ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 3, pp. 365–372. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © E.G. Sorokovikova, I.V. Tikhonova, O.I. Belykh, I.V. Klimenkov, E.V. Likhoshwai, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 3, pp. 412–420.

EXPERIMENTAL ARTICLES

Identification of Two Cyanobacterial Strains Isolated from the Kotel'nikovskii Hot Spring of the Baikal Rift

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Received February 1, 2007

Abstract—Two cyanobacterial strains, *Pseudanabaena* sp. 0411 and *Synechococcus* sp. 0431, were isolated from a sample collected in the Kotel'nikovskii hot spring of the Baikal rift. According to the results of light and transmission electron microscopy, as well as of the phylogenetic analysis of the 16S rRNA gene, these cyanobacteria were classified as *Pseudanabaena* sp. nov. and *Synechococcus bigranulatus* Skuja. The constructed phylogenetic tree shows that the studied strains are positioned in the clades of cyanobacteria isolated from hydrothermal vents of Asia and New Zealand, separately from marine and freshwater members of these genera, including those isolated from Lake Baikal.

Key words: cyanobacteria, hot springs, *Synechococcus, Pseudanabaena*, ultrastructure, 16S rRNA gene.

DOI: 10.1134/S002626170803017X

Cyanobacteria (or blue-green algae), whose estimated age is 3.5 billion years, are the most ancient organism on Earth [11]. They inhabit various ecological niches, including marine and freshwater ones, as well as Arctic and Antarctic ice [2]. Mineral hot springs, with physicochemical conditions similar to ancient ones, are one of the habitats of contemporary cyanobacteria. In hot springs, cyanobacterial mats consist of members of several cyanobacterial genera (mainly *Phormidium*, *Oscillatoria*, *Calothrix*, *Fischerella*, and *Microcoleus*) [3]. At the same time, each of these genera consists of species with different ecological properties. For example, both *Pseudanabaena* and *Synechococcus* include marine and freshwater thermo-, meso-, and psychrophilic cyanobacteria [4, 5]. Members of the genus *Synechococcus* are predominant in the Baikal picoplankton community [6], from which a total of 24 cultivable strains belonging to this genus have been isolated, which differ both in morphology and in 16S rRNA gene sequences [7].

The Kotel'nikovskii hot spring is located in the immediate vicinity of the northwestern shore of Lake Baikal, in the northern part of the Kotel'nikovskii Cape. Its water (pH 9.5) is of the fluoride–bicarbonate–sodium sulfate type, moderately mineralized (320 mg/l), with a high concentration of silicic acid (196 mg/l); the highest water temperature is 80°C [8, 9].

For determination of the species composition of cyanobacterial communities, light microscopy is insufficient, since the morphological properties often overlap or vary within a wide range. From the taxonomic point of view [10], precise identification of cyanobacteria requires data on their ultrastructural characteristics, such as the distribution of thylakoids and the cell wall structure, which, for a long time, have been used as important diagnostic indicators. Taxonomic studies of cyanobacteria carried out with consideration of the data on the cell ultrastructure correspond well with phylogenetic studies based upon the 16S rDNA gene sequences; the new comprehensive taxonomic classification of cyanobacteria is based primarily on molecular biological data [11].

Cyanobacteria belonging to the genera *Oscillatoria* Vaucher ex Gom., *Phormidium* Kütz. ex Gom., *Gloeocapsa* Kütz., and *Synechococcus* Näg. were previously isolated from the phototrophic communities of the Kotel'nikovskii hot spring and investigated by light microscopy [12].

In this work, we determined the taxonomic position of two cyanobacterial strains by light and transmission electron microscopy and carried out a phylogenetic analysis of their 16S rRNA gene sequences.

MATERIALS AND METHODS

Sampling and cultivation. Samples of cyanobacterial mats were collected in October, 2003. An enrichment culture of cyanobacteria was obtained in Z-8 liquid mineral medium [13] in a thermostat at 36°C in the daylight. The strains *Pseudanabaena* sp. 0411 and *Synechococcus* sp. 0431 were isolated and purified by subculturing in Z-8 medium with 1% agar.

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Light microscopy. To study the morphology of the cyanobacterial cells, the specimens were examined under a Zeiss Axiovert 200 light microscope (Germany). Microphotographs were obtained with a Pixera Penguin 600CL camera (DiRactor™) using the VideoTesT-Size 5.0 software package (www.videotest.ru). The cells were measured using the Image-Pro Plus 4.0 software package (www.mediacy.com).

Transmission electron microscopy (TEM). The cell preparations were fixed with 1% glutaraldehyde and 0.25% OsO_4 in 0.2 M cacodylate buffer (pH 7.4) for 30 min. Then they were postfixed with 1% OsO_4 in the same buffer for 2 h and washed three times with 30% ethanol. After dehydration in ascending concentrations of ethanol, in a mixture containing 100% ethanol and 100% acetone (1 : 1), and then in 100% acetone, the preparations were imbued with an Epon–Araldite–acetone mixture, embedded in Epon–Araldite, and polymerized at 60°C for 48 h. Ultrathin sections were obtained using an Ultracut K microtome (Leica Microsystems) and mounted on palladium-coated nylon grids. The ultrathin sections were stained with a saturated uranyl acetate solution in 70% alcohol for 10 min. The grids were washed with distilled water and additionally stained with Reynold's lead citrate for 10 min. The sections were examined under a Leo 906E transmission electron microscope (Zeiss, Germany) at an accelerating voltage of 80 kV. Microphotographs were obtained with a MegaView II digital camera using the MegaVision software (Soft Imaging System GmbH, Germany).

Molecular biological studies. DNA was extracted from the cells according to an accepted technique, using lysozyme, sodium dodecyl sulfate, and proteinase K, with subsequent phenol extraction [14]. The 16S rRNA gene fragment was amplified using the cyano-specific primers 106F and 781R corresponding to *E. coli* positions 106–805 [15]. The polymerase chain reaction included template denaturation during the first cycle at 94°C for 5 min and 35 amplification cycles. The thermal program was 1 min at 94°C, 1 min at 55°C, and 1 min 10 s at 72°C. During the last cycle, elongation was extended to 10 min. The PCR product was assayed by electrophoresis using 1% agarose gel. Bands of about 700 bp were cut, and the obtained nucleotide material was eluted by the freezing–thawing technique.

The nucleotide sequences of the 16S rRNA gene fragment were obtained using a Beckman CEQ™ 8800 sequencer (Beckman Coulter Inc., United States). The obtained sequences were aligned using the BLAST software package (GenBank). The phylogenetic tree was constructed with the neighbor-joining method using the Kimura model [16] with the MEGA 3.1 software package [17]. The nucleotide sequences determined in this work were deposited in the GenBank database (accession numbers, DQ408369 and DQ408367).

RESULTS AND DISCUSSION

Morphology and ultrastructure. Strain *Pseudanabaena* sp. 0411 formed reddish-brown or, rarely, brownish-green films. Old cultures were brown-colored. When grown in liquid media, trichomes tightly stick together; however, their loose ends exhibit oscillatory and rotational motions. The cells reproduce by trichome division into small motile fragments, hormogonia. Trichomes consist of isodiametric cells (separated by septa) with the length sometimes slightly exceeding the width. The cell size varies from 1.6 to 2.0 μm in width and from 2.0 to 2.5 μm in length. When present, the sheaths enclosing the trichomes are thin (Fig. 1a). The results of transmission electron microscopy demonstrate that the trichomes are enclosed in 100–260-nm thick polysaccharide sheaths (Fig. 1b–1f). Terminal cells are rounded, lacking calyptras (Fig. 1c). The cell wall is 40 nm thick and consists of four layers (L1–L4), including the electron-transparent layer L1 located just outside the plasmalemma, the peptidoglycan layer L2, and the outer membrane L4 separated by the electron-transparent layer L3 (Fig. 1d). Protrusions that are apparently involved in the sheath material secretion were detected on the outer membrane (Fig. 1e). The photosynthetic apparatus consists of six to eight thylakoids located at the cell periphery and in parallel with each other; their transverse sections are ring-shaped. Outside the thylakoids, phycobilisomes were detected. These are specific structures 18 nm in diameter consisting of subunits that contain phycobilins, accessory photosynthetic pigments (Fig. 1c–1d). The nucleoid is located in the center of the cell. The cytoplasm contains some inclusions whose size varies within a wide range. Cyanophycin granules whose average size is 260×200 nm are localized at the cell ends. Polyphosphate granules (270×210 nm) were detected both in the center and at the periphery of the cells. At the cell periphery, osmiophilic lipid inclusions (about 100 nm in diameter) are arranged along the thylakoids. All cells in the trichome are capable of division; no meristematic zones have been detected. The cells divide by invagination of the cell wall, followed by the growth of both septum ends towards each other (Fig. 1f). Gas vacuoles were not detected. All the properties of the studied strain, such as the variability of the pigment composition, the round shape of end cells, the 40-nm thick cell wall with a thin outer membrane, the thin polysaccharide sheath, peripheral thylakoids, and the unique division process are characteristic traits of cyanobacteria belonging to the genus *Pseudanabaena* [10].

Strain *Synechococcus* sp. 0431 isolated by us shows good growth in liquid medium where it forms a light green suspension which later solidifies into a film. On agarized media, no well-defined dense colonies were detected; motile cells were arranged chaotically on the surface. According to the data obtained by light microscopy, rodlike or slightly curved cells of the studied strain had rounded ends; the size of mature cells varied

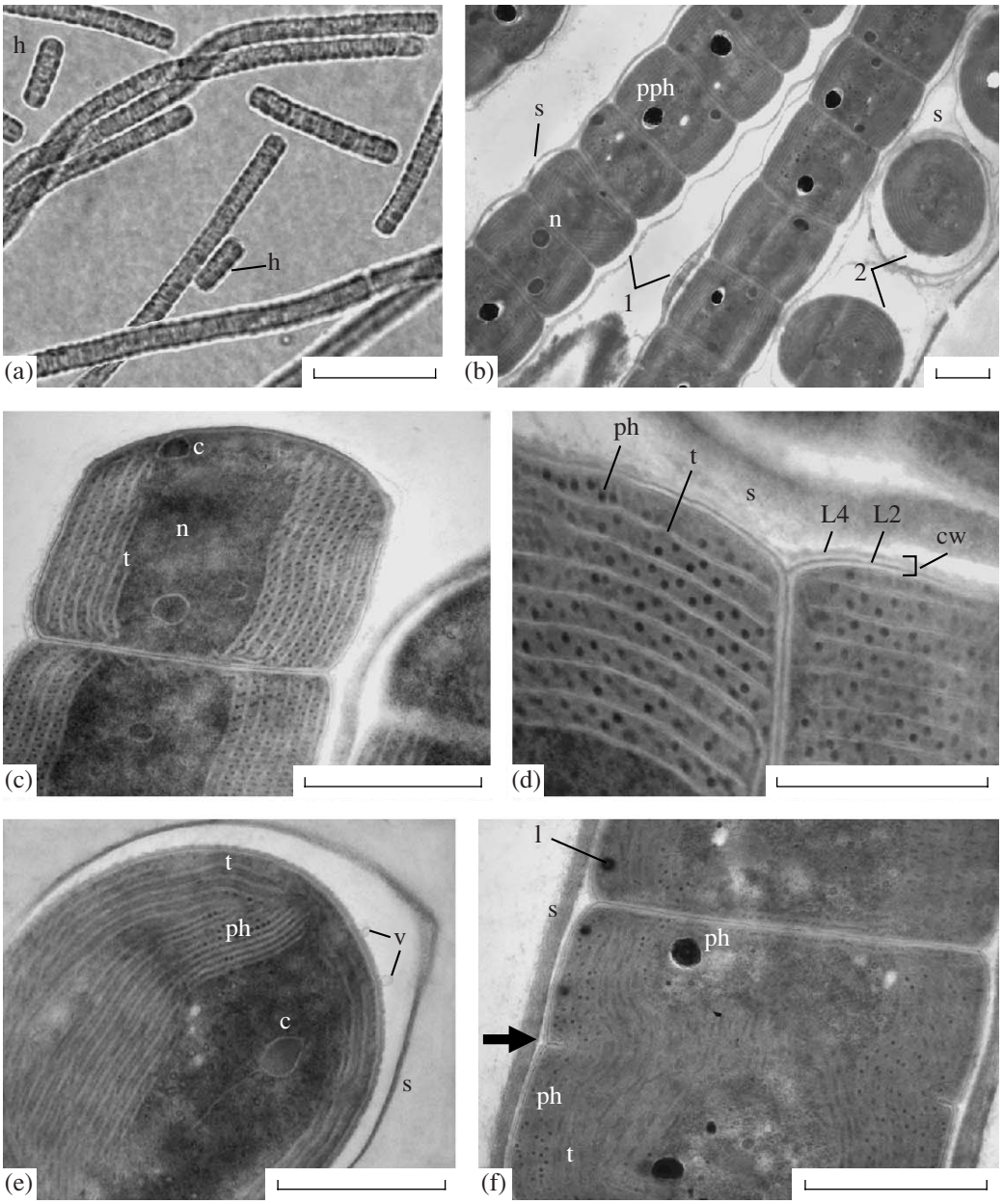

Fig. 1. Morphology and ultrastructure of *Pseudanabaena* sp. 0411 cells: (a) light microscopy; (b–f) TEM, ultrathin sections: (b) general view of trichomes: (1) longitudinal section and (2) cross-section; (c) trichome terminal cell; (d) structure of the photosynthetic apparatus and cell wall; (e) trichome cross-section; and (f) dividing cell. Designations: v, vesicles; h, hormogonia; cw, cell wall; l, lipid inclusions; n, nucleoid; pph, polyphosphate granules; t, thylakoids; ph, phycobilisomes; c, cyanophycin granules; s, sheath; L2, peptidoglycan layer of the cell wall; L4, outer membrane; an arrow points to a septum. Scale bars: (a) 10 μm; (b, c, e, and f) 1 μm; and (d) 500 nm.

from 1.5 to 2.0 μm in width and from 3.6 to 7.7 μm in length (Fig. 2a). The cells reproduce by binary division. At one end or both ends of all the studied cells, inclusions were detected, which, under a light microscope, look like pale areas due to their light-refracting properties (Fig. 2a). These bodies may be identified as large cyanophycin inclusions (390 × 240 nm on average), clearly seen in ultrathin sections (Fig. 2b). According to the data obtained by transmission electron microscopy, the cell wall consists of four layers; the outer membrane is undulating and loosely attached to the peptidoglycan layer. The cell contains three parallel peripheral thylakoids whose transverse sections are ring-shaped. A pale nucleoid area is clearly seen in the cytoplasm; polyhedral inclusions (120–220 nm in diameter) and polyphosphate granules (60 nm) were detected in the area between the nucleoid and thylakoids. Lipid inclusions (30–55 nm) were detected at the cell periphery

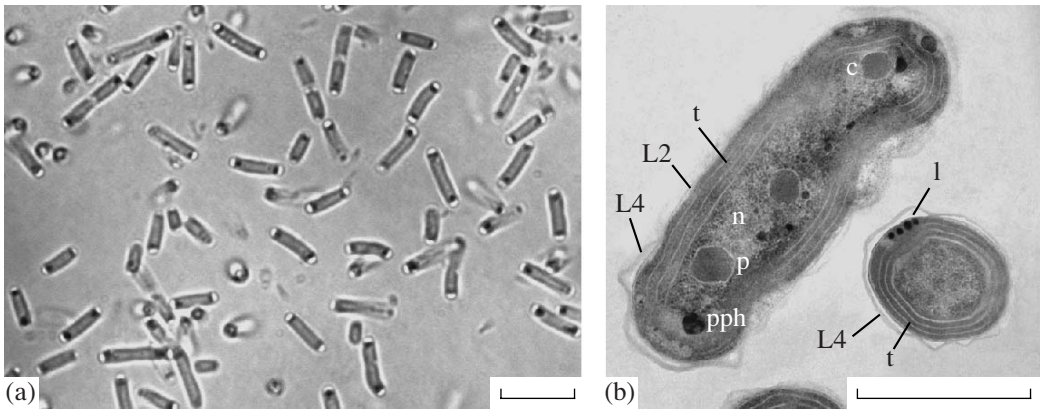

Fig. 2. Morphology and ultrastructure of *Synechococcus bigranulatus* 0431 cells: (a) light microscopy; (b) TEM, ultrathin sections. Designations: l, lipid inclusions; n, nucleoid; p, polyhedral inclusions; pph, polyphosphate granules; t, thylakoids; c, cyanophycin granules; L2, peptidoglycan layer of the cell wall; and L4, outer membrane. Scale bars: (a) 10 μm and (b) 1 μm.

(Fig. 2b). The mode of cell division of the studied strain and the arrangement of thylakoids are typical of the representatives of the genus *Synechococcus*. The cell size and the presence of specific polar inclusions and confinement to thermal habitats coincide with the taxonomic features of the species *S. bigranulatus* Skuja and thus indicate the affinity of strain *Synechococcus* sp. 0431 to this species [4].

Hence, the results of light and transmission electron microscopy indicate that the cell structures of the studied strains have well-pronounced genus- and species-specific characteristics.

Phylogenetic analysis. As a result of the amplification and sequencing of 16S rDNA gene fragments, partial nucleotide sequences, 640 bp for *Pseudanabaena* sp. 0411 and 430 bp for *S. bigranulatus* 0431, were determined.

The sequence of strain *Pseudanabaena* sp. 0411 showed high homology (99%) with the sequences of strains 19-2 (AF317504) and 11-3 (AF317503) isolated from the hot springs of New Zealand [18] and classified as *Oscillatoria amphigranulata* Van Goor=*Pseudanabaena amphigranulata* (Van Goor) Anagn. Comparative analysis of the determined nucleotide sequences revealed that, within the Gen-Bank database, only uncultivable cyanobacteria whose sequences were determined by molecular biological techniques were the closest relatives of *S. bigranulatus* 0431. The highest homology level (98%) was with strain DQ001391 isolated from the hot springs of Central Tibet, China [19] and strains AY787616 and AY787607 isolated from the hot springs of Thailand [20].

Phylogenetic analysis revealed that the genera *Pseudanabaena* and *Synechococcus* are polygenetic and form two and four clusters, respectively (Fig. 3 and 4). Strain *Pseudanabaena* sp. 0411 fell into a cluster with *P. amphigranulata* [18] with strong bootstrap support (100%) (Fig. 3). The resultant cluster (*Pseudanabaena* I) consists mainly of extremophilic cyanobacteria (thermophiles from hot springs and psychrophiles from Antarctic lakes). Bacteria with interesting properties were detected among the thermophilic representatives of *Pseudanabaena*. It is the authors' opinion that the cyanobacteria AB179527 and AY874091 whose sequences were determined by molecular analyses of silicon tuffs from the hot springs of Japan and Taiwan can be involved in silicon biomineralization and tuff formation [21]. During our laboratory experiment, we demonstrated that strain *Pseudanabaena* sp. 0411 was capable of precipitating silicon [22]. The cyanobacterial strains isolated from marine and freshwater ecosystems fell into the *Pseudanabaena* II cluster independent of extremophilic *Pseudanabaena* spp.

The high similarity level between the nucleotide sequences of strains *Pseudanabaena* sp. 0411 and *P. amphigranulata* 19-2 [18] was not sufficient to identify the studied strain as a representative of the latter species. According to the ecological characterization of the species *P. amphigranulata*, it does not inhabit hot springs; such descriptions are therefore incorrect [5]. Besides, while the presence of gas vacuoles arranged as polar aerotopes is a characteristic morphological trait of the above-mentioned species, these are not present in strain *Pseudanabaena* sp. 0411. *P. minima* (G.S. An) Anagn. and *P. thermalis* Anagn. are other species with cell sizes, shape of the terminal cells in the trichome, and habitats similar to those of the studied strain [5]. However, unlike *P. minima, Pseudanabaena* sp. 0411 lacks granular cell contents and pronounced septa within the trichome without thickened mid-cell septa. The lack of polar aerotopes and hyaline bridges between cells does not permit classification the studied strain as *P. thermalis.* The brownish-red color of *Pseudanabaena* sp. 0411 trichomes also differentiates it from the above-mentioned species forming blue-green colonies. The results of detailed analysis of the taxonomic properties indicate that strain *Pseudanabaena* sp. 0411 differs considerably from its close relatives

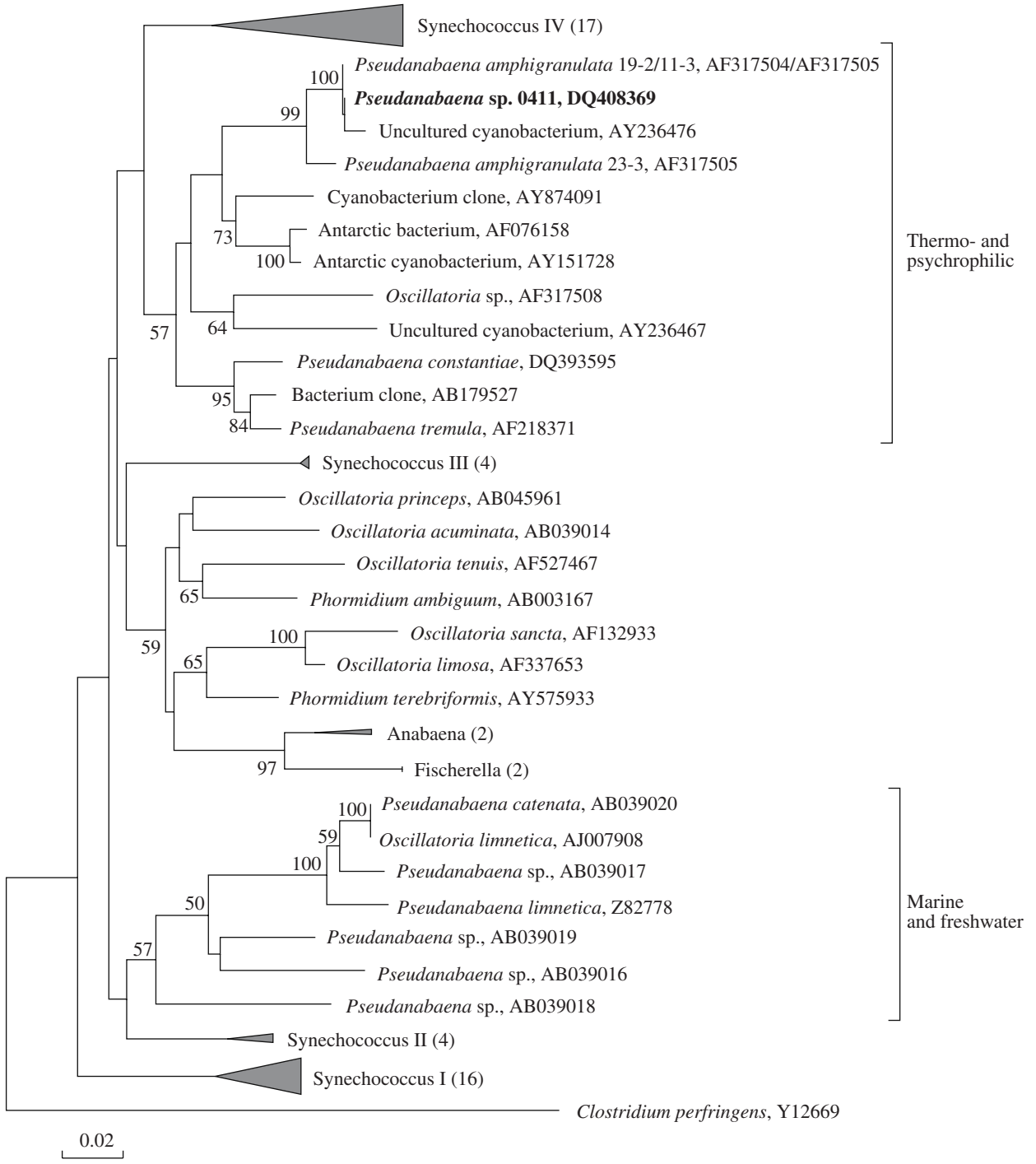

Fig. 3. Rooted phylogenetic tree of cyanobacteria of the genus *Pseudanabaena* based on 16S rDNA sequences and constructed using the neighbor-joining method. Evolutionary distances were calculated using the Kimura model. The numerals show the results of the bootstrap analysis (only bootstrap values above 50% were considered significant). The sequence in bold was determined in this work. The numbers of sequences that fell in the relevant cluster are given in parentheses. Scale bar, 2 nucleotide substitutions per 100 nucleotides.

within the genus *Pseudanabaena*; therefore can it be described as a new species.

The polyphyletic nature of the genus *Synechococcus* is well known [23]. According to the phylogenetic analysis, species *Synechococcus* spp. include four clusters consisting of the representatives of specific ecological groups (Fig. 4). The isolated strain *Synechococcus bigranulatus* 0431 and the uncultivable cyanobacterial clones from the hot springs of China and Thailand fell into a clade within cluster I which differs from *Syn-*

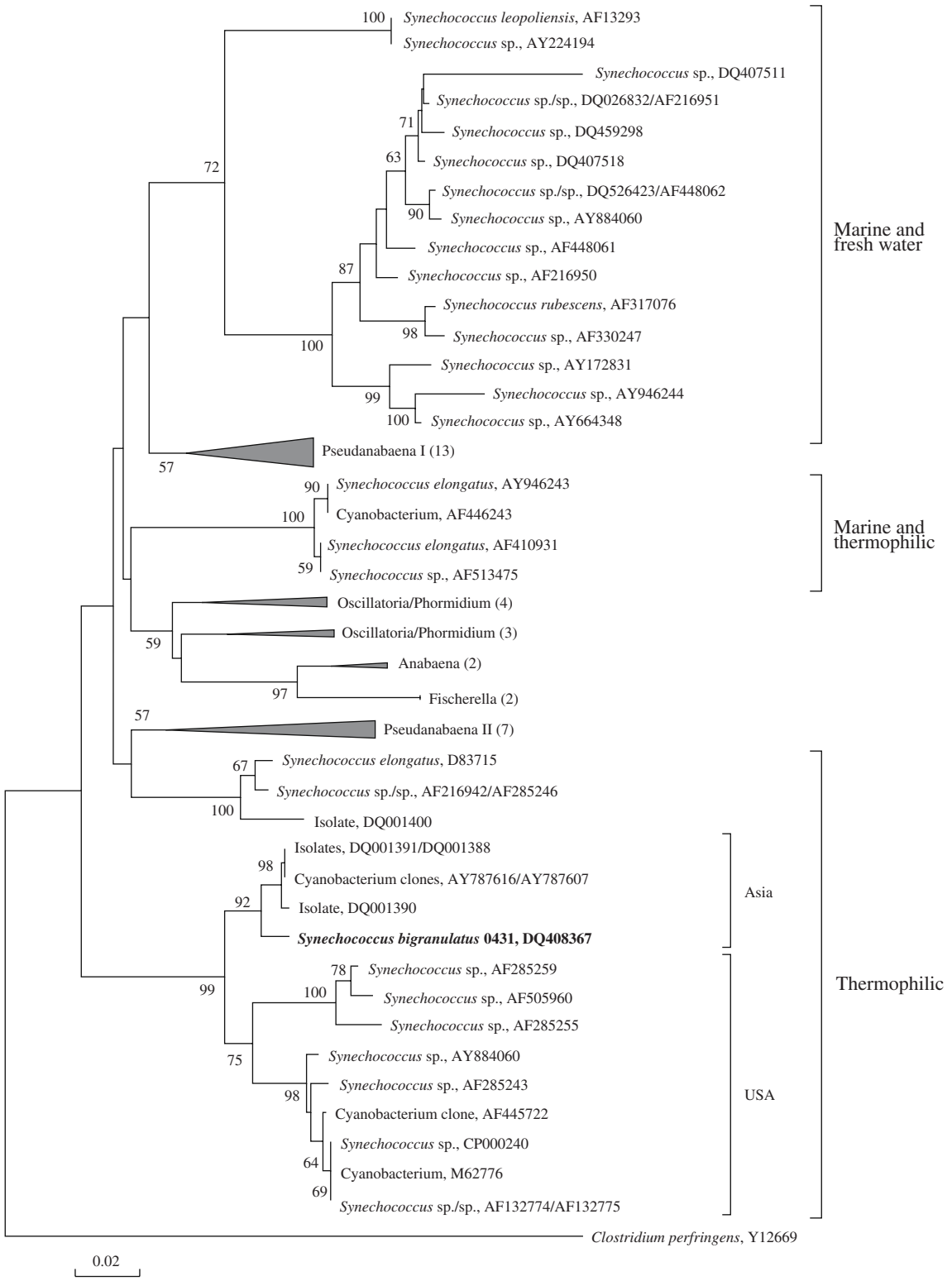

Fig. 4. Rooted phylogenetic tree of cyanobacteria of the genus *Synechococcus* based on 16S rDNA sequences and constructed using the neighbor-joining method. Evolutionary distances were calculated using the Kimura model. The numerals show the results of the bootstrap analysis (only bootstrap values above 50% were considered significant). The sequence in bold was determined in this work. The numbers of sequences that fell in the relevant cluster are given in parentheses. Scale bar, 2 nucleotide substitutions per 100 nucleotides.

echococcus isolates obtained from the hydrothermal vents of the United States with 99% bootstrap support. It should be noted that the *Synechococcus* spp. strains (DQ407511, DQ407518, and DQ459298) isolated previously from Lake Baikal [6] fall into the cluster together with freshwater *Synechococcus* species and are not closely related to the strain in question that was isolated from the hot spring on the lake shore. According to the published data, *S. bigranulatus* was found in hot springs ((20)–50–76°C) all over the world; it is known as "thermophilic *S. elongatus*," a model laboratory strain used for physiological and ecological experiments [4]. It was found that, within its cluster, the obtained nucleotide sequence of *S. bigranulatus* is the sole sequence of a cultivable microorganism, which makes it possible to use it for subsequent genetic experiments with the thermophilic representatives of the genus *Synechococcus*.

Hence, application of light and transmission electron microscopy as well as molecular techniques allowed us to determine the taxonomic position of the strains isolated from the Kotel'nikovskii hot spring. As a result, strain *Pseudanabaena* sp. 0411 is to be classified as a new species of the genus *Pseudanabaena*; strain *Synechococcus* sp. 0431 was affiliated with the species *S. bigranulatus*.

ACKNOWLEDGMENTS

The authors thank R.V. Adel'shin for the determination of nucleotide sequences and N.L. Bel'kova for discussing the results.

This work was supported by the Presidium of the Russian Academy of Sciences (grant no. 18.4) and by the Russian Science Support Foundation (grant for young scientists).

REFERENCES

1. *Bakterial'naya paleontologiya* (Bacterial Paleontology) Rozanov, Yu., Ed., Moscow: Paleontol. Inst. RAN, 2002.
2. Kondrat'eva, E.N., Maksimova, I.V., and Samuilov, V.D., *Fototrofnye mikroorganizmy. Ucheb. posobie* (Phototrophic Microorganisms. Tutorial), Moscow: Mosk. Gos. Univ., 1989.
3. Sergeev, V.N., Gerasimenko, L.M., and Zavarzin, G.A., The Proterozoic History and Present State of Cyanobacteria, *Mikrobiologiya*, 2002, vol. 71, no. 6, pp. 725–740 [*Microbiology* (Engl. Transl.), vol. 71, no. 6, pp. 623–637].
4. Komárek, J. and Anagnostidis, K., Cyanoprokaryota—1. Teil: Chroococcales, in *Süβwasserflora von Mitteleuropa,* Ettl, H., Gerloff, J., Heynig, H., and Mollenhauer, D., Eds., Band 19/1, Gustav Fischer, 1999.
5. Komárek, J. and Anagnostidis, K., Cyanoprokaryota—1. Teil 2: Oscillatoriales, *in Süβwasserflora von Mitteleuropa*, Buedel, B., Krienitz, L., Gaertner, G., and Schagerl, M., Eds., Band 19/2, Heidelberg: Elsevier/Spectrum, 2005.
6. Popovskaya, G.I. and Belykh, O.I., Stages of Investigation of the Lake Baikal Autotrophic Picoplankton, *Gidrobiol. Zhurnal,* 2003, vol. 39, pp. 12–24.
7. Tikhonova, I.V., Morphological and Genetic Properties of the Lake Baikal Picoplanktonic Cyanobacteria, *Extended Abstract of Cand. Sci. (Biol.) Dissertation*, Irkutsk: IGU, 2006.
8. *Baikal. Atlas* (Lake Baikal. An Atlas), Moscow: Roskartografiya, 1993, p. 160.
9. Pinneker, E.V., Kustov, Yu.I., Kalashnikova, E.B., and Pavlova, S.E., Geochemistry of Medicinal Mineral Waters of the Baikal Region, *Otechestvennaya Geologiya*, 1995, no. 12, pp. 42–48.
10. Anagnostidis, K. and Komárek, J., Modern Approach To the Classification System of Cyanophytes. 3—Oscillatoriales, *Arch. Hydrobiol.*, vol. 80, no. 1–4, pp. 327–472.
11. Hoffmann, L., Komárek, J., and Kaštovský, J., System of Cyanoprokaryotes (Cyanobacteria)—State in 2004, *Abstr. 16th Symp. Int. Ass. Cyanophyte Res.*, Luxemburg, 2004, p. 42.
12. Kompantseva, E.I. and Gorlenko, V.M., Phototrophic Communities in Some Thermal Springs of Lake Baikal, *Mikrobiologiya*, 1988, vol. 57, no. 5, pp. 841–846.
13. Rippka, R., Isolation and Purification of Cyanobacteria, in *Methods in Enzymology*, Packer, L. and Glazer, A.N., Eds., vol. 167, pp. 3–27.
14. Shmidt, T.M., DeLong, E.F., and Pace, N.R., Analysis of a Marine Picoplankton Community by 16S rRNA Gene Cloning and Sequencing, *J. Bacteriol.*, 1991, vol. 173, pp. 4371–4378.
15. Nübel, U., Garcia-Pichel, F., and Muyzer, G., PCR Primers To Amplify 16S rRNA Genes from Cyanobacteria, *Appl. Environ. Microbiol.*, 1997, vol. 63, pp. 3327–3332.
16. Kimura, M., A Simple Method for Estimating Evolutionary Rates of Base Substitution Through Comparative Studies of Nucleotides Sequences, *J. Molecular Evol.*, 1980, vol. 16, pp. 111–120.
17. Kumar, S., Tamura, K., and Nei, M., MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment, *Briefings in Bioinformatics,* 2004, vol. 5, pp. 150–163.
18. Miller, S.R. and Castenholz, R.W., Ecological Physiology of *Synechococcus* sp. Strain SH-94-5, a Naturally Occurring Cyanobacterium Deficient in Nitrate Assimilation, *Appl. Environ. Microbiol.*, 2001, vol. 67, pp. 3002–3009.
19. Lau, C.Y., Hongmei, J., Aitchison, J.C., and Pointing, S.B., Highly Diverse Community Structure in a Remote Central Tibetan Geothermal Spring Does Not Display Monotonic Variation to Thermal Stress, *FEMS Microbiol. Ecol.,* 2006, vol. 57, pp. 80–91.
20. Hongmei, J., Aitchison, J.C., Lacap, D.C., Peeraporn-pisal, Y., Sompong, U., and Pointing, S.B., Commu-

nity Phylogenetic Analysis of Moderately Thermophilic Cyanobacterial Mats from China, the Philippines and Thailand, *Extremophiles*, 2005, vol. 9, pp. 325–332.

21. Ng, C.C., Huang, W.C., Chang, C.C., Tzeng, W.S., Chen, T.W., Liu, Y.S., and Shyu, Y.T., Tufa Microbial Diversity Revealed by 16S rRNA Cloning in Taroko National Park, Taiwan, *Soil Biol. Biochem.*, 2006, vol. 38, pp. 342–348.
22. Likhoshwai, E.V., Sorokovikova, E.G., Bel'kova, N.L., Belykh, O.I., Titov, A.T., Sakirko, M.V., and Parfenova, V.V., Silicon Mineralization in the Culture of Cyanobacteria from Hot Springs, *Doklady AN*, 2006, vol. 407, no. 4, pp. 556–560 [*Doklady Biol. Sci.* (Engl. Transl.), vol. 407, pp. 201–204].
23. Schönhuber, W., Zarda, B., Eix, S., Rippka, R., Herdman, M., Ludwig, W., and Amann, R., In situ Identification of Cyanobacteria with Horseradish Peroxidase-Labeled, RRNA-Targeted Oligonucleotide Probes, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 1259–1267.