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EXPERIMENTAL ARTICLES

Isolation and Characterization of Nitrogen-Fixing Bacteria of the Genus *Azospirillum* from the Soil of a *Sphagnum* Peat Bog

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Abstract—he presence of nitrogen-fixing bacteria of the genus *Azospirillum* in the soils of acidic raised *Sphagnum* bogs is revealed for the first time. Three *Azospirillum* strains, B2, B21, and B22, were isolated as a component of methane-oxidizing enrichment cultures, whereas attempts to isolate them directly from peat samples have failed. The results of comparative analysis of the nucleotide sequences of 16S rRNA genes, DNA–DNA hybridization, and the analysis of the sequences of the functional genes encoding nitrogenase and ribulose-1, 5-bisphosphate carboxylase reveal that all the newly obtained strains can be classified as *Azospirillum lipoferum*. Yet, unlike *A. lipoferum*, the isolates do not require biotin and utilize sucrose, inositol, and glycerol for growth. The cell morphology of strain B2 differs from that of the type strain and strains B21 and B22. The results obtained indicate the variability of morphological, physiological, and biochemical properties in closely related *Azospirillum* strains and suggest the existence of metabolic relationships between methanotrophic bacteria and the representatives of the genus *Azospirillum* under peat bog conditions.

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Raised *Sphagnum* peat bogs are unique natural ecosystems characterized by high acidity and low buffering capacity, low mineral salt concentrations, and high levels of organic matter in an unavailable form. In such an ecosystem, the products of microbial fixation of atmospheric N_2 represent a major nitrogen source for plants and microorganisms. We have previously demonstrated that the nitrogen-fixing community of *Sphagnum* peat bogs is characterized by a relatively low activity and is active within a broad pH (3–7.5) and temperature range (5– 35°C) [1]. To date, there has been little published information on the composition of nitrogen-fixing microbial communities of bog soils. A number of bacterial strains belonging to the genera *Beijerinckia* [2] and *Burkholderia* [3] have been isolated from *Sphagnum* peat bog soils. Methanotrophic bacteria of *Sphagnum* peat bogs have been shown to exhibit nitrogenase activity [4]. The application of selective nutrient media and of the acetylene test have revealed the predominance of methylotrophic nitrogen fixers and facultatively anaerobic nitrogen fixers of the family *Enterobacteriaceae* in raised bogs [1].

Bacteria of the genus *Azospirillum* are a well-known example of so-called associative nitrogen fixers, which are widespread in the soils of tropical, subtropical, and temperate regions. These bacteria develop close relationships with the roots of various wild and agricultural plants [5, 6]. The investigations of these microorganisms carried out over the last few decades have primarily been aimed at gaining insight into the molecular nature of plant–microbial interactions in order to develop efficient modern biotechnologies (genetic, agricultural, etc.) [7, 8]. These studies have dealt to a considerably lesser degree with the distribution and ecological functions of these nitrogen fixers [9].

A. lipoferum and *A. brasilense* were long the only known members of the genus *Azospirillum* [10]. Other *Azospirillum* species have been described relatively recently. They were isolated from the rhizosphere of wild or agricultural plants growing in the tropical (*A. amazonense, A. halopraeferens, A. irakense*, and *A. largimobile*) or temperate zones (*A. doebereinerae, A. oryzae*). The free-living bacteria identified as *A. lipoferum* and *A. brasilense* were isolated from the soils of Finland [11] and Russia [12].

The application of semisolid nitrogen-free NFb medium [13] played a key role in the successful isolation of microaerophilic nitrogen-fixing bacteria and provided the basis for the isolation of all the new

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Azospirillum species, except for *A. largimobile*. Applying the NFb medium for enumeration and isolation of nitrogen fixers from the bog soils of various regions, we failed to detect bacteria of the genus *Azospirillum*. Yet analysis of the diversity of the *nifH* gene fragments in methanotrophic enrichment cultures obtained from the soil of an oligotrophic bog in central Russia has revealed the presence of clones with nucleotide sequences most closely related to the sequences of *Azospirilla* [14].

The goal of the present work was to isolate and characterize the nitrogen-fixing bacteria of the genus *Azospirillum* from these methane-oxidizing enrichment cultures.

MATERIALS AND METHODS

Object of study. The methane-oxidizing enrichment cultures SB26, SB31, and SB31A were isolated from peat samples taken from the Sosvyatskoye bog (Zapadnaya Dvina Field Station of the Institute of Forestry, Russian Academy of Sciences) in June 2000. The isolation and cultivation conditions were previously described in [14]. The isolation of *Azospirillum* cultures was carried out by inoculating 0.1 ml of the suspension of methane-oxidizing enrichments into the semisolid nitrogen-free NFb medium supplemented with sodium malate (1 g l^{-1}) [13]. The criteria for preliminary screening for *Azospirilla* were the presence of a dense film in the upper layer of the semisolid medium, high levels of nitrogenase (acetylene reductase) activity, and data obtained by light microscopy. To isolate pure *Azospirillum* cultures, the above-mentioned agarized malate medium with Congo red was used [15].

Cell morphology. The cell morphology was determined with a phase-contrast microscope (Lumam, Russia); the shape and size of the cells, as well as flagellation type, were studied by electron microscopy (JEM-100, Japan) of uranyl acetate-stained preparations.

Physiological and biochemical tests. The capacity of the isolates to utilize organic carbon was assessed by their growth after three successive transfers in liquid mineral NFb medium with potassium nitrate (0.2 g l^{-1}), supplemented with 1 g l^{-1} of various carbon sources. The effect of temperature, pH, and NaCl on the growth of isolates was assessed by the optical density of the cultures (λ = 600 nm, SF-26, Russia) inoculated into the liquid mineral medium with malate and nitrate. The rates of nitrogenase activity were determined by the acetylene test; protein concentrations were measured by the Lowry method.

Genotypic properties. The content of G+C base pairs in the DNA was determined according to the thermal denaturation curves in a spectrophotometer Pye Unicam SPI 800. DNA–DNA hybridization was performed by De Ley's optical reassociation method.

For the isolation and purification of the DNA preparations, we applied the method previously described in [16]. PCR amplification of the 16S rRNA gene fragments was performed with the universal bacterial primers [17]; the genes responsible for initial fixation of CO_2 (*cbbL*) and nitrogen (*nifH*) were amplified with the previously developed system of primers [16, 18].

Analysis of PCR products was carried out by electrophoresis in 2% agarose gel at 6 V cm^{-1}. Sequencing of PCR products was performed using a Silver Sequencing kit (Promega, United States), according to the manufacturer's recommendations with minor modifications.

The primary comparison of the de novo obtained sequences with the sequences within the GenBank database was performed using the NCBI BLAST software package [http://www.ncbi.nlm.nih.gov/BLAST/]. The nucleotide sequences and the deduced amino acid sequences of the studied genes were edited and aligned with the appropriate sequences from the closest relatives using the BioEdit software package [http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html]. The phylogenetic trees were constructed by the methods implemented in the TREECONW software package [http://bioc-www.uia.ac.be/u/yvdp/treeconw.html].

Sequence deposition. The 16S rRNA, *nifH*, and *cbbL* gene fragments of the strains B2, B21, and B22, as well as of the type strain *Azospirillum lipoferum* Sp 59b^T, have been deposited in the GenBank under the accession numbers DQ787328–DQ787330, DQ787331–DQ787334, and DQ787335–DQ787337, respectively.

RESULTS

Isolation of cultures. Within 7–12 days after inoculation of the methane-oxidizing enrichments into semisolid NFb medium, in three vials inoculated with the SB31 and SB31A suspensions, a thick surface film was formed, characteristic of the growth of *Azospirillum* species. The level of nitrogenase activity was high (over 100 nmol C_2H_4 h^{-1} per vial); phase contrast microscopic examination revealed the presence of vibrioid cells which exhibited writhing (snakelike) motion typical of azospirilla. By repeated plating onto agarized media, three pure *Azospirillum* cultures, designated B2, B21, and B22, were isolated from the three independent enrichment cultures.

When grown on potato agar, strains B21 and B22 produced light beige pearl slimy spherical colonies 2.0–6.0 mm in diameter. The size of the B2 colonies did not exceed 3.0 mm; in addition, they were not slimy. On an agarized Congo red medium, all colonies were purple.

Cell morphology and physiological and biochemical properties. When grown on a liquid nutrient medium, all the cells of strain B2 were vibrioid and were distinguished from other isolates by their short (0.7–0.9 μm) tapered cells (Fig. 1a). The cells of strains B21 and B22 had rounded ends and were 1.2–1.5 μm

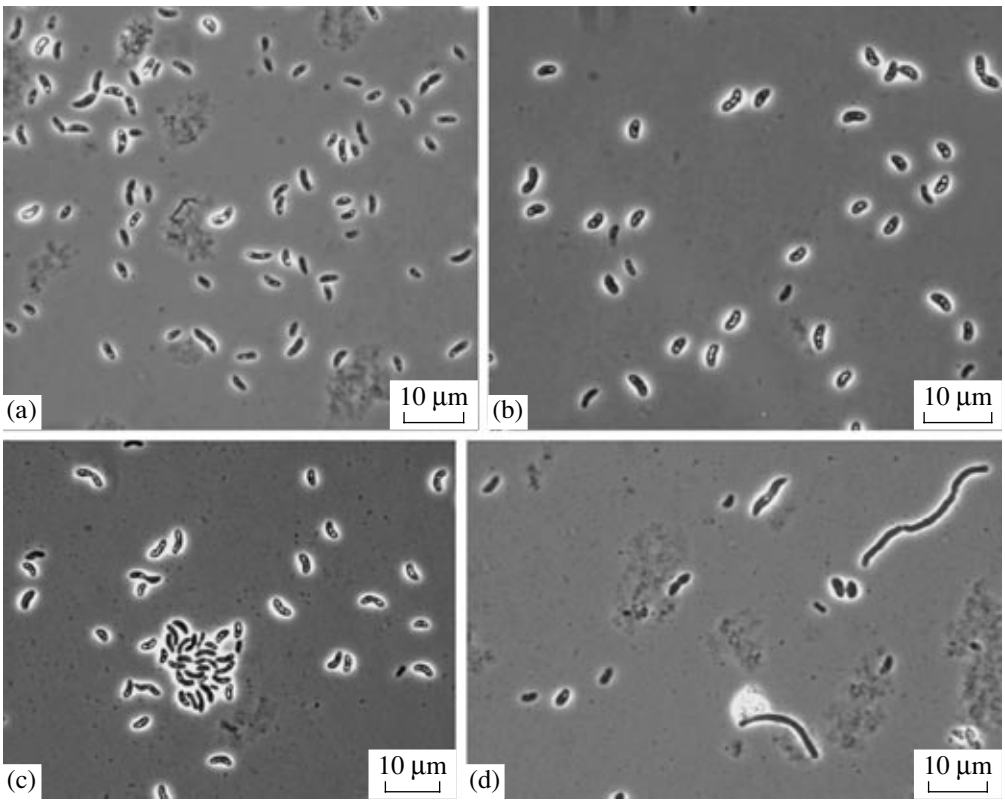

Fig. 1. Photomicrographs of the cells of B2 (a), B21 (b), and B22 (c) cultures grown in the liquid nutrient medium for 3 days and the 10-day B21 culture (d), phase microscopy.

long (Fig. 1b, 1c). In the case of long-term (more than five days) incubation in a liquid or semisolid NFb medium, cell polymorphism and the formation of long chains of unseparated cells were observed (Fig. 1d). Stationary phase cells of all three strains accumulated intracellular granules of poly-β–hydroxybutyrate. Electron microscopic observations revealed that, during growth in liquid media, all the investigated strains had one polar flagellum; during growth on agarized media, the cells had multiple peritrichous flagella. According to the cell wall structure, the bacteria were gram-negative.

Some important characteristics of the isolated cultures are given in Table 1. All the isolates had a respiratory type of metabolism and exhibited denitrifying activity under anaerobic conditions. Tests for catalase and urease were positive; the biotin requirement was not observed. The investigated cultures utilized a broad spectrum of carbon sources for aerobic growth, including sugars, organic acids, and alcohols.

The isolates grew at a pH range of 5.5–8.5 with an optimum at 6.5; at pH values higher than 7.0, the formation of film conglomerates was observed. The temperature limits for growth were 10–40°C with an optimum at 30°C for B2, and 10–50°C with an optimum at 37°C for the B21 and B22 strains.

The capacity for nitrogen fixation. The investigated *Azospirillum* strains were obtained by inoculation on the semisolid medium with malate, whereas further isolation, purification, and characterization were carried out with the use of nitrogen-containing media. However, growth on these media did not inhibit their nitrogenase activity.

Table 2 shows the results of measurements of the nitrogenase (acetylene reductase) activity of the isolated *Azospirillum* strains. The maximum activity rates (4.0–7.6 μg N g protein^{-1} h^{-1}) were detected in the presence of organic acids (malate and acetate). It should be noted that the pH ranges for nitrogenase activity were significantly narrower than those for the isolate growth. The nitrogen fixation activity of all the cultures was observed only in the pH range 6.0–7.0 and was not detected at higher and lower acidity values.

Genotypic identification of the isolates. The content of G+C base pairs in the DNA of all the newly isolated strains varied from 68 to 70%, which is typical for *Azospirillum* species [19]. To determine the phylogenetic position of the new strains B2, B21, and B22, a considerable number of their 16S rRNA genes (about 1350 nucleotides) were sequenced. Primary analysis of the similarity between the obtained nucleotide sequences and those deposited in the GenBank database with the BLAST analysis shows that the investi-

Table 1. Comparison of some differential properties of the type strain *Azospirillum lipoferum* and of the strains isolated from the *Sphagnum* bog

Property	*Azospirillum lipoferum* strains			
	59b^T	B2	B21	B22
Cell length, μm	1.0–1.5	0.7–1.2	1.0–1.5	1.0–1.5
Growth range*				
pH	5.7–6.8 (6.0)	5.5–8.5 (6.5)	5.5–8.5 (6.5)	5.5–8.5 (6.5)
temperature, °C	15–40 (37)	15–40 (30)	15–55 (30)	15–55 (30)
NaCl, %	1–3 (1.5)	1–2 (1.2)	1–3 (1.2)	1–3 (1.4)
Content of G+C base pairs (mol %)	69–70	68	68	70
Biotin requirement	+	–	–	–
Utilization of carbon sources:				
sucrose	–	+	+	+
inositol	–	+	+	+
lactose	–	+	+	+
cellobiose	–	+	+	–
maltose	–	+	+	+
rhamnose	+	+	–	–
mannitol	+	–	+	–
glycerol	–	+	+	+

* Optimum values are given in parentheses.

gated strains belong to the α subclass of *Proteobacteria* and are closest to members of the genus *Azospirillum*. On the phylogenetic tree (Fig. 2), the new strains form a compact cluster, together with the other *A. lipoferum* strains (including the type strain) and the representatives of the species *A. largimobile, A. doebereinerae*, and *A. oryzae*. The nucleotide sequences of the B21 and B22 strains were found to be identical. They were closest to the sequences of *A. lipoferum* F (99.8% similarity) and the type strain *A. oryzae* COC8^T (99.6% similarity); the strain B2 was closest to the strain *A. lipoferum* ATCC 29708 (99.6% similarity).

Since at this high level of relatedness, the resolving power of the 16S rRNA gene sequences is insufficient for precise strain identification, DNA–DNA hybridization was performed. The results revealed a high level of hybridization between the studied strains (62–76%), as well as between them and the type strain *A. lipoferum* 59b (65–75%), confirming the affiliation of the newly isolated strains to this species.

Table 2. Nitrogen-fixing activity of the three strains of *Azospirillum lipoferum* isolated from the *Sphagnum* bogs

Carbon source	Nitrogen-fixing activity, μg N g protein $^{-1}$ h^{-1}		
	B2	B21	B22
Glucose	2.2 ± 1.1	3.0 ± 0.9	2.4 ± 0.7
Malate	2.9 ± 1.3	4.1 ± 1.5	4.6 ± 0.9
Acetate	6.3 ± 2.1	7.0 ± 2.4	7.6 ± 3.3
Butyrate	0.9 ± 0.3	0.8 ± 0.4	0.4 ± 0.1
Cellobiose	0	0.2 ± 0.05	0.3 ± 0.1

Detection and analysis of the functional genes encoding nitrogenase and ribulose-1,5-bisphosphate carboxylase. Using the previously developed system of oligonucleotide primers [16], we detected and sequenced the *nifH* gene fragments (about 450 nucleotides) of all the studied *Azospirillum* strains. The obtained sequences of strains B2 and B21 were found to be almost identical (99.6% similarity), whereas the strain B22 sequences were somewhat different (96.6 and 97.1% similarity). Preliminary screening using the GenBank database showed the affinity of newly obtained nucleotide sequences with the *nifH* gene family.

We aligned the deduced *nifH* amino acid sequences of the studied strains with analogous sequences of the members of the genus *Azospirillum* and other nitrogen-fixing α-*Proteobacteria*, available from the GenBank database, and compared 150 positions of amino acid residues.

The constructed phylogenetic tree (Fig. 3) shows that the investigated strains form a cluster with *Azospirillum* strains, exhibiting a high level of sequence similarity with them (96.3–100% similarity). This level of

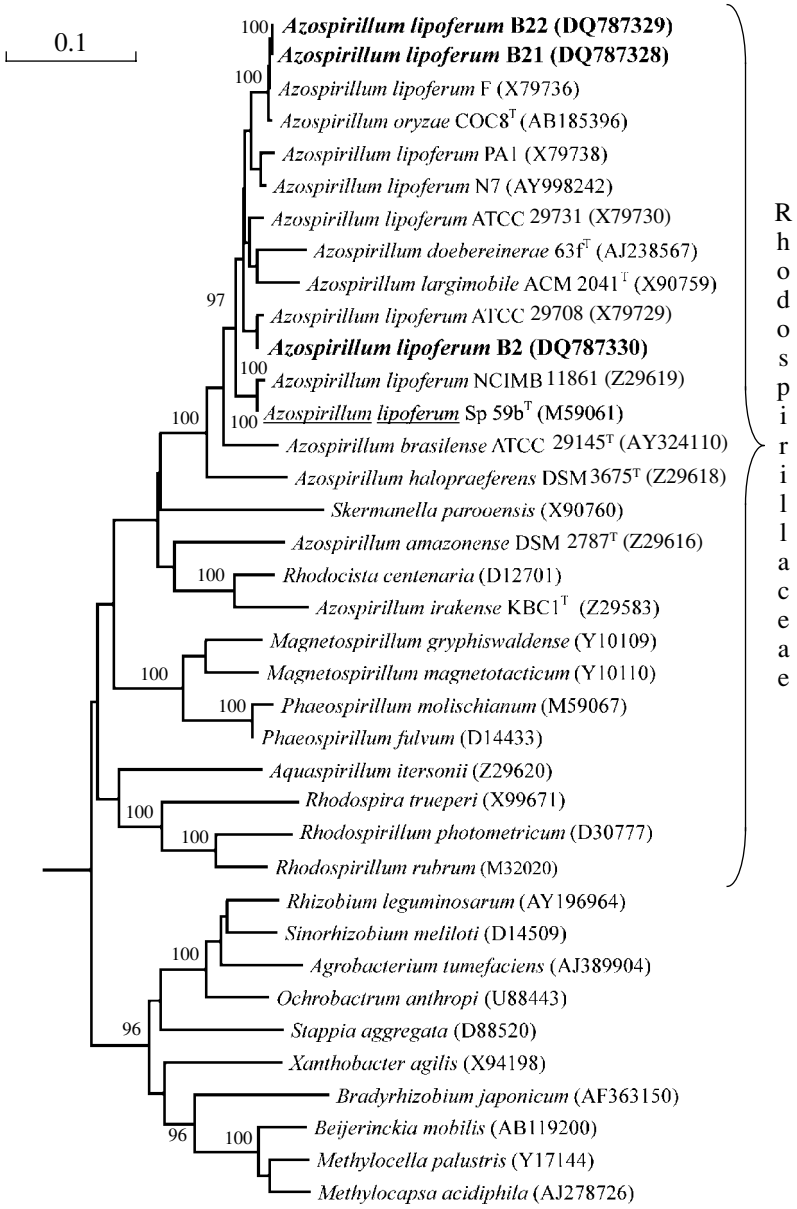

Fig. 2. Phylogenetic tree based on the nucleotide sequences of the 16S rRNA gene fragments. The sequences obtained in this study are shown in bold. The GenBank accession numbers of the gene fragment sequences used for the tree generation are given in parentheses. The scale bar corresponds to 10 substitutions per 100 nucleotides (evolutionary distances). The numbers indicate the statistical validity of the branching order determined by the bootstrap analysis of 500 alternative trees (values of less than 50 are not shown).

similarity approximately corresponds to that of the *nifH* amino acid sequences within the genus *Azospirillum* (96.3–99.4%) and is considerably higher than the similarity level between other diazotrophic α-*Proteobacteria* (86.6–94.5%). This fact suggests that the nitrogenase genes of various members of the genus *Azospirillum* have a common origin.

Using the previously developed system of primers [18], we amplified and sequenced the fragments (approximately 800 nucleotides) of the *cbbL* genes encoding form I (redlike) RuBisCO of the new isolates and of the type strain *A. lipoferum* 59b. The obtained sequences of all the strains studied were found to be close (93.8–97.4% similarity). Preliminary screening using the GenBank database showed the affinity of the newly obtained nucleotide sequences to the redlike *cbbL* gene family.

We aligned the deduced *cbbL* amino acid sequences with the analogous redlike form I RuBisCO sequences of other bacteria available from the GenBank database and compared 231 positions for amino acid residues. The resulting phylogenetic tree (Fig. 4) shows that the

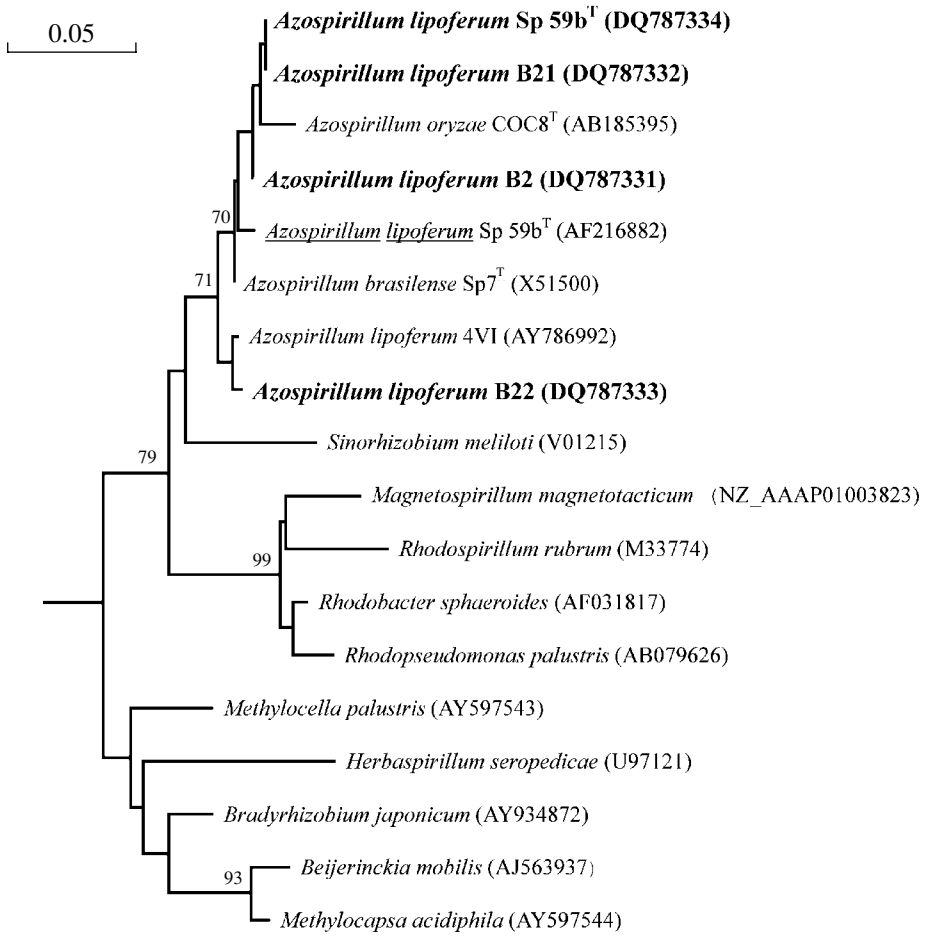

Fig. 3. Phylogenetic tree based on the comparative analysis of the deduced amino acid sequences of the *nifH* gene fragments. The sequences obtained in this study are shown in bold. The GenBank accession numbers of the gene fragment sequences used for the tree generation are given in parentheses. The scale bar corresponds to 5 substitutions per 100 amino acid residues (evolutionary distances). The numbers indicate the statistical validity of the branching order determined with the bootstrap analysis of 500 alternative trees (values of less than 50 are not shown).

studied strains form a single cluster with *A. lipoferum* GSF, the only strain available from the database, with a high level of sequence similarity (97.8–100%). This fact suggests that the RuBisCO genes of various members of the genus *Azospirillum* have a common origin. At the same time, the RuBisCO gene analysis did not reveal any close phylogenetic relatedness between the studied *Azospirillum* strains and other autotrophic α- and β-*Proteobacteria*, since the branching point position of *Azospirillum* on the constructed tree was unstable (with bootstrap support of 33).

DISCUSSION

Bacteria of the genus *Azospirillum* are widespread in the soils of various regions and usually occur in the rhizosphere of vascular plants. Their presence in the soil outside the plant rhizosphere has been observed in the soil of rice paddies, where their cell numbers did not exceed 2×10^5 cells g soil [20]. As far as we know, there have been no investigations of these microorganisms in the soils of peat bog ecosystems.

Despite our numerous attempts, we failed to isolate *Azospirilla* directly from the peat samples taken from the bogs of various types. However, molecular approaches demonstrated that they were present as a part of stable methanotrophic associations [14]; they have subsequently been isolated in pure culture. With the help of molecular techniques, it was demonstrated that azospirilla were a component of the epiphytic microbial communities of green algae of the order *Zygnematales* from the *Spahgnum* peat bog [21]. We failed to isolate any *Azospirillum* cultures from the rhizosphere of the bog grass species, cotton-grass and sedge (Doroshenko, unpublished data). This finding demonstrates that the distributional patterns of the azospirilla inhabiting bog ecosystems differ significantly from those of azospirilla from other studied ecosystems. In agrocenoses, the numbers of azospirilla in the rhizosphere soil may constitute up to 10% of the

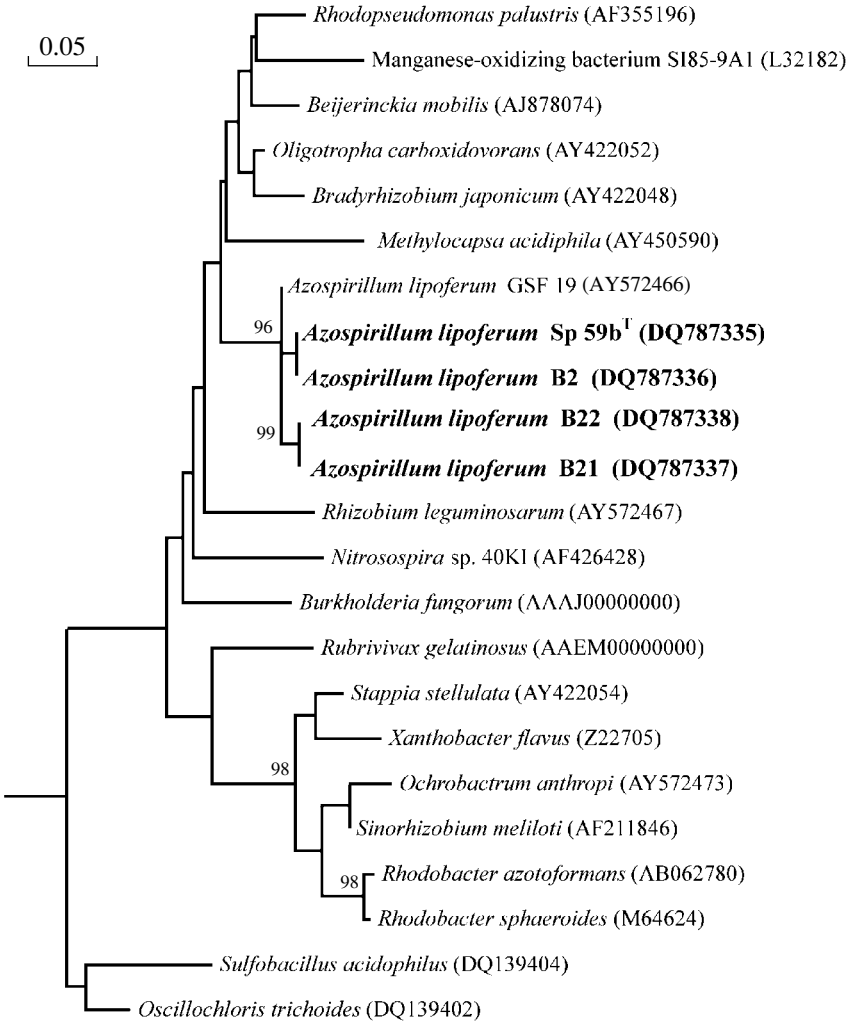

Fig. 4. Phylogenetic tree based on the comparative analysis of the deduced amino acid sequences of the *cbbL* gene fragments. The sequences obtained in this study are shown in bold. The GenBank accession numbers of the gene fragment sequences used for the tree generation are given in parentheses. The scale bar corresponds to 5 substitutions per 100 amino acid residues (evolutionary distances). The numbers indicate the statistical validity of the branching order determined with the bootstrap analysis of 500 alternative trees (values of less than 50 are not shown).

total number of bacteria, whereas, outside the rhizosphere, it does not exceed 0.1% [22].

An investigation of the temperature and pH growth ranges did not reveal any significant differences from other strains. It is notable that nitrogenase activity is extremely sensitive to changes in pH level, its optimum being limited to a very narrow interval. Organic acids are the main energy sources required for nitrogen fixation. This corresponds to the description of azospirilla as associative organisms utilizing the metabolic products of methanotrophic bacteria. It has been previously demonstrated that some members of the genus *Azospirillum* are capable of autotrophic growth through hydrogen oxidation and CO_2 assimilation with ribulose-1,5-bisphosphate carboxylase [23]. Our tests for the capacity of the isolated strains for autotrophic growth did not give an unambiguous answer, whereas analysis of the *cbbL* genes has confirmed the theoretical possibility of such growth for all the studied *Azospirillum* strains.

Phylogenetic analysis and DNA–DNA hybridization indicated that although the obtained isolates have some distinguishing morphological and physiological characteristics, including such traits as requirement for biotin and utilization of sucrose, which are essential for successful differentiation within the genus *Azospirillum*, they can be assigned to the species *A. lipoferum* due to the level of DNA similarity between themselves and with the type strain of this species. The results of the additional phylogenetic analysis of the *nifH* and RuBisCO gene sequences of the newly isolated strains do not contradict this conclusion.

This study will help us to broaden our understanding of the ecological niches occupied by azospirilla, which can no longer be considered inhabitants of the plant

rhizosphere only. Recent investigations have generally been focused on the interactions of *Azospirillum* species with the root systems of cereal crops [22]; however, there have been a number of publications concerning the isolation of *Azospirillum* strains from quite unusual habitats, such as the mycelium of phytopathogenic fungi or human skin [24]. The question of whether members of the genus *Azospirillum* are able to grow and exhibit activity in soil outside the plant rhizosphere is still open. A number of physiological mechanisms, including cyst formation, melanin production, and the synthesis of hydroxybutyrate and polysaccharides are known, which allow azospirilla to survive unfavorable environmental conditions. The formation of stable associations between azospirilla and methanotrophic bacteria may be considered as a population mechanism for survival, which, possibly, promotes nitrogen fixation in peat bog soil.

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