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EXPERIMENTAL ARTICLES

Genetic Diversity and Phylogeny of Root Nodule Bacteria Entering into Symbiosis with Bitter Peavine *Lathyrus vernus* (L.) Bernh.

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Abstract—The genetic diversity and phylogeny of root nodule bacteria entering into symbiosis with bitter peavine *Lathyrus vernus* (L.) Bernh. (*Fabaceae*) growing in various regions of the Republic of Bashkortostan were studied. RAPD analysis revealed a high degree of polymorphism of the DNA of the isolated strains giving evidence of the heterogeneity of the microorganisms in question. The study of the phylogeny of microsymbionts based on comparative analysis of the nucleotide sequences of 16S rRNA genes showed that the bacteria isolated from the plant nodules of *L. vernus* growing on the territory of Ufa and Beloretsk raions belonged to the species *Rhizobium leguminosarum*, whereas the microsymbionts of *L. vernus* growing on the territory of Tatyshly raion belonged to the species *Rhizobium tropici,*@ except for several strains of *Rhizobium leguminosarum*.

Keywords: bitter peavine, wood pea, legume–rhizobium symbiosis, root nodule bacteria, genetic diversity.

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The ability of leguminous plants to enrich soil with available nitrogen due to biological nitrogen fixation in symbiosis with root nodule bacteria is an essential factor not only for the maintenance of fertility of cultivated soils, but also for maintaining the balance of natural ecosystems.

While the root nodule bacteria entering into symbiosis with the legumes of agricultural significance have been characterized in detail, the microsymbionts of wild legumes remain poorly studied. In recent years, information appeared in the literature that, contrary to previous notions, not only bacteria of the family *Rhizobiaceae*, but also other bacteria not even belonging to the class *Alphaproteobacteria* are capable of efficient nitrogen-fixing symbioses with legumes [1–3]. In order to understand the formation of the specificity of nitrogen-fixing symbiosis, it is therefore necessary to have a full notion of the possible legume symbionts: their species composition, phylogeny, and genetic diversity. This especially concerns the microsymbionts of wild leguminous plants growing in temperate climates, where the most specialized and evolutionarily young representatives of the subfamily *Faboidae* dwell. These plants are characterized by highly specific interaction of the "cross-inoculation group" type, in which the rhizobia of a certain species or even biotype form an efficient symbiosis only with the representatives of a certain genus or several closely related genera of plants [4]. In the course of evolution, this increase in the specificity of the interaction of the partners was accompanied by an increase in nitrogen-fixing activity [5].

Plants of the genus *Lathyrus* L. are the most widespread wild legumes on the territory of the Republic of Bashkortostan (RB).

Bitter peavine *Lathyrus vernus* (L.) Bernh. (*Fabaceae*) is a perennial rhizomatous herb with a wide area of prevalence. It grows in shady forests and shrubs throughout Europe, in the European part of Russia, and in Belarus and Ukraine, as well as in certain areas in the Caucasus and Siberia. It is of interest as a fodder and vernal nectariferous plant (a bee plant); it is also used in traditional medicine. In recent years, it has been used widely as an ornamental plant in landscaping.

The aim of this work was to look into the genetic diversity and phylogeny of root nodule bacteria entering into symbiosis with *Lathyrus vernus* growing in various regions of the RB.

MATERIALS AND METHODS

Root nodules were collected from the roots of *L. vernus* before flowering in plants spaced at least 10 m apart. The nodular surface was sequentially sterilized for 2 min in 70% ethyl alcohol and for 2 min in 10% sodium hypochlorite. The nodules were then

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washed off repeatedly with sterile tap water. The tips of the distal part of the nodules were removed with sterile needles of disposable 5-ml syringes. Another sterile needle was then used to scrape off the nodule content at the side of the section and to plate it on solid TY nutrient medium (0.1% yeast extract, 1% bacto-tripton, 0.1% $CaCl_2$, 1.5% agar) was inoculated.

Bacterial DNA was isolated by cell lyzing in 1% Triton X100. For this purpose, a small amount of the bacterial mass was placed into 1.5-ml test tubes with 100 μl of 1% Triton X100, resuspended, and incubated for 10 min at 95°C. The cell debris was precipitated by centrifugation at 12000 *g* for 3 min. The supernatant was used as a template for PCR.

The PCR was carried out on a Tertsik MS2 amplifier (DNK-Tekhnologiya, Russia) and T1 Thermocycler amplifier (Biometra, Germany) using the standard kits for DNA amplification.

The genetic diversity of the strains harvested was studied with RAPD (Random Amplified Polymorphic DNA) analysis [6] using the following random primers: (1) 5'-gggcgctg-3', (2) 5'-caggcccatc-3', (3) 5'-gcgtccattc-3', and (4) 5'-acggtggacg-3'.

PCR restriction fragment length polymorphism analysis [7] of the 16S rRNA gene was carried out using the finely cleaving restriction endonucleases *Kzo*9I and *Hae*III. The primers PdrfF (5'-tggctcagaacgaacgaacgctggcggc-3') and PdrfR (5'-taccttgttacgacttcaccccagtc-3') flanking the gene fragment (about 1400 bp) were used for 16S rRNA gene amplification.

Phylogenetic analysis of the strains was carried out based on the data of multiple alignment of the sequenced 16S rRNA gene fragments also amplified using the primers PdrfF and PdrfR. The nucleotide sequences were determined using an automatic ABI PRISM 310 sequencer (Applied Biosystems, Inc., United States) with Big Dye Terminator v.3.1 kits.

Analysis of the plasmid profile of the microorganisms was carried out by the method described by Eckhardt [8]. The plasmids were separated in 0.8% agarose gel using a PPI-200 current inverter (MJ Research, United States) at 3–4 V/cm gel.

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

The search for similar sequences in the GenBank database was carried out using the Megablast program available at the NCBI website (www.ncbi.nlm.nih.gov).

The 16S rRNA nucleotide sequences of the strains studied were deposited in the International Nucleotide Sequence Databank under the numbers GQ118964, GQ118966, and GQ118965.

RESULTS AND DISCUSSION

In order to study the phylogeny and genetic diversity of the root nodule bacteria nodulating *L. vernus* growing on the territory of Tatyshly (A), Ufa (B), and Beloretsk raions of the RB (Fig. 1), the nodules were collected from roots of these plants. Later, pure bacterial cultures capable of forming nodules on the roots of *L. vernus* were isolated from them: 167 strains from the nodules of the plants growing in Tatyshly rayon (group A); 50 strains, from Ufa rayon (group B); and 50 strains, from Beloretsk raion (group C).

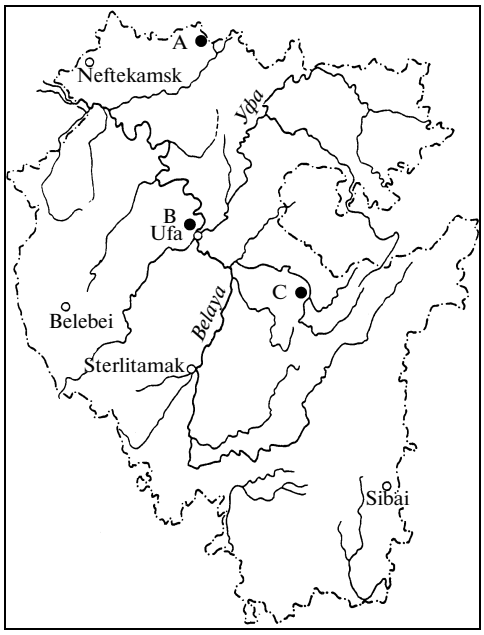

Fig. 1. Map of the RB with designations of the regions of collecting the nodules from the roots of *L. vernus*: in Tatyshly raion (A) and Ufa (B) and Beloretsk raion (C) of the RB.

The genetic diversity of the strains obtained was studied by RAPD analysis using several random primers. The strains from all the three groups were found to have a high degree of DNA polymorphism. Figure 2 exemplifies the RAPD profiles of group B strains for two random primers.

However, it was possible to reveal several strains with identical RAPD profiles. Since complete coincidence of the RAPD profiles for several random primers implied strain identity, one sample from each homogeneous group was used for subsequent study. Thus, 109, 42, and 40 strains remained in groups A, B, and C, respectively.

To determine the phylogenetic relationship between the root nodule bacteria, RFLP analysis of 16S rRNA genes was carried out using finely cleaved restriction endonucleases *Kzo*9I and *Hae*III. It was found that, despite the high strain heterogeneity, bacteria within the groups were sufficiently homogeneous according to the RFLP profile of 16S rRNA genes. Thus, for example, all group A strains formed one homogeneous group, except for two differing strains, and the bacteria of groups B and C could further be

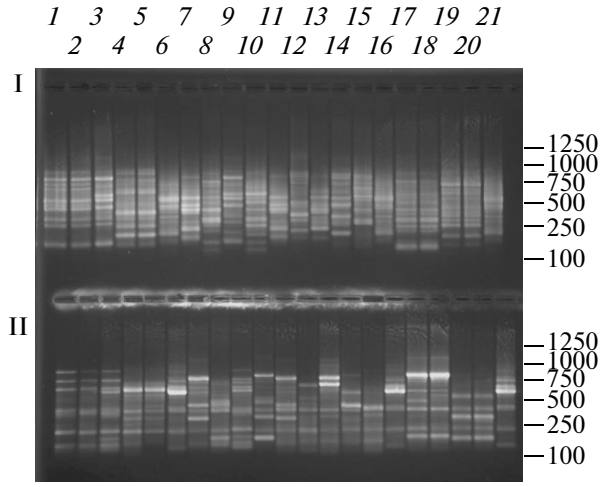

Fig. 2. Electrophoretogram of the RAPD analysis of group B root nodule bacteria using the primers 5'-gggcgctg-3' (I) and 5'-caggcccatc-3' (II). The size of the DNA fragments in the nucleotide pairs is indicated on the right. Strain numbers are designated by digits.

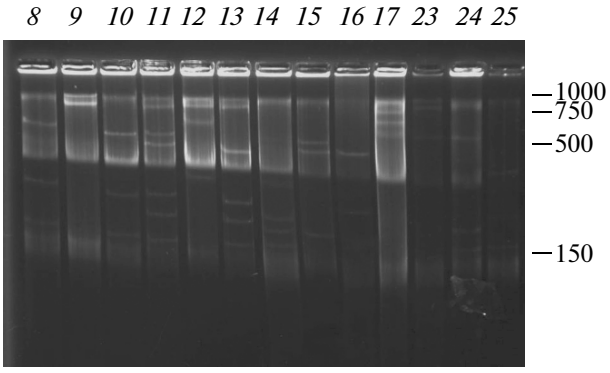

Fig. 3. Plasmid profiles of group B root nodule bacteria. The size is given in Kb (thousands of nucleotide base pairs). Strain numbers are designated by digits.

subdivided into two subgroups. For group B bacteria, the between-subgroup ratio was 71 (B1) and 29% (B2); for group C bacteria, 65 (C1) and 35% (C2). Thus, it was found that, in each sampling region, bitter peavine entered into symbiosis with a group of closely related strains characterized, nevertheless, by a high degree of DNA polymorphism, which could have been caused by a different plasmid composition of the bacteria studied. In order to check this suggestion, the plasmid profiles of the root nodule bacteria were investigated. Indeed, all the microsymbiont strains exhibiting DNA polymorphism were found to differ in plasmid composition. The number of plasmids among the strains varied between two and eight, and their size varied from 150 to 1000 Kb (Fig. 3).

The considerable plasmid diversity of the strains nodulating a single plant species suggests a sufficiently high rate of genetic recombination, resulting in the emergence of a large number of strains with high DNA polymorphism capable of entering into symbiosis with a certain plant species.

To investigate the phylogenetic position of the strains of the strains under study, the sequences of 16S rRNA gene fragments (approximately 1400 bp) were determined and compared with the known similar sequences from the GenBank database. For this purpose, two strains from each group were analyzed.

The search for similar sequences in the GenBank database demonstrated that the sequences of the 1300- to 1400-bp fragments from the microorganisms assigned to groups B and C were almost completely (98% homology) identical to the 16S rRNA gene sequence of *Rhizobium leguminosarum* (Fig. 4). Most of them appeared to be closer to *R. leguminosarum* bv. *trifolii* (B1, B2, C1), while group C2 bacteria were closer to *R. leguminosarum* bv. *viciae*. As for group A microorganisms, the 16S rRNA gene sequences exhibited 99% homology to the sequence of *R. tropici* (GQ118964), while the few strains differing in the 16S RFLP profile (GQ118966 and GQ118965) were related to the species *R. leguminosarum* bv. *viciae*.

The plants of the genus *Lathyrus* were considered to comprise one cross-inoculation group with the plants of the genera *Vicia, Pisum*, and *Lens* and to enter into symbiosis with *R. leguminosarum* bv. *viciae* [9, 10]. Our results show that, under the soil and climatic conditions of the RB, *L. vernus* enters into symbiosis with strains related not only to *R. leguminosarum* bv. *viciae*, but also to *R. leguminosarum* bv. *trifolii*. Still more unexpected were the results of the study of group A root nodule bacteria, which, according to the 16S rRNA gene sequences, appeared to be close to *R. tropici*, which are usually the symbionts of *Phaseolus vulgaris* L. Although these species are closely related and even in the 1984 edition of *Bergey's Manual of Determinative Bacteriology* all these bacteria were classified within one species *Rhizobium leguminosarum*, which included three biovars—*phaseoli, trifolii*, and *viciae*—the division into the biovars was, however, based on host specificity. *R. leguminosarum* bv. *trifolii* comprised the strains inoculating clover Trifolium spp.; *R. leguminosarum* bv. *viciae*, the strains inoculating the species identified with or related to the tribe *Vicieae*, such as *Pisum*, *Vicia, Lens,* and *Lathyrus* spp.; and *R. leguminosarum* bv. *phaseoli*, the strains nodulating *Phaseolus* spp. Later, the obvious heterogeneity among the strains of *R. leguminosarum* bv. *phaseoli* led to this biovar being divided with the description of two new species: *R. tropici* and *R. etli*. Since *L. vernus* is capable of entering into symbiosis with *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, and *R. tropici*, other plant species of the tribe *Vicieae* are probably also able, under certain conditions, to enter into symbiosis with all these species of bacteria. This finding may be of some practical value. For example, *R. tropici* is more resistant to such unfavorable environmental

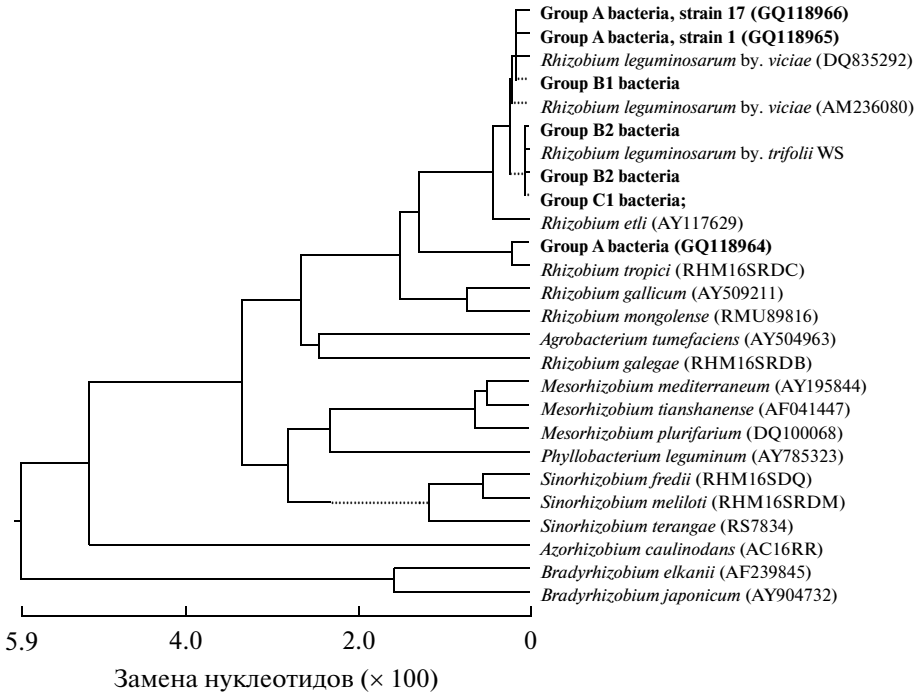

Fig. 4. Phylogenetic tree of the root nodule bacteria constructed on the basis of comparative analysis of 16S rRNA gene sequences.

conditions as high temperature, low humidity, and low pH, despite being a less effective nitrogen-fixer than *R. leguminosarum* due to the presence of only one copy of the *nifH* gene [11]. *R. leguminosarum* cells contain multiple copies of this gene [12]. Nevertheless, this provides good material for the selection of effective strains of root nodule bacteria which may be utilized as symbionts for leguminous cultures grown under unfavorable conditions.

Thus, under the RB soils conditions, *L. vernus* enters into symbiosis with genetically heterogeneous strains of *R. leguminosarum* (both bv. *viciae* and bv. *trifolii*) and, in certain cases, depending on the soil and climatic conditions, with *R. tropici* strains, which was previously not considered be typical of these plants. The genetic heterogeneity of the strains is mainly due to the differences in their plasmid composition, which, in turn, is indicative of a sufficiently high genetic recombination rate between the rhizosphere bacteria.

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