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EXPERIMENTAL ARTICLES

Nitrogen-Fixing Cyanobacterium *Trichormus variabilis* of the Lake Baikal Phytoplankton

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Abstract—A new filamentous cyanobacterial strain BAC 9610 was isolated from the lake Baikal pelagial. Data obtained by light, scanning, and transmission electron microscopy, along with 16S rRNA gene sequence analysis, allowed the bacterium identification as *Trichormus variabilis*, previously known as *Anabaena variabilis. Trichormus* is a cyanobacterial genus not presented in the list of Baikal plankton algae; *A. variabilis* also hasn't been previously detected in Baikal phytoplankton. *T. variabilis* nitrogen fixation ability was demonstrated. The gene responsible for nitrogen fixation, *nif*H, was identified by PCR and was partially sequenced. No hepatotoxin synthesis genes were revealed in the strain.

Key words: cyanobacteria, nitrogen fixation, *Trichormus variabilis*, toxins, 16S rRNA gene, *nif*H gene.

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Cyanobacteria, previously named blue-green algae, are an ancient and widespread group of prokaryotes. They possess a unique ability to combine two vital processes of the biosphere, namely, oxygenic photosynthesis and nitrogen fixation. Filamentous cyanobacteria capable of dinitrogen fixation, have heterocysts, specialized cells containing nitrogenase, the major enzyme of nitrogen fixation. Nitrogenase is a binary metalloprotein, (Mo–Fe-protein) which uses the energy obtained by photosynthesis to bind molecular nitrogen and reduce it to ammonia. The enzyme is a part of the nitrogenase complex, comprising one more Fe-protein (nitrogenase reductase) responsible for supply of low-energy electrons for nitrogenase. It is only the complex of these two enzymes which is capable of nitrogen fixation. Nitrogenase reductase is encoded by the *nif*H gene, while two genes are responsible for nitrogenase synthesis, *nif*D and *nif*K. Identification of the genes responsible for nitrogen fixation, particularly the *nif*H gene, allowed the discovery of a whole number of potentially diazotrophic organisms including cyanobacteria, proteobacteria, and anaerobic bacteria in a variety of ecosystems [1, 2].

In freshwater basins, cyanobacteria are among the major planktonic nitrogen fixers, gaining therefore, a significant advantage under conditions of nitrogen deficiency [3]. The most common species belong to the genera *Anabaena* Bory, *Aphanizomenon* Morren, *Gloecapsa* (Kütz.) Hollerb., *Nostoc* Vauch. *ex* Born. *et* Flah., *Oscillatoria* Vauch., and *Trichormus* Rales *ex* Born. *et* Flah.

Several cyanobacterial genera, known as nitrogen fixing bacteria in other water basins, are enlisted among Baikal plankton algae [4]. Works on the lake Baikal phytoplankton have shown that cyanobacteria are rich in species diversity and numbers, both in the pelagial and in shallow water, especially during summer time [5, 6]. However, despite the importance of cyanobacteria for the lake ecosystem, no study has been focused on nitrogen fixation so far.

Massive growth of nitrogen-fixing species often causes water bloom. It may be accompanied by synthesis of toxins indeed hazardous to human and animal health and life. Quite a number of cyanotoxins are known today, hepatotoxins are the best studied among them. Molecular biological techniques for detection of cyanotoxin synthesis genes allowed the revealing of toxic cyanobacteria in many water basins of the world. Using genetic markers, we have shown the presence of cyanobacteria capable of hepatotoxin synthesis in the Baikal water reservoirs. No toxic strain has been detected in Baikal, although potentially toxic species have been revealed among the lake phytoplankton [7].

The goal of the present work was to determine the taxonomic position of a new cyanobacteria strain isolated from the lake Baikal pelagial using methods of microscopy and 16S rRNA gene sequence analysis and to study the ability of the strain to fix nitrogen and to synthesize toxins.

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MATERIALS AND METHODS

In August 2005, plankton samples were collected from the 0–25 m layer of the South Baikal pelagial to isolate the culture. Enrichment cultures were grown in a Z8 liquid mineral medium [8]. Multiple transfers of the culture in agarized Z8 media were performed in order to isolate the pure culture. The culture was maintained in a New Brunswick G25 incubator (New Brunswick Scientific, United States) under the following illumination conditions: 14 h light (intensity of 750 lx) and 10 h darkness, at 12°C. The isolated strain is stored in the collection of cyanobacteria and algae of the Limnological Institute (Siberian Branch, Russian Academy of Sciences) under the record number BAC 9610.

In the experiments on the influence of illumination and stirring on the growth characteristics of the isolated strain, a Z8 medium was used. A picoplankton strain *Synechococcus* sp. BAC 98111 at the Limnological Institute collection was used as a control. Experiments on the ability for nitrogen fixation were carried out in a nitrate-free Z8 medium. Culture growth dynamics was evaluated by optical density measurements using an SF-46 spectrophotometer (LOMO, Russia) at 700 nm, every three days.

Morphology and pigment characteristics of unfixed cells were observed under an Axiovert 200 microscope (Zeiss, Germany), equipped with an HBO 50 mercury lamp and three sets of filters: G 365, BP 450–490, and BP 546/12. Microphotographs were obtained using a Penguin 600CL camera (Pixera Corp., United States) and the VideoTest-Razmer 5.0 software package (www.videotest.ru). This software was also used for the cell dimensions measurements and statistical treatment of the results.

To prepare the cells for scanning electron microscopy (SEM), they were fixed with glutaraldehyde (at the final concentration of 1%), placed on Millipore polycarbonate membrane filters with the pore diameter of 0.2 μm, and dehydrated in an ascending series of alcohols. After critical point drying (Balzers CPD 030 Critical Point Dryer, Bal-Tec AG, Lichtenstein), the preparations were coated with gold (Blazers SCD 004) and examined in a Philips SEM 525M scanning electron microscope (Philips, The Netherlands). Microphotographs were obtained using the system of electron image scanning (Limnological Institute).

For transmission electron microscopy (TEM), the cells were fixed in 1% glutaraldehyde and 0.25% OsO_4 solution in 0.1 M cacodylate buffer. Then the cells were postfixed with 1% OsO_4, dehydrated in an ascending series of alcohols and in 100% acetone, embedded into Epon–Araldite mixture, and left to polymerize. Ultrathin sections were cut with an Ultracut K microtome (Leika Microsystems, Germany). The sections were contrasted with uranyl acetate and lead citrate as described by Reynolds [9]. The sections were examined in a Leo 906E electron microscope (Zeiss, Germany). Microphotographs were obtained using a MegaView II digital camera (Leo Elektronenmikroskopie GmbH, Germany) and the MegaVision software package (Soft Imaging System GmbH, Germany).

DNA was isolated by silica gel adsorption, using a RiboSorb set (FGUN TsNIIE Rospotrebnadzora, Russia), following the producer's instructions. Polymerase chain reaction was carried out in a Mini Cycler apparatus (MJ Research, Bio-Rad, United States) using Amplisens reagents (Central Research Institute of Epidemiology, Moscow, Russia) in a total volume of 15 μl (DNA volume 1 μl, 200–300 ng/μl). Amplification of the 16S rRNA gene was carried out using the cyanobacteria-specific primers 106F and 781R (a) [10] corresponding to *E. coli* positions 106–127 and 781–805. The reaction was performed under the following conditions: one cycle of initial denaturing at 94°C for 5 min; 35 three-step cycles: denaturing at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and the finishing synthesis step at 72°C for 10 min. DNA from a green alga *Choricystis* sp. BAC 9708 was used as a negative control. CNF and CNR primers, specially designed for cyanobacteria [1], were used for amplification of the *nif*H gene sequence. The following PCR parameters were used: one cycle at 95°C for 10 min, 30 three-step cycles: 95°C for 1 min, 57°C for 30s, and 72°C for 40s, and the finishing synthesis step at 72°C for 6 min. DNA of *Synechococcus* sp. BAC 98111 was used as a negative control.

Universal primers HepF and HepR [11] were used to identify the genes of hepatotoxin (microcystins and nodularins) synthesis. PCR was carried out under the following conditions: one cycle at 92°C for 2 min, 35 three-step cycles: 92°C for 20 s, 52°C for 30 s, and 72°C for 1 min except for the last cycle elongation step being 6 min longer. DNA of a toxic *Mycrocystis aeruginosa* CALU 972 strain, kindly provided by L.N. Voloshko (Komarov Botanical Institute, Saint-Petersburg), was used as a positive control, and DNA of *Synechococcus* sp. strain BAC 98111, as a negative control.

PCR products were analyzed by horizontal electrophoresis in 1% agarose gel using 0.5% TAE buffer (20 mM Tris–OH, 0.5 mM EDTA, 7.8 mM CH_3COOH, pH 7.6). The gels were stained with ethidium bromide. Phage λ DNA treated with *Pst*I restriction endonuclease (SibEnzim, Novosibirsk, Russia) were used as molecular weight markers. DNA bands were excised from the gel by a sterile scalpel and placed into microtubes.

PCR products were eluted by freezing–thawing with subsequent centrifugation. The products purity and concentration were analyzed in agarose gel. Nucleotide sequences were determined on a Beckman CEQ^{tm} 8800 instrument (BeckmanCoulter, Inc., United States). The Chromas software package was used to process the obtained chromatograms (www.technelysium.com.au/chromas.html). Nucleotide sequences were compared to the GenBank, EMBL, and DDBJ data banks using the BLAST program for high homology sequences search

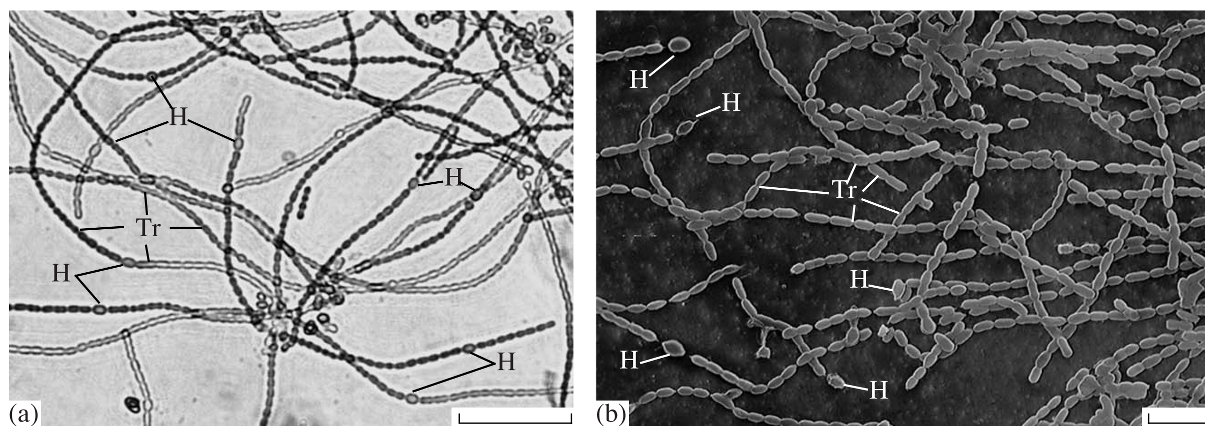

Fig. 1. Light (a) and scanning electron micrographs (b) of *T. variabilis* BAC 9610; Tr, trichomes and H, heterocysts. Scale bar is 50 μm (a) and 100 μm (b).

(www.ncbi.nlm.nih.gov/BLAST). For phylogenetic analysis, the sequences were aligned with those from the databases. Phylogenetic trees were constructed according to Kimura's two-parameter model by the neighbor-joining method using the MEGA software, version 3.1 [12]. Bootstrap analysis was used to evaluate confidence limits of branching. The sequences obtained were deposited in the data base under the record numbers, EU031807 and EU031808.

RESULTS AND DISCUSSION

A culture of filamentous cyanobacteria BAC 9610, was isolated from the Lake Baikal pelagial. It formed blue-green mucous colonies on agarized media. Cells viewed under the light microscope formed elongated, curved trichomes, with deep constrictions, slightly narrowing towards the ends, without sheaths, and with heterocysts (Fig. 1a). Vegetative cells of the strain were of ellipsoid shape, 3.92 μm wide (SD = 0.52, $n = 50$) and 5.75 μm long (SD = 0.94, $n = 50$) according to the light microscopy data. Heterocysts of immature trichomes had terminal locations, while those of mature trichomes, have intercalary localization. Completely formed heterocysts are of ellipsoid shape with a diameter of 4.97 μm (SD = 0.54, $n = 25$). When examined by epifluorescence microscopy under the green filter (545 nm wavelength), vegetative cells emitted a bright orange-red fluorescence owing to phycobilins (additional photosynthesis pigments), while mature heterocysts lacked autofluorescence due to the absence of these pigments.

On the basis of morphological characteristics, the isolated strain was identified as *Anabaena variabilis* Kütz in accordance with traditional botanical classification [13]. *A. variabilis* is a freshwater cosmopolitan species capable of nitrogen fixation first described in 1888 [14] and has been thoroughly studied. According to Gollerbakh [13], the genus *Anabaena* belongs to the family *Anabaenaceae*, order *Nostocales.* Other presently accepted classifications assign this genus to the order *Nostocales*, family *Nostocaceae* [15] and IV.I subgroup in bacteriological classification [16]. The akinete position, shape of terminal cells, and width of vegetative cells are important taxonomic characteristics of the genus *Anabaena* [17]. These morphological traits are preserved under various light and temperature regimes, and even in salty water. *A. variabilis* is characterized by high morphological variability and exists in several forms. The description of the isolated strain fits into the species diagnosis limits; however, the relationship between the dimensions of heterocysts and vegetative cells doesn't match any of the five forms listed in the manual. Thus, BAC 9610 may be a representative of a novel form of the species.

Within the modern, comprehensive classification, combining botanical and bacteriological approaches, the genus *Trichormus* was segregated from the genus *Anabaena* within subfamily *Nostocoidae*. From this new point of view, *A. variabilis, A. azollae* Strasb., *A. doliolum* Bharadw., and a number of other species belong to the genus *Trichormus* [15]. Thus, the names *A. variabilis* and *T. variabilis* (Kütz.) Kom. *et* Anagn. are considered synonyms. Transfer of the species *A. variabilis* to a new genus was supported by the characteristics of akinete formation. The genus *Trichormus* is characterized by akinete formation from vegetative cells in the middle, between two heterocysts. In the cells of *Anabaena*, akinetes are formed in close proximity to heterocysts (right beside or several cells apart from them); their growth is directed away from heterocysts. No akinetes were found close to heterocysts in strain BAC 9610, which allows us to assign it to the genus *Trichormus*, which had not been previously revealed in Baikal.

SEM examination data showed the strain to have trichomes consisting of ellipsoidal cells with rounded ends, and well distinguished constrictions between divided cells. The cell surface is smooth, without any additional structures (Fig. 1b). In contrast to the vege-

tative cells, the heterocyst surface is well structured and is surrounded by a thick mucus with distinct boundaries.

TEM allowed examination of the ultrathin structure of *T. variabilis* vegetative cells and heterocysts at various developmental stages. Trichomes containing mature, separated cells and young, recently divided cells, still in close pairs, are presented in Fig. 2a. Young intercalary cells are of a wide-ellipsoid shape and are 2.5 μm in diameter; the mucus envelope is thin and practically invisible. Young terminal cells are typically elongated ellipsoids, 1.5 μm in diameter (Fig. 2c). A thicker mucous envelope surrounds the old cells (Fig. 2d). The cell wall consists of four layers, as is typical of cyanobacteria, and is located close to the cytoplasmic membrane. The overall view of the cell is presented in Fig. 2b. The nucleoid region is well distinguished in the center of the cell. The photosynthetic apparatus consisting of a system of thylakoid membranes in young cells is usually located on the periphery of the cell, or penetrates irregularly through the whole cell randomly (Figs. 2c and d).

The intrathylakoid space is not augmented and is filled with an electron-dense matter; phycobilisomes and polysaccharide granules can be seen in the interthylakoid stroma. Ribosomes mainly reside close to the nucleoid region, cyanophycin granules are found in various regions except for the nucleoplasm (Figs. 2b and c). Polyhedral bodies were observed in mature cells. Polyphosphate inclusions usually occurred as cavities, formed in the course of sectioning (Fig. 2a and b). Most of the cyanophycin granules, which represent inclusions of an arginine–asparaginate copolymer and serve as a nitrogen reservoir, were found in heterocysts (Fig. 2f). The largest amount of cyanophycin granules, was previously reported after the nitrogenase activity peak [18].

Heterocyst formation was observed in *T. variabilis* cultures grown in a nitrogen-free media; the stages of this process were investigated using ultrathin sections (Figs. 3a–d). Heterocysts were coated by a typical envelope of a complex structure, consisting of three layers, fibrillar, homogeneous, and lamellar, preventing oxygen penetration from the outside. Immature heterocysts are of a more round shape, smaller, and with a thylakoid structure close to that of vegetative cells (Fig. 3a). In more mature heterocysts, thylakoid outgrowth is observed and an electron-transparent layer appears in the interthylakoid space. Further development is accompanied by interthylakoid space augmentation, and by thylakoid deformation and fragmentation (Figs. 3b–d). Typically, large lipid droplets are observed among the inclusions at every developmental stage. Aggregation of the thylakoid membranes is noticed around the pore as presented in Fig. 3b. Here the thylakoids are partially disintegrated and an electron-thick matter is formed, which later fills the pore and a part of the basal space as a fairly large homogeneous body (Fig. 3b). A mature terminal heterocyst is presented in Fig. 3c. An intercalary heterocyst between two vegetative cells is shown in Fig. 3d; here, the third lamellar layer forming after the fibrillar and the homogeneous layers, is seen distinctly in the polar zones of the heterocyst.

Therefore, according to the TEM data, the *T. variabilis* BAC 9610 cell and the heterocyst structure is similar to the *A. variabilis* (= *T. variabilis*) ultrathin structure, described previously in a number of works [19–21].

Culture growth curves in various media are presented in Fig. 4. *T. variabilis* BAC 9610 and *Synechococcus* sp. The BAC 98111 control strain grew well in a Z8 mineral medium; doubling of cell numbers of the strain under study was observed after three days of cultivation, while *Synechococcus* sp. culture transferred to the logarithmic growth phase on the ninth day. When grown in a nitrate-free Z8 medium, *T. variabilis* BAC 9610 culture passed rapidly into the logarithmic phase, which was accompanied by a significant increase in heterocysts numbers, up to 5–6 per one filament. The control strain didn't exhibit the ability to grow in a medium lacking bound nitrogen. The ability to grow in a nitrate-free medium demonstrates that *T. variabilis* BAC 9610 is a nitrogen-fixing strain and implies the presence of functioning genes of the nitrogenase complex.

Being a component of the Lake Baikal phytoplankton, the strain under study is probably contributing to the water enrichment with nitrogen. The peak of autotrophic picoplankton development occurs in summer, and nitrate concentration in the epilimnion decreases considerably, sometimes below detection limits, by July–August [6, 22]. Shortage of nitrates, the major form of mineral nitrogen in the Baikal pelagial, and the maximum season temperature favor development of nitrogen-fixing cyanobacteria in the lake, as it has been pointed out previously by Popovskaya [5]. During the summer months, *A. lemmermannii* P. Richter is the most actively vegetating species, generating wide blooming fields, up to several kilometers long [23]. *A. lemmermanii* concentration in such fields may reach $50–100 \times 10^6$ cells/l. In the lake's shallow water, mass development of *A. scheremetievi* Elenk., *A. flos-aquae* (Lyngb.) Breb., *A. spiroides* Kleb., and *A. hassalii* (Kütz) Wittr. occurs. According to the modern classification [15], these species are not moved to the genus *Trichormus* and belong to the genus *Anabaena*.

To confirm the taxonomic position of the strain BAC 9610, a nucleotide sequence analysis of a 16S rRNA gene fragment, 618 bp long, was performed. Comparative analysis of the obtained sequence revealed a 98% homology with *A. variabilis* (which is presently classified as *T. variabilis*) and with uncultured bacteria from the database. These are nitrogen-fixing strains *A. variabilis* 0441 (DQ408368), *A. variabilis* ATCC 29413 (AY768387), *A. variabilis* KCTC AG10273 (DQ234828), *A. variabilis*

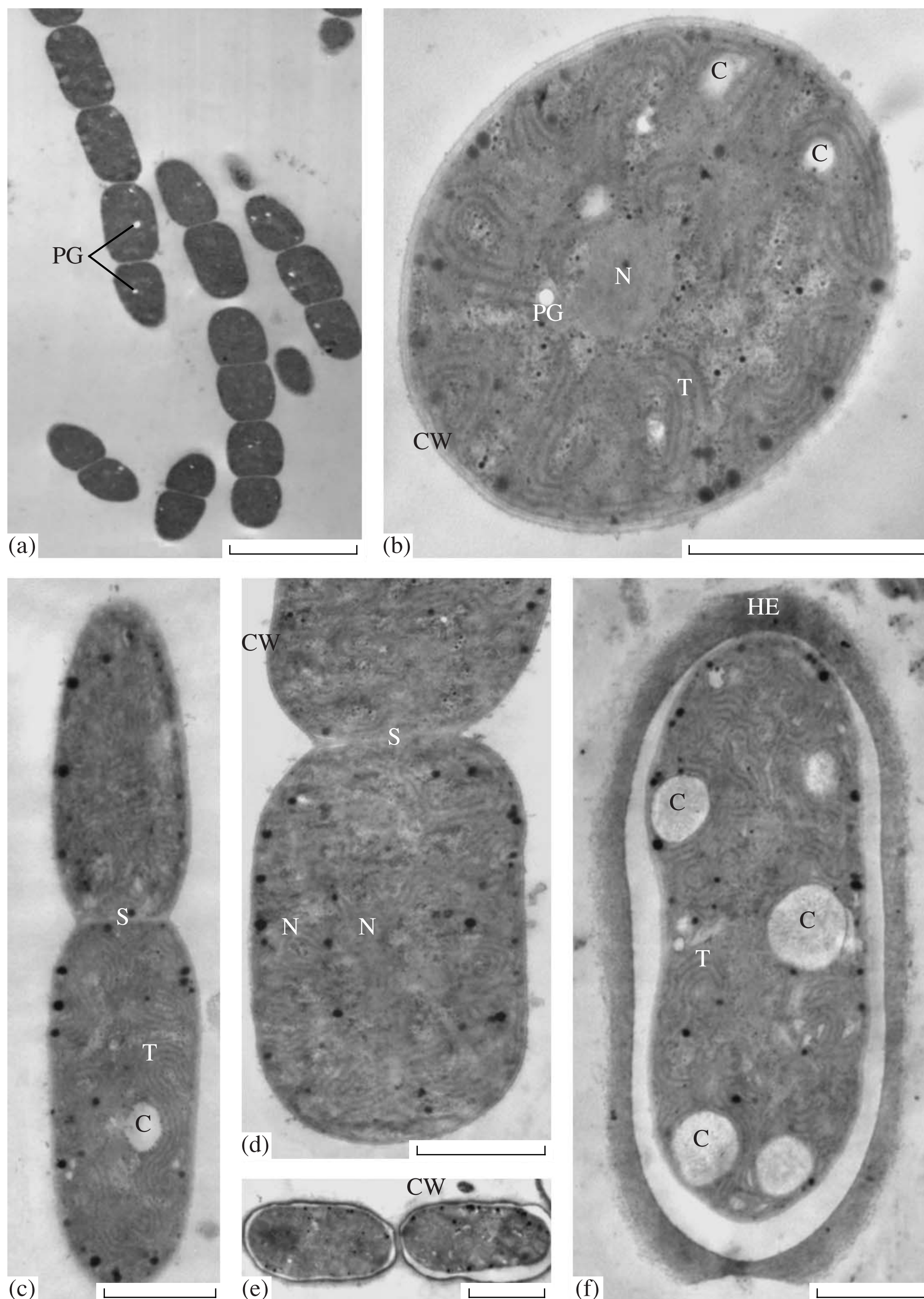

Fig. 2. Ultrastructure of *T. variabilis* BAC 9610 vegetative cells and heterocyst according to TEM of ultrathin lengthwise (a, c–f) and cross sections (b): a, general view of the trichomes; b, general view of a mature cell; c, young terminal cells; d, mature cells; e, old cells; f, heterocyst with large cyanophycin granules. CW, cell wall; N, nucleoid; PG, polyphosphate granules; T, thylakoids; C, cyanophycin granules; HE, heterocyst envelope; S, septum. Scale bar is 5 μm (a), 1 μm (b–d, f), and 2 μm (e).

KCTC AG10059 (DQ234826), *A. variabilis* KCTC AG10064 (DQ234827), and *A. variabilis* IAM M-204 (AB74502), isolated from freshwater basins, and uncultured bacterial clones SILK50 (EF467481) and SILK9 (EF467518) isolated from the biofilms of sulfide caves. Eight one-letter replacements were revealed in the gene

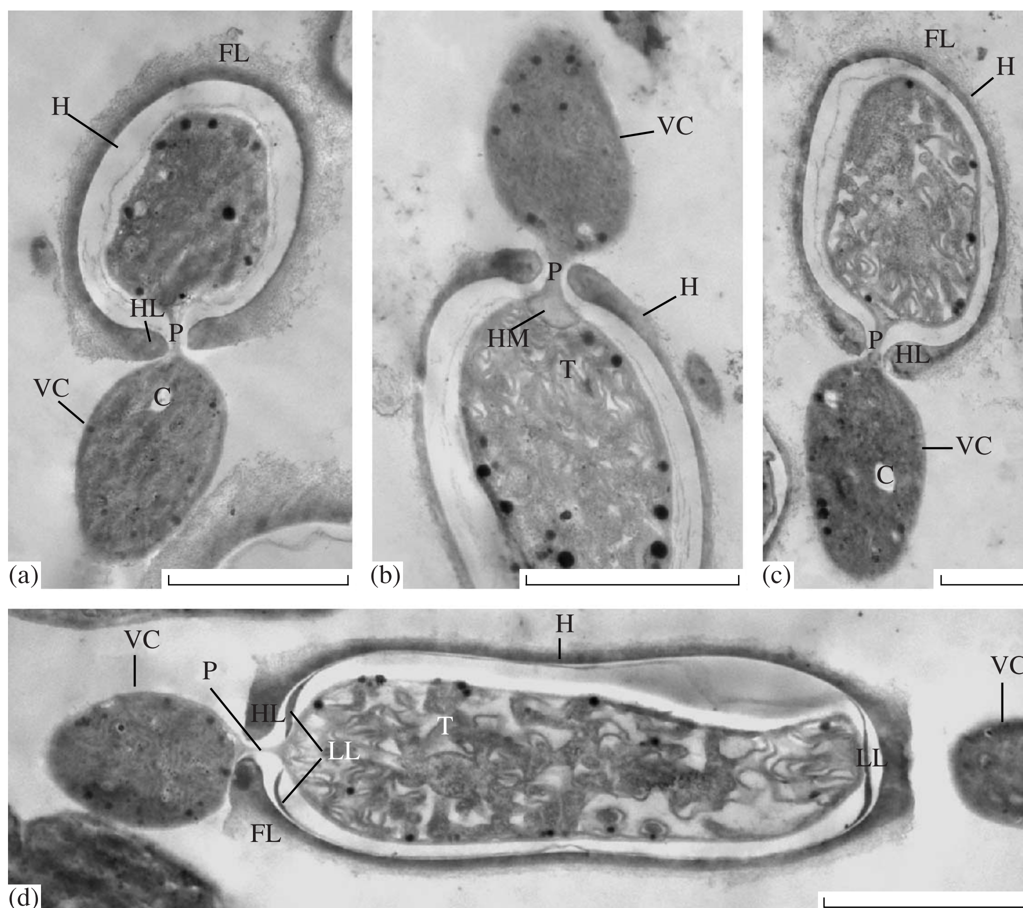

Fig. 3. Ultrastructure of heterocysts at various developmental stages according to the TEM of ultrathin sections: a, rounded heterocyst with unchanged thylakoid structure; b, thylakoid aggregation near the pore and generation of the homogeneous matter in the pore; c, heterocyst with disintegrated thylakoids; d, mature heterocyst with a three-layer envelope surrounded by two vegetative cells. VC, vegetative cell; H, heterocyst; P, pore; HM, homogeneous matter; T, thylakoids; C, cyanophycin granules; HL, homogeneous layer of the heterocyst envelope; FL, fibrillar layer of the heterocyst envelope; LL, lamellar layer of the heterocyst envelope. Scale: 2 μm (a, b, d) and 1 μm (c).

fragment of strain BAC 9610, in comparison with its closest relatives.

Results of the phylogenetic analysis, on the basis of the 16S rRNA gene sequence is presented in Fig. 5. Strain BAC 9610 belongs to the freshwater *A. variabilis (T. variabilis)* strain cluster with a medium bootstrap support. The species *T. variabilis* is polyphyletic, forming two clusters on the tree. *T. variabilis* strains are well distinguished from the *Anabaena* species and the *Nostoc* generic group.

Therefore, the results of the16S rRNA gene fragment sequencing agree with the taxonomic identification of the strain based on its morphological characteristics. Undoubtedly, the isolated strain belongs to the species *T. variabilis.*

Experimentally demonstrated, the nitrogen-fixing capacity of *T. variabilis* BAC 9610 was confirmed by identification of the nitrogenase complex genes (*nif*H). A 359-bp long fragment was obtained on amplification.

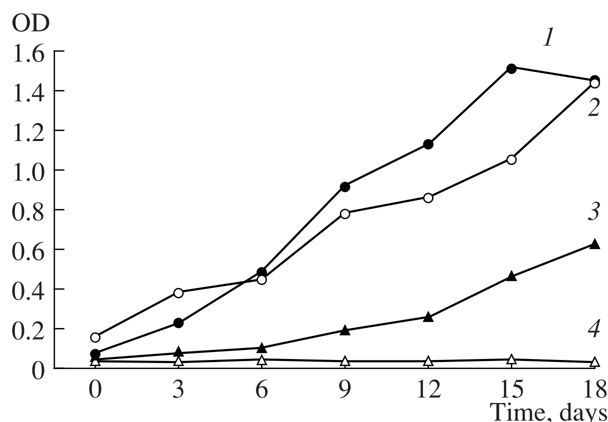

Fig. 4. Growth curves of strains *T. variabilis* BAC 9610 and *Synechococcus* sp. BAC 98111 on media Z8 and nitrate-free Z8: BAC 9610, Z8 (*1*); BAC 9610, nitrate-free Z8 (*2*); BAC 98111, Z8 (*3*); BAC 98111, nitrate-free Z8 (*4*).

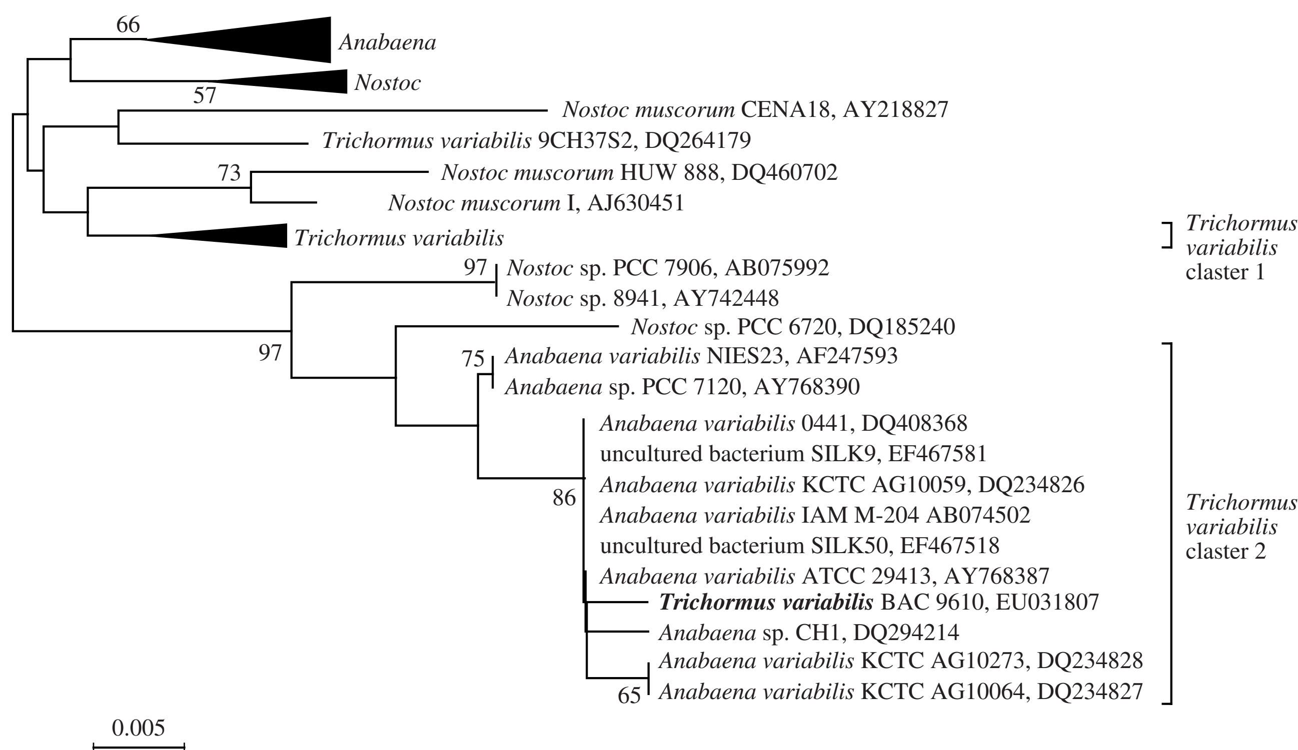

Fig. 5. Phylogenetic position of strain *T. variabilis* BAC 9610 determined on the basis of the 16S rRNA gene sequence analysis using the neighbor-joining method. Evolution distances are determined using Kimura's model. Bootstrap analysis results are marked by figures. The scale corresponds to 5 nucleotide replacements per 1000 nucleotides.

Direct sequence analysis and BLAST analysis revealed a 96% homology with nitrogen-fixing cyanobacteria *Anabaena* sp. CH1 (DQ294216) isolated from a Chinese rice field. A freshwater strain *A. variabilis* ATCC 29413 (CP000117), a soil strain *Nostoc* sp. PCC 6720 (V01428), and a lichen symbiont *A. azollae* (L34879) were also found to be closely related. Exact phylogenetic identification of the species according to the *nif*H gene sequence analysis was not possible owing to the low number of sequences present in the world database.

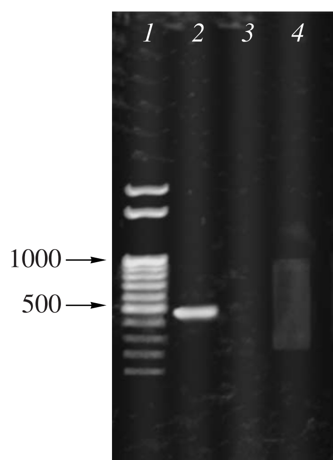

Fig. 6. Gel electrophoresis of the *mcy*E gene after PCR amplification: DNA marker (*1*); *Microcystis aeruginosa* CALU 972 (*2*), *Synechococcus* sp. BAC 98111 (*3*); and *Trichormus variabilis* BAC 9610 (*4*).

In an amplification test with a pair of primers HepF–HepR in order to identify the genes of hepatotoxins synthesis, the strain under study displayed a negative response (Fig. 6), which means *T. variabilis* is a nontoxic species and its mass development in Lake Baikal is of no danger.

Thus, a representative of a new genus of Baikal organisms, *Trichormus*, was isolated and characterized. Based on light and electron microscopy data and 16S rRNA gene sequence analysis, the strain was identified as *T. variabilis.* Strain BAC 9610 is nontoxic and nitrogen-fixing, as it contains genes of nitrogenase complex.

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