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EXPERIMENTAL ARTICLES

Evaluation of the Diversity of Nitrogen-Fixing Bacteria in Soybean Rhizosphere by *nifH* Gene Analysis

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Abstract—Biological nitrogen fixation plays an important role in the nitrogen balance of agricultural ecosystems and provides an essential part of nitrogen nutrition for plants, even in conditions of intensive fertilization. The main agrobiotechnological method for soybean cultivation (*Glycine max* (L.) Merril) is an application of microbial preparations based on *Bradyrhizobium japonicum*. Successful inoculation strongly depends on the interactions between the introduced microorganism and the aboriginal rhizosphere microorganisms. To evaluate the composition of diazotrophic communities, a study of the diversity of the molecular marker for nitrogen fixation, the *nifH* gene, in the samples of soybean rhizosphere soil was carried out. Experiments were performed in the variants when soybean was cultivated without inoculation and after adding bacterial preparations, as well as in enrichment cultures of diazotrophs. The revealed diazotrophic microorganisms demonstrated low level of similarity to the known microorganisms (74–95% identity by nucleotides), and were identified as species of the phyla *Firmicutes* and *Proteobacteria*. In the composition of nitrogen-fixing communities in the rhizosphere soil, the microorganisms of the genera *Clostridium, Paenibacillus,* and *Spirochaeta* were shown to prevail.

Keywords: nitrogen fixation, *nifH* gene, molecular ecology, rhizosphere

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Nitrogen-fixing microorganisms are widespread in soil biocenoses and are involved in one of the most important parts of the nitrogen cycle which, together with photosynthesis, ensures the productivity of the biosphere. Since accessible nitrogen sources are quite limited in most natural ecosystems, biological fixation of nitrogen plays an important role in the circulation of biogenic elements [1].

To enhance the plant productivity and crop quality and to preserve natural fertility of the soil avoiding deterioration of the environment, application of the biological preparations containing nitrogen-fixing microorganisms is a most common approach. The application of microbial preparations makes it possible to regulate the amount and activity of useful microorganisms in the rhizosphere of cultivated plants, and to supply plants with nitrogen fixed from the atmosphere. If soybean is cultivated in a soil for the first time, this soil usually lacks specific root nodule bacteria or contains them in a very small amount [2]. Selection of the new highly efficient strains of root nodule bacteria and determination of the complementary plant partners may be promising in the enhancement of the efficiency of symbiotic nitrogen fixation and legumes productivity [3, 4]. To obtain new strains of rhizobia, apart from analytical selection, genetic engineering [5] methods including transposon mutagenesis have been used in the last decades [6].

The interrelation between higher plants and soil microorganisms is one of the most interesting and complicated questions of biological science. Symbiosis of legumes with root nodule bacteria (rhizobia) is practically important and therefore well-studied. Nevertheless, the information regarding the diversity of diazotrophic bacteria in the "soil–leguminous plant" system is incomplete and is mainly based on the results obtained by traditional cultivation methods. Successful inoculation with rhizobia strongly depends on survival of the introduced bacteria in soil and their relations with the aboriginal microorganisms. Currently rhizobia are studied not only as legumes symbionts but also as associative and endophytic nitrogen fixers [7]. To control the activity of microbial communities, the internal structure of the system must be evaluated, the properties of the dominant microbial populations studied, and the stability threshold determined.

Development of methods based on the analysis of nucleic acids extracted from soil led to significant progress in studying soil microbial diversity. These methods include integral estimations resulting from the study of the kinetics of DNA reassociation [8], nucleotide sequence analysis of 16S rRNA [9] or special functional genetic markers [10], and in situ hybridization with fluorescent probes (FISH) [11].

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Table 1. Soil samples of the field experiment with the Gorlitsa variety of the soybean (2008) used in the study

No.	Variant of the field experiment	Description
1	P 1	Control (without inoculation)
2	P 2	Inoculation with *B. japonicum* UKM B-6035 (71t)
3	P 3	Inoculation with *B. japonicum* UKM B-6018 (2c)
4	P 8	Inoculation with *B. japonicum* UKM B-6018 + *Bacillus megaterium* UKM B-5724
5	P 9	Inoculation with *B. japonicum* UKM B-6035 (sensibilized with genistein)

In recent years, some data have been published regarding the *nifH* gene analysis: *nifH* gene is a convenient phylogenetic gene marker for the study of nitrogen-fixers in natural ecosystems. PCR analysis of the *nifH* gene fragments was successfully applied for evaluation of composition of nitrogen-fixing communities of various soils [12–19].

The goal of the present work was to study the diversity of nitrogen-fixing microorganisms in the rhizosphere soil of soybean after inoculation with rhizobia using the methods of molecular ecology and microbiology.

MATERIALS AND METHODS

Subjects of the study. Field experiments were carried out during the vegetation period (2008) at the Experimental Station of the Institute of Agroecology, National Academy of Sciences of Ukraine, Kiev region. The variants of field experiments are described in Table 1. Pre-sowing treatment of the control variant of soybean seeds (Gorlitsa variety) was carried out with sterile tap water. Experimental variants were treated with the inocula containing highly efficient soybean rhizobia strains which have been selected in the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine: *Bradyrhizobium japonicum* UKM B-6018 or *B. japonicum* UKM B-6035 (monoinoculum); a mixture of *B. japonicum* UKM B-6018 and *Bacillus megaterium* UKM B-5724 (binary inoculum); sensitized culture of *B. japonicum* UKM B-6035 obtained after cultivation of rhizobia with genistein, a signal flavonoid of the soybean–rhizobia symbiosis, in a concentration of 10^{-11} M (Sigma, Germany). Bacterial load during inoculation was 10^7 cells per seed.

Soil was characterized as a gray forest soil. Agrochemical properties of the 0–20 cm arable layer were the following: content of the humus by Tyurin, 1.3%; pH 5.4; hydrolytic acidity, 2.4 mg-eq./100 g; content of the easily hydrolyzed nitrogen by Cornfield, 125 mg/kg soil; content of labile phosphorus (P_2O_5) by Kirsanov, 220 mg/kg soil; exchange potassium (K_2O) by Kirsanov, 164 mg/kg soil.

The samples of rhizosphere soil were taken during the stage of soybean flowering at the plots with introduction of different bacterial preparations (Table 1).

Enrichment cultures of diazotrophic bacteria. Initial enrichment cultures of diazotrophs were obtained by adding of 2 g of rhizosphere soil to 500 mL Erlenmeyer flasks containing 200 mL of nitrogen-free mineral Ashby culture medium with mannitol. Cultivation was carried out under cotton plugs, but without shaking at 28°C for 7 days.

Isolation of DNA from soil and enrichment cultures. For isolation of DNA from soil samples, Power Soil DNA Isolation Kit (MoBio, United States) was used according to the manufacturer's recommendations with insignificant modifications. DNA concentration and degree of preparations purity were assessed as OD_{260}/OD_{280}, and OD_{260}/OD_{230} by spectrophotometry (Smart Spec Plus, BioRad, United States).

DNA was isolated from enrichment cultures using the Wizard Genomic DNA Purification Kit (Promega, United States), according to the manufacturer's recommendations with insignificant modifications.

Increased yield of microbial DNA was achieved by the treatment of soil samples and the biomass of enrichment cultures with lysing buffer at 80°C with shaking.

Amplification of the 16S rRNA and *nifH* fragments. The sequences of the primers used in this study are shown in Table 2. The 16S rRNA gene fragment was amplified using the universal primers 341F and 907R. Further separation of the amplicons by DGGE was carried out using the direct primer containing a 3'-terminal GC-clamp [20]. Amplification reaction mixture (25 μL) contained: DNA polymerase buffer (2 mM of $MgCl_2$; 17 mM of $(NH_4)_2SO_4$; 67 mM of Tris-HCl, pH 8.8), 6 nM of each dNTP; 20–50 ng of DNA template; 6.25 pM of the direct and reverse primers, and 1.5 U of BioTaq DNA polymerase (Dialat Ltd., Russia). Amplification conditions were as follows: initial cycle at 94°C for 9 min, 50°C for 1 min, 72°C for 2 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and final elongation at 72°C for 7 min.

For amplification of the *nifH* gene fragment of DNA isolated from soil, five systems of degenerative primers were tested. The F1–R6 primer system was developed in the Winogradsky Institute of Microbiology, Russian Academy of Sciences and Bioengineering Centre, Russian Academy of Sciences [21], the size of a fragment was 370 bp. Amplification was carried out in 25 μL of the reaction mixture containing: DNA polymerase buffer (67 mM of Tris-HCl, pH 8.8; 17 mM of $(NH_4)_2SO_4$; 1.5 mM of $MgCl_2$); 5 nM of each dNTP; 6.25 pM of each primer, 20–50 ng of

Table 2. Sequences and specificity of the primer systems used in the study

Primer	Target gene	Sequence (from 5' to 3' end)	Specificity	Reference
341F	16S rRNA	GTTTGATCMTGGCTCAG	All *Bacteria*	Muyzer et al., 1993
341F-GC	16S rRNA	GTTTGATCMTGGCTCAG (GCCCGCCGCGCCCCGCGCCCGTC CCGCCGCCCCCCGCCCG)	All *Bacteria*	Muyzer et al., 1993
907R	16S rRNA	TACGGYTACCTTGTTACGACTT	All *Bacteria*	Muyzer et al., 1993
F1	*nifH*	TAYGGIAARGGIGGIATIGGIAARTC	Nitrogen-fixing *Bacteria* and *Archea*	Marusina et al., 2001
R6	*nifH*	GCCATCATYTCICCIG	Nitrogen-fixing *Bacteria* and *Archea*	Marusina et al., 2001
*nifH*for	*nifH*	TAYGGNAARGGNGGHATYGGYATC	Nitrogen-fixing *Bacteria* and *Archea*	Sarita et al., 2008
*nifH*rev	*nifH*	ATRTTRTTNGCNGCRTAVABBGCCATCAT	Nitrogen-fixing *Bacteria* and *Archea*	Sarita et al., 2008
nifH-f	*nifH*	GGHAARGGHGGHATHGGNAARTC	Nitrogen-fixing *Bacteria* and *Archea*	Mehta et al., 2003
nifH-r	*nifH*	GGCATNGCRAANCCVCCRCANAC	Nitrogen-fixing *Bacteria* and *Archea*	Mehta et al., 2003
PolF	*nifH*	TGCGAYCCSAARGCBGACTC	Nitrogen-fixing *Bacteria* and *Archea*	Poly et al., 2001
PolR	*nifH*	ATSGCCATCATYTCRCCGGA	Nitrogen-fixing *Bacteria* and *Archea*	Poly et al., 2001
nifH-F	*nifH*	AAA GGY GGW ATC GGY AAR TCC ACC AC	Nitrogen-fixing *Bacteria* and *Archea*	Rösch et al., 2002
nifH-R	*nifH*	TTG TTS GCS GCR TAC ATS GCC ATC AT	Nitrogen-fixing *Bacteria* and *Archea*	Rösch et al., 2002

DNA template; and 1.5 U of BioTaq DNA polymerase (Dialat Ltd, Russia). Amplification conditions were as follows: 94°C for 3 min, 53°C for 3 min, 72°C for 30 s, 5 cycles at 94°C for 30 s, 53°C for 2 min, 72°C for 30 s, 27 cycles at 94°C for 30 s, 45°C for 30 s, 72°C for 20 s, and final elongation at 72°C for 7 min.

For the primer system *nifH*for–*nifH*rev [22], amplification conditions were as follows: initial denaturation at 94°C for 3 min, 30 cycles at 94°C for 1 min, 50°C for 45 s, 72°C for 1 min, and final elongation at 72°C for 5 min. The expected fragment size was 420 bp.

For the primer system *nifH*-f–*nifH*-r [23], amplification conditions were as follows: initial denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and final elongation at 72°C for 10 min. The expected fragment size was 400 bp.

For the primer pair PolF–PolR [13], amplification conditions were as follows: initial denaturation at 94°C for 2 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and final elongation at 72°C for 7 min. The expected fragment size was 360 bp.

For the primer system *nifH*F–*nifH*R [24], amplification conditions were as follows: 96°C for 20 s, 65°C for 30 s, 72°C for 30 s, 2 cycles at 96°C for 20 s, 62°C for 30 s, 72°C for 35 s, 3 cycles at 96°C for 20 s, 59°C for 30 s, 72°C for 40 s, 4 cycles at 96°C for 20 s, 56°C for 30 s, 72°C for 45 s, 5 cycles at 96°C for 20 s, 53°C for 30 s, 72°C for 50 s, 25 cycles at 94°C for 20 s, 50°C for 45 s, 72°C for 1 min; and final elongation at 72°C for 10 min.

The PCR products were separated on a 1.2% agarose gel in 1× TAE buffer containing ethidium bromide at 6 V/cm^2 and were digitalized using a GelDoc system (BioRad, United States). As a standard for the fragment size, commercial 1 kb and 100 bp DNA molecular markers were used (Fermentas, Lithuania).

Analysis of the composition of rhizosphere microbial communities by denaturing gradient gel electrophoresis (DGGE) was carried out using a DCode Universal Mutation Detection System (BioRad, United States) at the constant temperature of 60°C. The 16S rRNA fragments were separated at 100 V at denaturants gradient from 40 to 70% for 16 h. The resulting gels were stained with ethidium bromide and registered using a Gel Doc system (BioRad, United States). Similarity coefficients of the stained samples were calculated using the TREECONW software package. Material of the typical bands was eluted, purified, reamplified,

and used for determination of the nucleotide sequences in the service laboratory using the Big Dye Terminator v3 kit and an ABI Prism 3100 automatic sequencer (Applied Biosystems Inc., United States) with the 907R universal primer.

Cloning and sequencing. Prior to cloning, the PCR products were separated from the impurities (primer residues, oligonucleotides) on a 1.2% low-melt agarose in 1× TAE buffer with the PCR-Preps kit (Promega, United States). *E. coli* DH10B competent cells were transformed with these purified PCR products using commercial reagents pGEM-T Easy vector system I (Promega, United States). For each soil clone library and the clone libraries of enrichment cultures, 50 and 30 positive colonies, respectively, were randomly chosen. PCR reaction using the universal plasmid primers M13F and M13R was carried out with the colony material, and the presence of the insert was detected by electrophoresis on an agarose gel.

The resulting product was purified using the Ultra Clean PCR Clean-Up kit (MoBio, United States).

The *nifH* gene fragments from enrichment cultures were sequenced in the Macrogen service laboratory (Korea), and the *nifH* gene fragments from the soil DNA were sequenced in Syntol, Russia. Sequencing was performed on an ABI 3730 automatic sequencer (Applied Biosystems Inc., United States) with the reverse primer M13.

Analysis of nucleotide and protein sequences. Nucleotide sequences were analyzed using the BLAST network server (http://www.ncbi.nlm.nih.gov/BLAST). The alignment of nucleotide sequences was carried out by the BioEdit program tool (http://jwbrown.mbio.ncsu.edu/BioEdit.html). After detailed analysis of the results (BLAST-analysis and translation to protein sequences), the clones were grouped into the sequence types within which amino acid sequences shared 95–100% similarity.

Deduced amino acid sequences were inspected using the TREECONW software package (http://bioc-www.uia.ac.be/u/yudp/treeconw.html) and a dendrogram reflecting the degree of similarity between the studied and referent *nifH* gene sequences was constructed.

Deposition of the nucleotide sequences. The obtained *nifH* gene sequences were submitted to GenBank under accession numbers JQ032619–JQ032628.

RESULTS

DGGE analysis of microbial communities. Soil DNA preparations were successfully used for amplification of the 16S rRNA gene fragment. The fingerprints obtained after DGGE separation of the amplicon mixture and their cluster analysis results are shown in Fig. 1. In the composition of the dominant microbial populations of the soybean rhizosphere soil after different seed treatment procedures, some variations were revealed. In the composition of all communities, six to eight major bacterial groups were identified. Four groups were found in all the samples and could be regarded as the widespread aboriginal organisms whose presence did not depend on microbes introduced. In soil samples inoculated with rhizobia and the binary preparation, additional bands were detected (Figure 1). Cluster analysis revealed high degree of similarity (60%) between P2 and P8 communities.

Results of the typical bands sequence analysis are shown in Table 3. All nucleotide sequences demonstrated maximal similarity to the sequences of natural clones from various ecosystems. The degree of similarity ranged from high (99%) to low (73–76%). Importantly, no similar sequences were found in the database for 5 out of 11 sequences. One of the possible reasons could be incomplete separation of the amplicons during DGGE: this was registered earlier for environmental samples [24]. The nucleotide sequence of the typical band no. 10 of P1 control soil (without inoculation) demonstrated maximal similarity to environmental clones of *Firmicutes* which were found during the analysis of microbial communities of agricultural soils of Kansas, United States [25]. Similar bands were present in all other samples. Sequence analysis of the band no. 2 (P2 soil) located at the same distance from the start confirmed high degree of similarity to *Firmicutes* (99% similarity to environmental clones). Moreover, members of the *Acidobacteria* were found in microbial communities. The nucleotide sequence of the band no. 3 (P2 soil) demonstrated high degree of similarity (99%) to the sequences of environmental clones obtained in the study of microbial communities of agricultural soils of the Western Amazon river [26].

Among the dominant communities of plant rhizosphere inoculated with bacterial preparations, introduced (*B. japonicum*) was not found. It was probably displaced with aboriginal microorganisms and only remained in the material of soybean root nodules.

Amplification of the *nifH* gene fragments from rhizosphere soil and enrichment cultures. To amplify the fragments of the key nitrogen fixation genes, 5 primer systems frequently used in the studies of the composition of soil nitrogen-fixing communities were tested. The products of the necessary size were obtained only with the primer system *nifH*for–*nifH*rev proposed by Sarita et al. [23]: 400 bp both for pure cultures of diazotrophs (positive control) and soil DNA. The amplification product of the *nifH* gene fragment from a pure culture of *Azospirillum* sp. was analyzed and the obtained sequences were shown to be the *nifH* gene fragments of this microorganism.

Analysis of the clone libraries of soil DNA *nifH* fragments. On the basis of PCR products of the *nifH* gene from soil samples, seven clone libraries were constructed: one for each soil (50 clones in each) and two for enrichment cultures (30 clones in each). Separa-

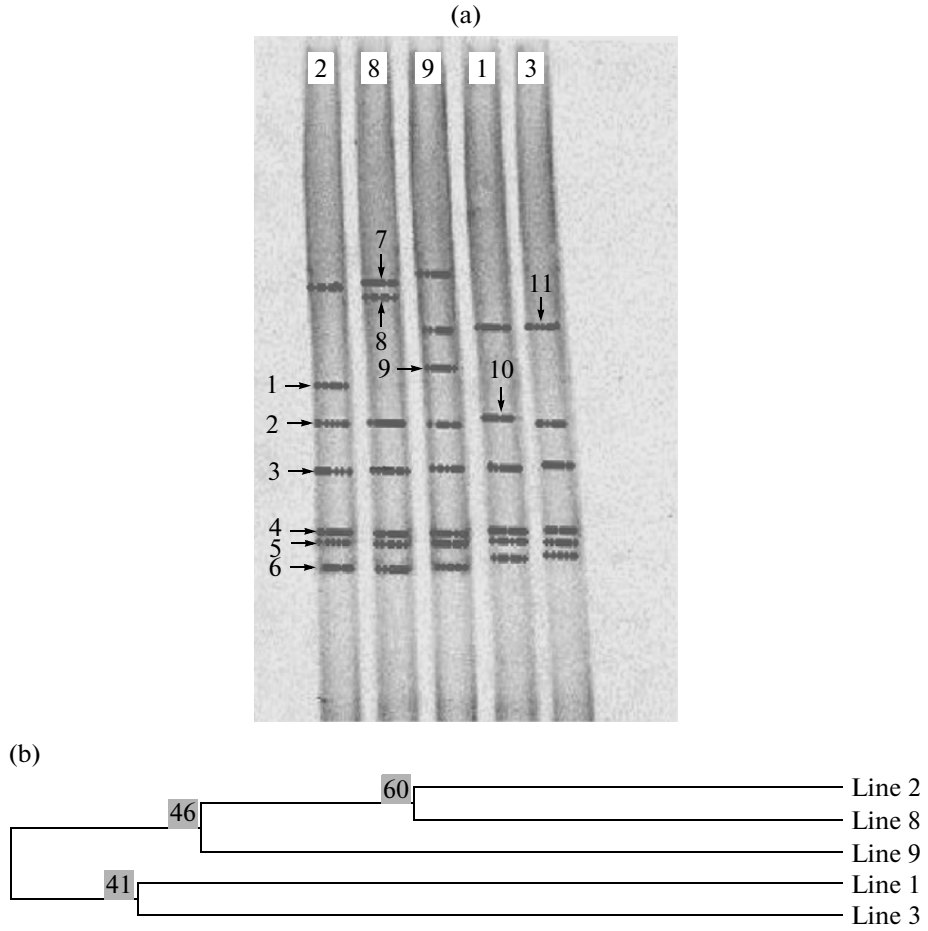

Fig. 1. Profiles of separation of the 16S rRNA gene fragments by DGGE (a) and cluster analysis of the obtained fingerprints (b). Designations of the lines indicate the variants of the experimental samples in Table 1. Arrows and numerals indicate the bands used for the further sequence analysis.

tion in agarose gel (data not shown) demonstrated all of the chosen clones to contain an insertion in the plasmid. After sequencing of the corresponding insertions, the sequences sharing 75% and more similarity with the GenBank *nifH* gene sequences were selected.

The sequences obtained for each community were analyzed using the BioEdit program and several groups were determined. Initial BLAST analysis of the nucleotide sequences revealed that most of the sequences were similar to those from the phylum *Firmicutes*, domain *Bacteria*. The sequences of only one group belonged to the phylum *Proteobacteria*. Members of this phylum are frequently identified in microbial communities of rhizosphere by microbiological and molecular methods [13–17, 22].

Translation was carried out, and the sequences were grouped into the sequence types, with 95–100% amino acid sequence similarity within each sequence type. Distribution of the clones within five soil clone libraries according to their sequence types is shown in Table 4.

Composition of soil nitrogen-fixing bacteria in the variants of the experiment was shown to be different. Most frequently S2 (all soils except P2) and S3 (all soils except P8) sequence types were registered. The diversity of nitrogen-fixing microorganisms was highest in P2 and P3 soils (three phylotypes). In the rest of soil types only two phylotypes were indicated. Alignment of amino acid sequences of each phylotype with the corresponding *nifH* gene sequences from the GenBank was carried out. The results of the phylogenetic analysis are shown in Fig. 2.

Most of the *nifH* gene sequences obtained both for the soils and enrichment cultures formed a compact cluster together with the obligate and facultative anaerobic bacteria (*Clostridium, Paenibacillus*). However, the representatives of the S2 phylotype were included in the same cluster as the *Alphaproteobacteria* species and demonstrated similarity to members of the genera *Leptospirillum, Derxia*, and *Azohydromonas.* These nitrogen-fixing bacteria were identified in all variants of soil except P2 (inoculation with rhizobia). The second big group included the clones of the

Table 3. Results of BLAST analysis of the nucleotide sequences of the typical bands obtained after DGGE separation of 16S rRNA gene fragments

Band no.	Most close relative (GenBank accession no.)	Nucleotide sequence similarity, %
2	Uncultured *Firmicutes* bacterium clone GASP-KC2S3_D10 16S ribosomal RNA gene (EU299794)	99%
3	Uncultured *Acidobacteria* bacterium clone U000107404 16S ribosomal RNA gene (FJ037313)	99%
4	Uncultured *Nitrosomonadaceae* bacterium clone Elev_16S_442 16S ribosomal RNA gene (EF019260)	84%
	Uncultured betaproteobacterium clone GASP-MB1W2_D05 16S ribosomal RNA gene (EF664861)	86%
7	Uncultured *Acidobacteria* 16S rRNA gene, clone ARN15 (AM936586)	89%
8	Uncultured *Firmicutes* bacterium clone GASP-45KB-172-D10 16S ribosomal RNA gene (EU044364)	73%
	Uncultured *Acidobacteria* bacterium clone BN3-34 16S ribosomal RNA gene (FJ870622)	74%
10	Uncultured *Firmicutes* bacterium clone GASP-KA1S3_D07 16S ribosomal RNA gene (EU297129)	76%

Table 4. Distribution of the clones of various sequence types in the studied samples of soybean rhizosphere soil

Soil	Clones number, %				
Sequence type Closest organism (GenBank accession number)	S1 *Paenibacillus massiliensis* (AY373370)	S2 *Leprospirillum ferrooxidans* (AY204398)	S3 *Clostridium pasteurianum* (XO7474XO6756)	S4 *Spirohaeta stenos-trepta* (AF325793)	S5 *Clostridium pasteurianum* (XO7474XO6756)
P 1	0	67	33	0	0
P 2	35	0	35	0	30
P 3	0	32	40	28	0
P 8	35	65	0	0	0
P 9	0	50	50	0	0

S3 sequence type, which were most similar to *Clostridium pasteurianum*. The sequences of the S3 type exhibited high similarity to the sequences of the S1 type (analysis of the enrichment cultures). The cluster of anaerobic nitrogen-fixers of the genus *Clostridium* also included members of the S5 phylotype. Sequences of the S4 phylotype were identical (100%) to those obtained by analysis of the enrichment cultures and were similar to the *Spirochaeta stenostrepta* sequences. The clones from the S1 group demonstrated identity (100%) to the *nifH* gene sequences of *Paenibacillus massiliensis*, and the S1 phylotype was therefore identified as a representative of this species.

Analysis of the *nifH* clone libraries of enrichment cultures. Results of the initial analysis of the sequences determined that 8 clones of SP1 and 6 clones of SP3 clone libraries were chimeric. These clones were excluded from the subsequent analysis. The remaining clones were classified into the sequence types within which nucleotide sequence similarity was 95–100%. Distribution of the clones between the sequence types (ST) is shown in Table 5. The sequences of the

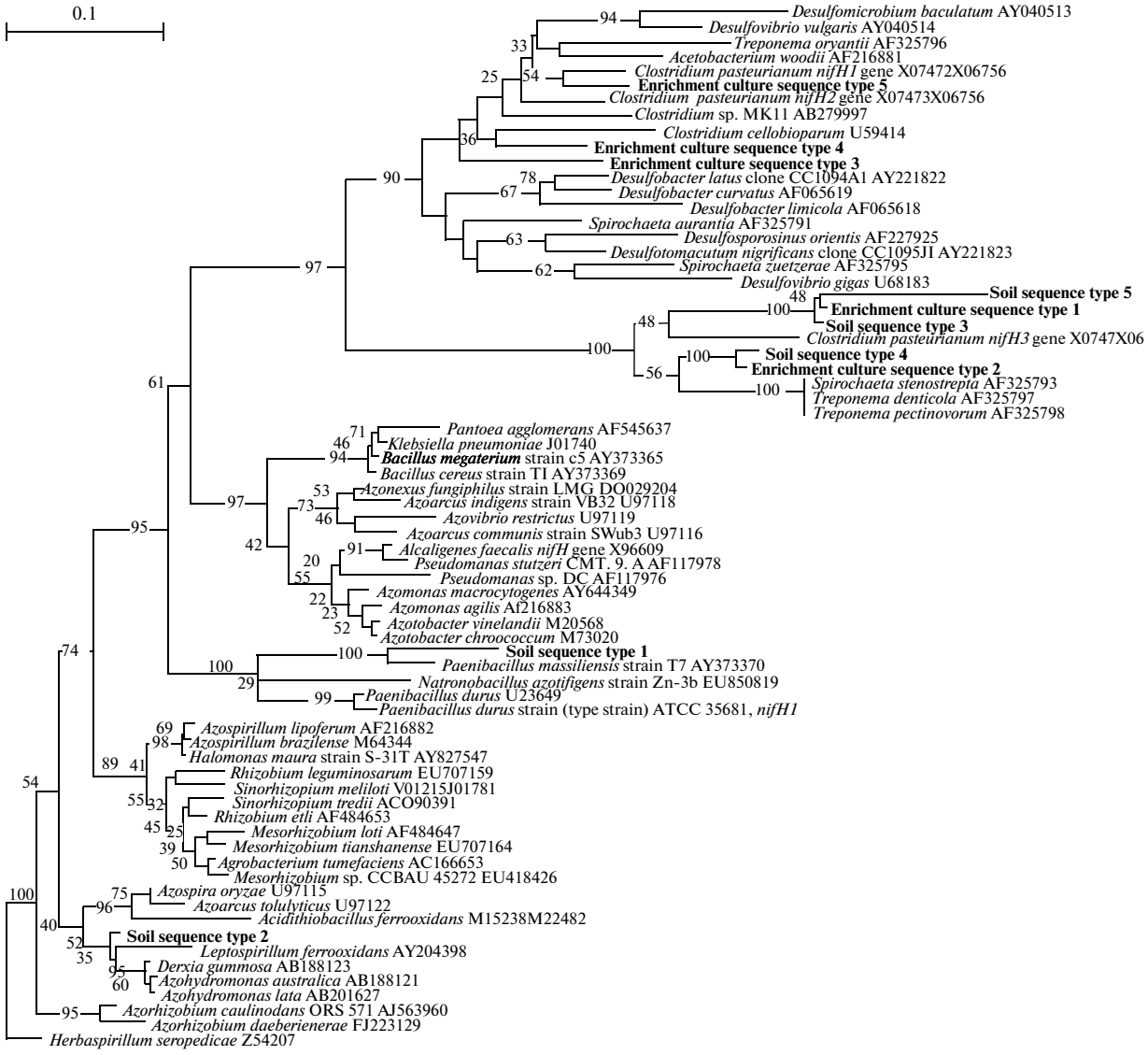

Fig. 2. Phylogenetic tree constructed based on the translated amino acid sequences of the *nifH* gene fragments, obtained by analysis of soil samples and enrichment cultures. The sequences obtained in the present work are shown in boldface. Scale bar corresponds to 10 replacements per 100 amino acid residues (evolutionary distance). The numerals show the significance of the branching order determined using "bootstrap"-analysis of 500 alternative trees (only bootstrap values over 50 are shown).

sequence groups ST1 and ST5 shared 74–95% similarity to the widespread obligate anaerobes of the class *Clostridia*, phylum *Firmicutes*. Sequences of the other groups exhibited the highest similarity (92–95%) to the sequences of environmental clones with uncertain taxonomy. The data obtained are in accord with the literature data. In the natural communities no more than 30–40% of the clones of the functional genes exhibit high similarity (more than 70%) to the known cultivated microorganisms [24].

Diversity of nitrogen-fixing microorganisms of the SP1 enrichment culture was significantly higher than that of the initial P1 soil. Two dominant sequence types, ST2 and ST3 (54 and 27% of the total clone number in the clone library, respectively), were determined, as well as three minor sequence types presented by single clones: ST4, ST5, and ST6. In the SP3 enrichment culture 96% of the clones were related to the ST1 sequence type and just one clone belonged to the ST2 type.

DISCUSSION

Abundant evidence exists regarding the composition of nitrogen-fixing microbial communities of the rhizosphere of various plants studied using microbiological and molecular methods. In most of the studies, members of the *Proteobacteria* were identified as the dominant species [22]. Though plant roots are able to conduct oxygen, anaerobic diazotrophs were found in

the rhizosphere [24]. This fact may be explained by the presence of anaerobic microniches. In the early works on microbial community analysis applying cultivation approaches, predominant development of clostridia in the rhizosphere of maize was determined [25]. These results were subsequently confirmed by Russian researchers for various wild-growing and agricultural plants. Legumes, especially perennial, were shown to stimulate *Cl. pasteurianum* growth more effectively than cereals [26]. Notably, in the soybean rhizosphere in the Primorskii krai numerous vegetative cells of bacteria of the genus *Clostridium* were detected [27]. The study of the communities of nitrogen-fixing bacteria of potato rhizosphere using the *nifH* gene analysis revealed the dominance of facultative anaerobes of the class *Bacilli*, closely related to the members of *Paenibacillus* and *Natronobacillus* [28]. In the present work, the predominating nitrogen fixers of the soybean rhizosphere were shown to be anaerobes. According to the phylogeny constructed basing on the *nifH* gene analysis, all sequences described in this study belonged to the group of anaerobes, which includes clostridia, sulfate-reducers, and methanogens [29]. The domination of anaerobes in the rhizosphere soil may be due to the specific composition of its organic compounds, mechanical properties of the soil, and specific features of microbial communities.

In recent decades, analysis of the evolutionary, conservative *nifH* gene has been widely used for detection of nitrogen-fixing microorganisms in natural microbial communities. Comparative analysis of this gene sequences with the known nucleotide sequences may provide important information regarding the phylogenetic position of nitrogen-fixing bacteria. Molecular analysis undertaken in the present work demonstrated that the composition of nitrogen-fixing communities of the analyzed soils included, besides known bacteria, unidentified nitrogen-fixing bacteria belonging to obligate anaerobes. These data improve our knowledge of the biodiversity of soil diazotrophs in these soils, which was previously analyzed using cultivation methods. The results of the current study improve our understanding of the biodiversity of rhizosphere nitrogen-fixing microorganisms, the structure of diazotrophic communities of these agrocenoses, and existing conception of effective symbiosis and interrelations of microorganisms in the legumes rhizosphere.

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