locust (*Robinia pseudoacacia*) were collected from two different coastal forests in Chiba Prefecture (35°20′N, 140°24′E) and Nigata Prefecture (35°21′N, 140°27′E), Japan, and from an experimental research station at the University of Tokyo (35°44′N, 139°15′E) in Tokyo, Japan. In Chiba, the forest vegetation was mainly composed of *Pinus thunbergii* and black locust. The soil condition was sandy, and the soil pH was 6.5. The mean annual temperature at this forest is 15.1 °C, and the mean annual precipitation is 1617 mm. The coastal forest in Nigata also has sandy soil, the annual mean temperature is 13.5 °C, and the annual precipitation is 1776 mm. The experimental station of the University of Tokyo has approximately 50 cm black soil in the upper

* Corresponding author at: Department of Tropical Plant and Soil Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, St. John Science Lab, Room 102, 3190 Maile way Honolulu, HI 96822, USA.
Tel.: +81 3 5841 5206; fax: +81 3 5841 7554.
E-mail address: ayamis@hawaii.edu (A. Shiraishi).

0723-2020/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2010.04.005

Table 1
Sets of primers used in the study.

Gene		Reference
nodA	5′-TCACARCTCKGGCCCGTTCCG-3′ 5′-TGGGCSGGNGCNAGRCCBGA-3′	[9]
nodC	5′-AGGTGGTYGAYGACGGTTC-3′ 5′-CGYGACAGCCANTCKCTATTG-3′	[6]
nodCI	5′-CCATACGCACCGTGG TGCTCTTGC-3′ 5′-TCTTCCATVAWRTGVGTNGTCA-3′	[9]
nodDC	5′-GTACAGGAGGGCATCGCGAA-3′ 5′-CTGCAGCTGCAGCGAATCTG-3′	[23]
nifH	5′-TACGGNAARGGSGGNATCGGCAA-3′ 5′-AGCATGTCYTCSAGYTCNTCCA-3′	[6]
nifHD	5′-GCCWTCTAYGGNAARGGNGG-3′ 5′-ATCAGGCCGATCGGGCATT-3′	[9]

layer and a red soil layer beneath. The annual mean temperature of the station is 13.7 °C, and the annual mean rainfall is 1400 mm.

Collection and isolation of bacteria

The collected nodules were surface-sterilized with approximately 3% sodium hypochlorite for 20 min, rinsed three times with sterilized distilled water, and then crushed with a sterilized glass stick. The nodule contents were spread on a yeast mannitol agar (YMA) plate [18] and incubated at 25 °C for 3–7 days. One colony was retrieved from each plate, as the plates were colonized by the same colony type, which was white and sticky.

PCR amplification

A single colony from each YMA plate was transferred to 2 ml of liquid yeast mannitol (YM) medium and cultured at 23 °C for 3–7 days. Proliferating bacteria in the YM medium were collected by centrifugation at 10,000 × *g* for 5 min, and DNA was extracted using the cetyltrimethylammonium bromide method [21].

Almost the full-length 16S rRNA gene was amplified with the fD1 and rD1 primers [19] using the Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The PCR reactions were performed using the following protocol: initial denaturation at 95 °C for 85 s; 35 cycles at 94 °C for 35 s, 54 °C for 55 s, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Additionally, the *nod* and *nif* genes were amplified with *nodA*, *nodC*, *nodCI*, *nodDC*, *nifH*, and *nifHD* primers as described each references (Table 1).

Restriction fragment length polymorphism (RFLP) analysis

The PCR products were digested with 2.5 units the *Afa* I, *Hha* I, *Hinf* I, and *Msp* I endonucleases according to the manufacturer's instructions (Fermentas, Glen Burnie, MD, USA). After the restricted DNA fragments were separated by electrophoresis on a 3% agarose gel (certified low-range ultra agarose; Bio Rad Laboratories, Hercules, CA, USA) at 100 V for 3 h, the gels were stained with ethidium bromide and photographed on a UV illuminator (FAS III system, Toyobo, Osaka, Japan). The restriction fragment lengths were calculated with 1D Image Analysis Software (Kodak, New York, NY, USA) or the Sequencher program (http://www.genecodes.com/, Gene Codes, Ann Arbor, MI, USA). A 100-bp DNA molecular size marker (Toyobo) was used as the size standard.

Cloning and sequencing

The PCR products amplified with the *nif* and *nod* primers (Table 1) were separated on 1% SeaKem GTG agarose gels (Cambrex, Rockland, ME, USA) and recovered with SUPREC EZ (Takara Bio, Shiga, Japan), following the manufacturer's instructions. Each DNA sample was inserted into the pT7 Blue T-Vector (Novagen, Madison, WI, USA) using the DNA Ligation Kit Version 2.1 (Takara Bio) and transformed into *Escherichia coli* JM109 competent cells (Takara Bio). Then, the nucleotide sequences of the DNA samples were determined with a Beckman Coulter CEQ8800 sequencer. Amplification for the sequencing reaction was conducted with M13F and M13R primers. The DNA sequences obtained in this study were deposited in GenBank (Table 2). After sequencing, the positions of the enzyme recognition sites and an accurate length for the restriction fragments lengths were determined with the Sequencher program (Gene Codes).

Phylogenetic analysis

The rhizobial 16S rRNA gene and *nodA* sequences were aligned with those of rhizobia obtained from GenBank (see Appendix Table A1 for species and accession numbers) using Codoncode Aligner (CodonCode Co., Dedham, MA, USA) and Bioedit (http://www.mbio.ncsu.edu/BioEdit/). A dendrogram was constructed from the aligned sequences using the neighbor-joining method in Clustal W [13]. Other *nod* and *nif* genes were examined with the BLASTN program (NCBI, Bethesda, MD, USA).

Light microscopy

Plants roots were washed with tap water, cut into segments, and fixed in 20% (v/v) formaldehyde solution, 10% dimethyl sulfoxide, 0.1% phosphate buffer (pH 7.0), and 0.1% Nonidet P-40 overnight. Then, the samples were dehydrated in an ethanol series (50%, 70%, 85%, 95%, and 99.5% ethyl alcohol). After dehydration, the roots were penetrated with 50% ethanol, 50% Technovit 7100, and 100% Technovit 7100 for 2–3 h each, in order. The samples were embedded in Technovit 7100, cut into 3-μm sections with a Leica RM2145 microtome, stained with 0.05% toluidine blue for 5 min, and then washed with tap water. An OLYMPUS BX50 light microscope (Olympus, Tokyo, Japan) was used to observe the sections.

Inoculation test and Koch's postulates

Bacterial strains (Table 2) isolated from the nodules collected from the three sites were grown on YMA plates. *Mesorhizobium loti* strain Ch90 was used as a positive control, and the negative control was YM medium. A single colony from each plate was transferred to liquid YM medium and shaken at 23 °C for 1 week. Black locust seeds were surface-sterilized with boiling water and germinated in a sterilized test tube containing autoclaved filter paper [11] and nitrogen-free medium [1]. One milliliter of each liquid culture medium was applied to a black locust seedling. Each bacterial isolate was inoculated on ten black locust seedlings. The plant roots were observed 4 weeks after the inoculation, and bacteria were re-isolated from the nodules that formed.

Results

16S rRNA gene analysis

A total of 757 bacterial strains were isolated from 757 surface-sterilized nodules. The isolates were classified into ten groups based on PCR-RFLP analysis (Appendix Tables A1 and A2). The most dominant, RFLP type 1, occupied 50% of all isolates, 10% were type 2, 5% were type 3, and 4% were type 4. Each of these four types comprised 10% of the strains isolated from one tree, whereas the remaining six