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EXPERIMENTAL ARTICLES

Influence of the Introduction of *Caragana arborescens* on the Composition of Its Root-Nodule Bacteria

An. Kh. Baymiev[1], K. G. Ptitsyn, and Al. Kh. Baimiev

Institute of Biochemistry and Genetics, Ufa Scientific Center, Russian Academy of Sciences, Ufa, Bashkortostan, Russia

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Abstract—The genetic diversity and phylogeny of root-nodule bacteria entering into symbiotic relations with *Caragana arborescens* introduced on the territory of the Republic of Bashkortostan (RB) were studied. Analysis of the 85 strains isolated from root-nodules showed that, according to the DNA polymorphism revealed by RAPD analysis, they can be divided into 12 groups. It was found that, both in natural habitats and on the territory of the RB, *Caragana arborescens* is primarily nodulated by *Mesorhizobium* bacteria whose 16S rRNA gene sequences differ to some degree from those of the bacteria described earlier by Chinese authors. Bacteria assigned to the genus *Phyllobacterium* based on their 16S rRNA gene sequences were also revealed in plants growing on the territory of the RB.

Key words: *Caragana arborescens*, root-nodule bacteria, leguminous-rhizobial symbiosis, genetic biodiversity, RAPD analysis, gene 16S rRNA.

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Caragana arborescens is a decorative legume widespread on the territory of the Republic of Bashkortostan (RB). It has several horticultural forms. It is a shrub commonly used for creating thick live hedges in landscaping and melioration, improving soil, and anchoring sands and gully slopes.

Caragana arborescens was introduced to the territory of RB. Its natural habitat is western Siberia, Altai, the Sayan Mountains, Kazakhstan, China, and Mongolia.

As in most legumes, the biological productivity of *Caragana arborescens* depends significantly on the effectiveness of symbiosis with root-nodule bacteria. Hence, one of the limiting factors in legume introduction a new growth site is the presence of suitable root-nodule bacteria capable of entering into symbiosis with a given plant or the ability of the bacteria introduced with seeds to adjust to new soil and natural–climatic conditions.

Earlier, Chinese researchers revealed that most root-nodule bacteria entering into symbiosis with *Caragana* plants in natural habitats (Liaoning Province, China) belong to the genus *Mesorhizobium* [1, 2]. This genus of bacteria was described by Jarvis [3] based on their cultural properties as root-nodule bacteria with a growth rate on nutrient media intermediate between the quickly growing root-nodule bacteria of the genus *Rhizobium* and the slowly growing bacteria of the genus *Bradyrhizobium*. Later, 16S rRNA gene sequencing revealed that this genus comprises a sufficiently homogeneous group of microorganisms close to the representatives of the genus *Phyllobacterium* [4, 5].

The aim of this work was to study the genetic diversity and phylogeny of root-nodule bacteria entering into symbiotic relations with *Caragana arborescens* growing on the territory of the RB and look into the effect of the introduction of this plant on the composition of its microsymbionts.

MATERIALS AND METHODS

The nodules were collected from the roots of young one- to two-year-old *Caragana arborescens* plants growing in Tatyshlinsky raion, RB. The nodules were oblong, 7–10 mm long and 3–5 mm wide. We collected approximately 100 nodules from ten plants growing at an interval of 15–20 m from one another on a area of approximately 500 m^2.

The bacteria were isolated from the nodules as follows. The nodule surface was sterilized sequentially with 70% ethyl alcohol (2 min) and with 10% sodium hypochlorite solution (2 min). The nodules were then washed off with sterile tap water. The uppermost distal tip of a nodule was cut off with sterile needles of disposable 5-ml syringes. Another sterile needle was used to sample the material by scraping off the section, and the solid nutrient TY medium was inoculated (0.1% yeast extract, 1% bacto tryptone, 0.1% $CaCl_2$, and 1.5% agar).

The DNA was isolated from bacteria by lysing the cells in 1% Triton X100. For this purpose, a small

[1] Corresponding author: e-mail: baymiev@anrb.ru

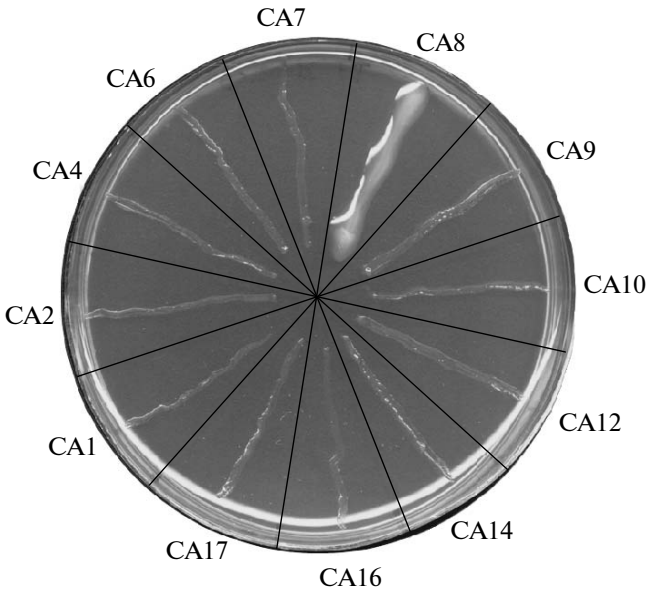

Fig. 1. Petri dish with TY medium with slowly and quickly growing strains of root-nodule bacteria entering into symbiosis with *Caragana arborescens* (5 days).

amount of bacterial mass was placed in 1.5-ml test tubes with 100 μl of Triton X100, resuspended, and incubated at 95°C for 10 min. The cell debris was removed by centrifugation at 10000 *g* for 3 min. The supernatant was taken as a template for PCR.

The PCR was performed on Tertsik MC2 (DNK-Tekhnologiya, Russia) and T1 Thermocycler (Biometra, Germany) amplifiers using the standard kits for DNA amplification.

The genetic diversity of the strains sampled was studied with Random Amplified Polymorphic DNA(RAPD) analysis [6] using the following "random" primers:

1. 5'-gggcgctg-3'
2. 5'-caggcccatc-3'
3. 5'-gcgtccattc-3'
4. 5'-acggtggacg-3'.

The PCR–RAPD analysis (the restriction fragment length polymorphism) [7] of 16S rRNA genes was carried out using the finely cleaving restriction endonucleases *Alu*I and *Msp*I. Primers PdrfF (5'-tggctcagaacgaacgctggcggc-3') and PdrfR (5'-taccttgttacgacttcaccccagtc-3') flanking the gene fragment of approximately 1400 bp were used for amplification of the 16S rRNA gene.

The strain phylogeny was studied by sequencing the 16S rRNA gene fragments amplified with the primers PdrfF and PdrfR. The nucleotide sequences were determined on an ABI PRISM 310 automatic sequencer (Applied Biosystems Inc., United States) with Big Dye Terminator v.3.1. kits.

Computer-aided analysis of the nucleotide sequences was carried out using the Lasergene software package (DNASTAR, Inc., United States).

RESULTS AND DISCUSSION

A total of 85 strains of root-nodule bacteria were isolated from *Caragana arborescens* nodules in the course of the experiment. The capacity of the isolates for nodule formation on the *Caragana* roots was tested by inoculating the sprouts of this plant. All the strains formed morphologically normal nodules one month after inoculation. On the agarized TY nutrient medium, all the microorganisms studied formed similar colonies, except for five strains, which were markedly different from the rest in morphology and colony growth rate (Fig. 1).

While the bulk of the strains formed established colonies on solid nutrient medium 5 days after inoculation, those that differed formed distinctly visible colonies already after 24 h.

The genetic diversity of the strains was studied with RAPD analysis using several random oligonucleotide primers. It was found that the root-nodule bacteria of *Caragana arborescens* were characterized by a relatively high degree of heterogeneity.

Nevertheless, based on RAPD analysis, the samples could be divided into several groups by similarity in the distribution of the amplified DNA bands. It appeared that quickly growing strains form a separate group with absolutely identical RAPD profiles. In the group of slowly growing root-nodule bacteria, it was also possible to single out several groups; all the root-nodule bacteria studied were therefore arbitrarily subdivided into a group of quickly growing (CA8) and 11 groups of slowly growing microorganisms (CA1, CA2, CA4, CA6, CA7, CA9, CA10, CA12, CA16, CA14, and CA17) (Fig. 2). The quantitative distribution of the strains in the groups is shown in the table.

Later, only one sample from each group was taken for study, because the total coincidence of the RAPD

Quantitative distribution of the strains in the groups

Group	CA1	CA2	CA4	CA6	CA7	CA9	CA10	CA12	CA14	CA16	CA17	CA8
Number of strains	5	2	5	7	22	3	12	3	2	13	6	5

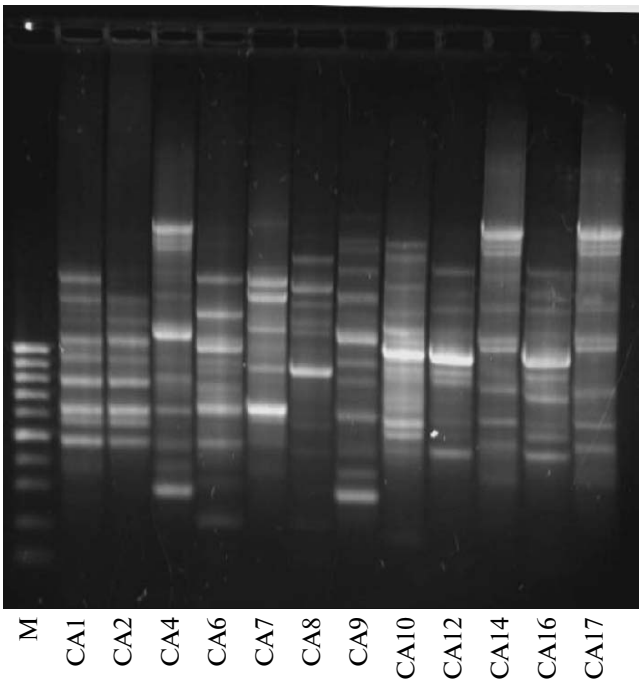

Fig. 2. Electrophoregram of the RAPD analysis of DNA of the microbial strains analyzed using the 5'-gggcgctg-3' sequence primer. M is a 100 bp marker.

profiles by several random primers enabled us to suggest strain identity within each group.

In order to study the phylogeny of the strains, 16S RAPD analysis was used, which provides preliminary information on the phylogenetic relations between microorganisms.

It is noteworthy that, with the use of the *Alu*I finely cleaving restriction endonuclease, the RAPD profiles of slowly growing strains appeared to be absolutely identical; and with the use of *Msp*I, we observed differences in some bands. The RAPD profiles of quickly growing strains differed from those of the slowly growing strains in all the cases (Fig. 3). This is most probably an indication of a close relationship between slowly growing strains.

In order determine to the phylogeny of these strains more exactly, 16S rRNA gene fragments of these microorganisms (approx. 1400 bp) were sequenced.

Comparative analysis of the sequences revealed high homology of 16S ribosomal genes in all the microorganisms studied. The homology within the group of slowly growing root-nodule bacteria was higher than between quickly growing and slowly growing ones, although certain differences also existed between slowly growing root-nodule bacteria, except for two pairs of groups (CA4 and CA14, CA10 and CA12), which appeared to be identical.

The search for similar sequences in the gene database using the Megablast software package available on the NCBI website (www.ncbi.nlm.nih.gov) showed that the sequenced fragments of all slowly growing root-nodule bacteria exhibited high homology to the sequences of *Mesorhizobium*; quickly growing isolate were similar to the species *Phyllobacterium myrsinacearum*.

Based on multiple alignment of the 16S rRNA gene sequences we obtained and those from the international EMBL/GenBank/DDBJ databases, a phylogenetic tree of bacteria of the family *Rhizobiaceae* was constructed. It was found that the investigated strains

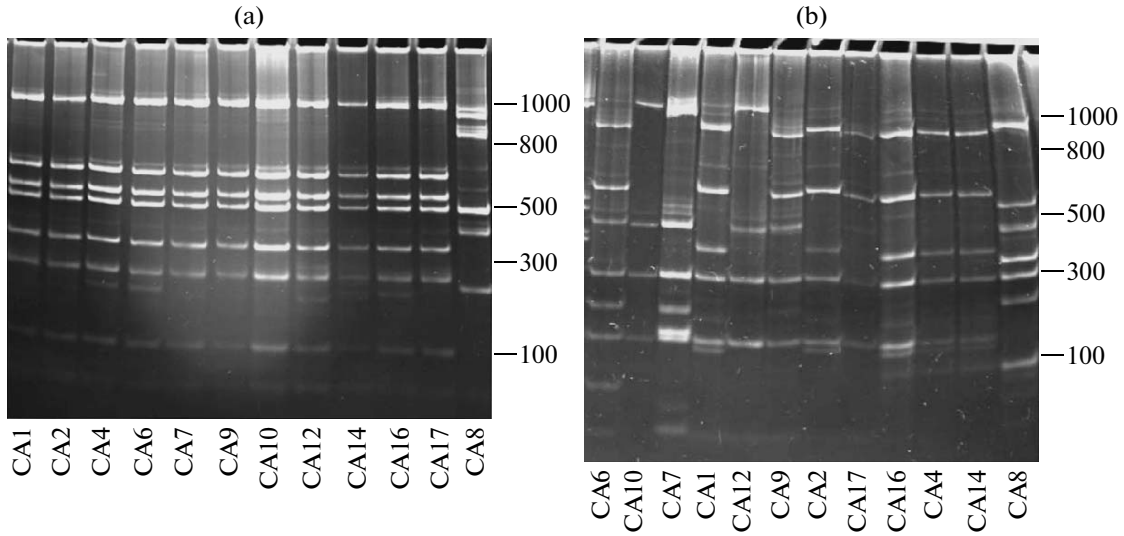

Fig. 3. Electrophoretogram of the RAPD analysis of 16S rRNA genes of the strains analyzed using fine-cleavage restriction endonucleases *Alu*I (a) and *Msp*I (b).

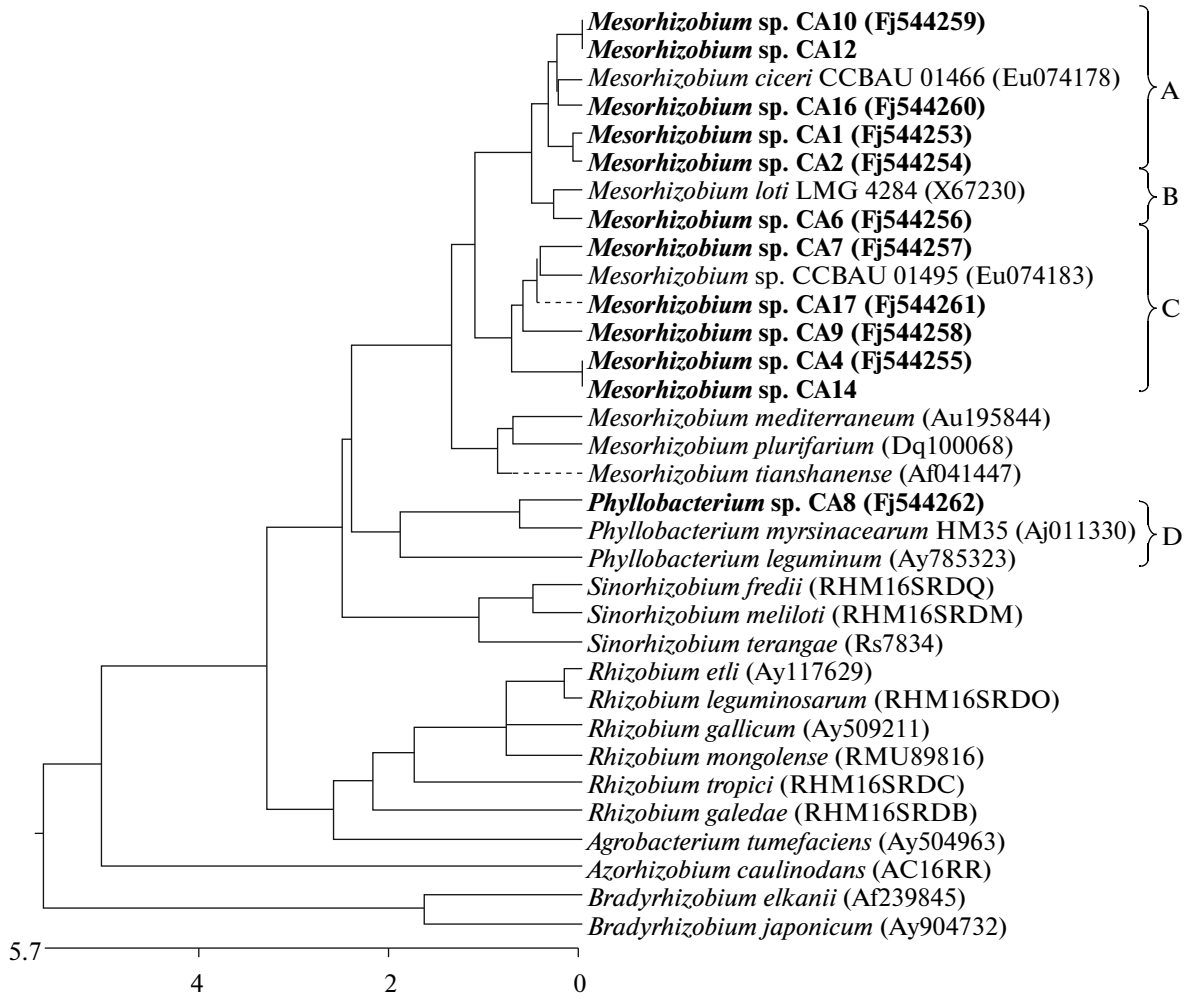

Fig. 4. The tree of similarity in the 16S rRNA gene nucleotide sequences of *Rhizobiaceae* constructed using the Lasergene MegAlign program (DNASTAR, United States). The horizontal axis shows the weight of this alignment expressed as the number of nucleotide substitutions (×100).

of root-nodule bacteria formed four clusters: three clusters of slowly growing microorganisms and a separate cluster of quickly growing ones (Fig. 4).

Cluster A of slowly growing root-nodule bacteria, which comprises bacteria from five groups (CA1, CA2, CA10, CA12, CA16), is close to the 16S rRNA gene sequence to *Mesorhizobium ciceri* strain CCBAU 01466 (EU074176) isolated from the root nodules of arboreal legumes of the genus *Caragana* growing in China.

The strains of four groups (CA4, CA7, CA9, and CA17) combined in cluster B are close to the species *Mesorhizobium caraganae* (Eu074183) recently described by Chinese researchers [2] and forming symbiotic relationships with *Caragana* plants in natural habitats (Liaoning Province, China).

Strain CA6 exhibited high similarity to *Mesorhizobium loti* (X67230) (cluster C).

The 16S rRNA gene sequences of quickly growing root-nodule bacteria exhibited high similarity to the species *Phyllobacterium myrsinacearum* (Fj544262) (cluster D).

Thus, in the introduced plants growing thousands of kilometers away from their natural habitat, the composition of root-nodule bacteria was nearly identical to the composition described by Yan [2]. This testifies to a high degree of specificity of symbiosis of a given plant.

Such a strict symbiosis characteristic of evolutionarily young legumes from the subfamily *Papilionoideae* growing in temperate latitudes is the most effective one from the point of view of its capacity for nitrogen fixation. The evolution of leguminous–rhizobial symbiosis is believed to have followed the direction of increasing the specificity of partner interaction accompanied by an increase in nitrogen-fixing activity [8].

The new microbiological surroundings in plant rhizosphere that appeared in a new habitat also affected the change in the symbiont composition. Thus, the emergence of the symbiosis with *Phyllobacterium*, which was not revealed by Yan in China, was most probably the consequence of the plant introduction.

Although the genes *sym* and *nif* of most *Mesorhizobium* strains are situated within the symbiotic (Sym) island on the chromosome [3, 9, 10], these genes were also found to be capable of horizontal transfer between strains in soil and under laboratory conditions [11, 12]. These islands are believed to be site-specific conjugation transposons capable of integrating into a phenylalanine tRNA gene on the chromosome of a recipient bacterium [9, 12, 13]. *Phyllobacterium* could be suggested to be endowed with the property of entering into symbiosis with *Caragana arborescens*, because they acquired the necessary genes via horizontal transfer. However, our RAPD analysis of the *NodC* gene (the data are not shown) showed the difference between *Mesorhizobium* and *Phyllobacterium* by this gene. The emergence of symbiosis between *Caragana arborescens* and *Phyllobacterium* is most probably not related to horizontal gene transfer.

To date, the genus *Phyllobacterium*, which is phylogenetically close to *Mesorhizobium*, has not been included among root-nodule bacteria, although the question of reclassification was raised as early as 1998 [4]. The fact that the *Phyllobacterium* bacteria are capable of entering into symbiosis with *Caragana arborescens*, another species along with the already known [14, 16] legumes, is an additional prerequisite for this species being classified as a root-nodule bacterium.

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