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EXPERIMENTAL ARTICLES

Anoxygenic Phototrophic Bacteria from Microbial Communities of Goryachinsk Thermal Spring (Baikal Area, Russia)

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Abstract—Species composition of anoxygenic phototrophic bacteria in microbial mats of the Goryachinsk thermal spring was investigated along the temperature gradient. The spring belonging to nitrogenous alkaline hydrotherms is located at the shore of Lake Baikal 188 km north-east from Ulan-Ude. The water is of the sulfate–sodium type, contains trace amounts of sulfide, and salinity does not exceed 0.64 g/L, pH 9.5. The temperature at the outlet of the spring may reach 54°C. The cultures of filamentous anoxygenic phototrophic bacteria, nonsulfur and sulfur purple bacteria, and aerobic anoxygenic phototrophic bacteria were identified using the *puf*LM molecular marker. The *fmo*A marker was used for identification of green sulfur bacteria. Filamentous cyanobacteria predominated in the mats, with anoxygenic phototrophs comprising a minor component of the phototrophic communities. Thermophilic bacteria *Chloroflexus aurantiacus* were detected in the samples from both the thermophilic and mesophilic mats. Cultures of nonsulfur purple bacteria similar to *Blastochloris sulfoviridis* and *Rhodomicrobium vannielii* were isolated from the mats developed at high (50.6–49.4°C) and low temperatures (45–20°C). Purple sulfur bacteria *Allochromatium* sp. and *Thiocapsa* sp., as well as green sulfur bacteria *Chlorobium* sp., were revealed in low-temperature mats. Truly thermophilic purple and green sulfur bacteria were not found in the spring. Anoxygenic phototrophic bacteria found in the spring were typical of the sulfur communities, for which the sulfur cycle is mandatory. The presence of aerobic bacteriochlorophyll *a*-containing bacteria identified as *Agrobacterium* (*Rhizobium*) *tumifaciens* in the mesophilic (20°C) mat is of interest.

Keywords: Goryachinsk, Baikal area, hydrotherms, anoxygenic phototrophic bacteria, *puf*LM, *fmo*A

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Thermal springs attract attention of microbiologists primarily as habitats of thermophilic microorganisms representing a variety of physiological and taxonomic groups. Microbial mats developed in spring beds, with cyanobacteria, unicellular algae, and anoxygenic phototrophic bacteria (APB) acting as producers, are considered the systems most resembling the ancient phototrophic communities [1]. The temperature of surface thermal waters gradually changes from high, favorable for thermophilic microorganisms, to moderate. Thus, thermal springs represent a natural model enabling investigation of the transition states between thermophilic and mesophilic microbial communities.

Thermal springs are may be divided into several types [2], the most common type being alkaline nitric thermal waters. Alkaline nitric thermal water provinces cover large areas in Central Asia, eastern Siberia, India, eastern and southern Africa, South America, Europe, western United States, as well as the western and eastern (but not central) areas of Iceland. Geochemically, the features of nitric thermal waters are determined by the processes of silicate hydrolysis and oxygen losses for oxidation; as a result, the predominating gaseous compound is nitrogen, and sulfates are partially reduced to hydrosulfides. In the Baikal rift zone, there is a large number of nitric thermal springs with temperatures up to 84°C and pH ranging from 6.1 to 9.3. They are formed independently from magmatic and thermometamorphic processes, which makes them different from thermal waters of the areas of active volcanism [3].

Microbial communities of the Baikal rift thermal springs have long been studied; however, the species composition of phototrophic communities has previously been investigated only by conventional microbiological methods, without using molecular genetic

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techniques. The most comprehensively studied phototrophic communities are those of the springs located on the Kotelnikovskii peninsula, the Bolsherechensk spring 30 km from the northern border of the Barguzin nature reserve, and the Uro spring 40 km from the settlement of Barguzin [4, 5]. General hydrochemical and microbiological characterization of thermal waters of the Baikal rift zone is provided, for instance, in [6]. The Goryachinsk thermal spring has been known for over 200 years, but its microbial community has not been comprehensively studied.

The goal of the present work was to investigate the APB species diversity in microbial mats of the Goryachinsk thermal spring using both conventional microbiological techniques and molecular genetic methods.

MATERIALS ANS METHODS

Sources. Bacteria were isolated from algobacterial mats that developed in different temperature zones the Goryachnsk spring. To characterize the sampling sites, the temperature was registered with an electric thermometer (HANNA HI 8314, Romania), pH, with a portable pH-meter (HANNA HI 8314), and total salinity, with a TDS-4 meter (Singapore). Sulfide concentrations were determined using the standard colorimetric technique. Mat specimens and microbial cultures were examined by light microscopy using an Olympus BX-41 microscope (Olympus, Japan).

Absorption spectra of the cell pigments were analyzed after disruption of the cells from the samples and pure cultures using a Soniprep 150 plus ultrasonic disintegrator at 14.5 kHz. Cell debris was precipitated by centrifugation at 7000 *g* for 5 min, and the supernatant containing membrane fragments and chlorosomes was used for spectrometry.

Taxonomic classification of oxygenic phototrophs was based on their morphological traits. Anoxygenic phototrophs were registered by culturing in selective media. Filamentous APB (FAPB) were grown in the medium containing the following (g/L): KH_2PO_4, 0.4; NH_4Cl, 0.5; $MgCl_2$, 0.4; KCl, 0.5; NaCl, 0.5; $Na_2S_2O_3$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.3; $NaHCO_3$, 0.5; vitamin B_{12}, 10 μg/L; and the trace element solution according to Pfennig and Lippert.

The basic culture medium was modified in several ways to sustain the growth of phototrophic bacteria of different taxonomic groups. For thermophilic *Chloroflexus* species and purple non-sulfur bacteria, growth media were supplemented with 0.5 g/L sodium acetate, 0.5 g/L sodium malate, 0.5 g/L sodium pyruvate, 0.1 g/L yeast extract, and 0.1 g/L $Na_2S \cdot 9H_2O$. Bacterial species of the families *Chromatiaceae* and *Chlorobiaceae* were grown in the basic medium supplemented with 0.5 g/L $Na_2S \cdot 9H_2O$, 0.5 g/L sodium thiosulfate, and 0.5 g/L sodium acetate. Aerobic bacteriochlorophyll *a*-containing bacteria were grown on agar plates with a heterotrophic medium [7].

APB species growing in the cultures were identified based on cell morphology, bacteriochlorophyll and carotenoid types, capacity for autotrophic and heterotrophic growth on sulfide, ability to grow in the darkness, and the effects of temperature and pH.

Molecular genetic identification of APB was performed in pure cultures using PCR with primers to the group-specific molecular markers *puf*LM and *fmo*A. Isolated pure cultures of aerobic bacteria containing bacteriochlorophyll *a* were identified based on their 16S rRNA gene sequences.

DNA was isolated from FAPB cultures using the CTAB method [8] with minor modifications. From all other bacterial cultures, DNA was isolated as described previously in [9].

Fragments of the *puf*LM operon were amplified and sequenced using two group-specific primer sets. The set of primers specific for purple sulfur and non-sulfur bacteria was described in [10]. The other pair of primers specific for chlorosome-containing FAPB was designed for the present study: forward, 5'-CGAGCCGGARTAYAAGATCAA-3', and reverse 5'-AGAAGATCGAGAGCATGTG-3'. For both systems of primers, the PCR protocol included the initial cycle of 2 min at 94°C, 30 s at 56°C, and 90 s at 72°C, 42 cycles of 30 s at 94°C, 30 s at 56°C, 90 s at 72°C, and the final elongation for 5 min at 72°C.

The primers constructed for detection of the *puf*M gene in *Agrobacterium tumifaciens* were also used: 5'-GCACCTGGACTGGA-3' (forward) and 5'-CCATGGTCCAGCGCCAGA-3' (reverse). The PCR protocol was as follows: initial denaturation at 94°C for 3 min, 30 cycles of 50 s at 94°C, 50 s at 55°C, and 50 s at 72°C; final elongation at 72°C for 10 min.

Amplification of the 16S rRNA gene fragments and subsequent sequencing of PCR products were performed with universal bacterial primers 27f and 1492r [11].

Amplification and subsequent sequencing of a *fmo*A fragment were performed with the primers described in [12].

The PCR mixture (25 μL) for amplification of target gene fragments contained 1× buffer for *BioTaq* DNA polymerase (17 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; 2 mM $MgCl_2$), 12.5 nmol each dNTP, 50 ng DNA template, 5 pmol of each relevant primer, and 3 U *BioTaq* DNA polymerase (Dialat LTD, Russia).

PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, United States) according to the manufacturer's instructions and sequenced using a BigDye Terminator v. 3.1 Cycle Sequencing Kit on an automated DNA Analyzer 3730

Physicochemical characteristics and species composition of the phototrophic microbiota of the Goryachinsk thermal spring

Sampling site	Temperature, °C	pH	Species composition
Gor-1	50.6	8.8	*Leptolyngbya* sp., *Oscillotoria* sp., *Chloroflexus aurantiacus, Blasrochloris sulfoviridis*
Gor-2	49.4	9.0	*Leptolyngbya* sp., *Oscillotoria* sp., *Chloroflexus aurantiacus, Blasrochloris sulfoviridis, Rhodomicrobium vannielii*
Gor-3	45.8	9.0	*Leptolyngbya* sp., *Oscillotoria* sp., *Chloroflexus aurantiacus, Allochromatium* sp., *Thiocapsa* sp.
Gor-4	25	9.0	*Leptolyngbya* sp., *Oscillotoria* sp., *Navicula* sp., *Chloroflexus aurantiacus, Blasrochloris sulfoviridis, Chlorobium* sp., *Agrobacterium tumifaciens, Rhodopseudomonas faecalis*

as recommended by the manufacturer (Applied Biosystems, United Sates).

The obtained sequences were edited using the BioEdit software package [13]. For APB identification and phylogenetic analysis, the resulting nucleotide sequences of *puf*LM fragments were translated *in silico* into amino acid sequences of the L subunit of the APB photosystem reaction center. Preliminary comparison with the sequences retrieved from the GenBank database was performed using the BLAST software [http://www.ncbi.nlm.nih.gov/blast]. Alignment of both nucleotide sequences and amino acid sequences obtained by *in silico* translation was performed using CLUSTAL W [14]. Phylogenetic trees were constructed using the maximum likelihood algorithm implemented in the MEGA 5.0 software package [15].

The obtained nucleotide sequences were deposited into the GenBank database with accession numbers KF888726–KF888735.

RESULTS AND DISCUSSION

Characterization of the Goryachinsk thermal spring. The Goryachinsk thermal spring is located at a balneotherapy resort on the shore of Lake Baikal, 188 km north-west of Ulan-Ude (52.59.245' N, 108.18.475' E). Samples were collected in September 2012: the sodium sulfate spring water contained traces of sulfides and salinity was less than 0.64 g/L. In the discharge zone, the water temperature was 54°C, pH 9.5. It should be noted that in a previous publication, lower pH values of 6.8–8.6 were reported for the spring, probably due to seasonal pH fluctuations [4]. In the spring bed, a dark green cyanobacterial mat 1–4 mm thick developed. Microbial mat samples were collected so as to represent two thermal areas: thermophilic (sites Gor-1, 50.6°C, Gor-2, 49.4°C, and Gor-3, 45.8°C) and mesophilic (site Gor-4, 25°C) (table).

Species Composition of Phototrophic Microbiota of Algobacterial Mats

Oxygenic phototrophs. An algobacterial mat is growing in the bed of the Goryachinsk spring. In this spring, the numbers of unicellular cyanobacteria in both thermophilic and mesophilic mats were insignificant. The principal mat-forming microorganisms were filamentous cyanobacteria (Figs. 1a, 1b). The thermophilic mat was up to 4 mm thick. Microscopic investigation showed that it was predominantly composed of a cyanobacterium *Leptolyngbya* sp. (trichomes approximately 2 μm in diameter). Two large forms of *Oscillotoria* sp., 20 and 10 μm in diameter, were codominant species. In the mesophilic zone, the algobacterial mat had a mosaic structure and was less than 1 mm thick. In addition to the cyanobacterial species described above, the moderate-temperature area (below 20–30°C) contained diatoms.

Anoxygenic phototrophic bacteria. Absorption spectrum of the pigments present in the natural microbial mat at Gor-1 site confirmed that the phototrophic community was dominated by oxygenic phototrophs containing chlorophyll *a* with an absorption peak at 670 nm (Fig. 2). The large peak at 618 nm probably corresponded to phycocyanin, which is typical for most cyanobacteria. A smaller peak at 744 nm was suggestive of the presence of anoxygenic phototrophs containing bacteriochlorophyll *c*. Bacteriochlorophyll *a* could not be reliably identified in this absorption spectrum.

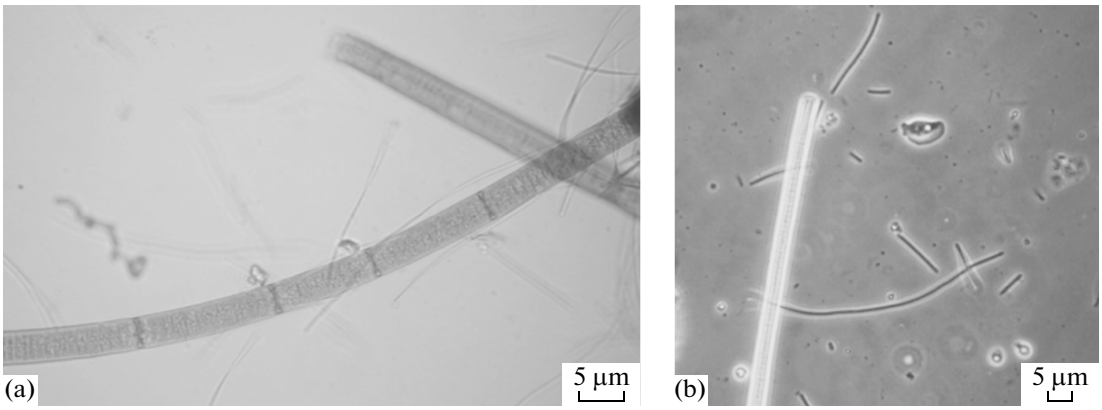


Fig. 1. Morphology of phototrophic microorganisms of microbial mats of the Goryachinsk spring. Sample collection sites: (a), Gor-1 (50.1°C); (b), Gor-4 (25°C).

Incubation of material of all samples, including the mesophilic ones, under illumination at 50°C promoted growth of thermophilic filamentous green bacteria. The presence of thermophilic FAPB in the mesophilic mat was confirmed by direct PCR-based detection of the *puf*LM operon, which is specific for chlorosome-containing FAPB. Importantly, the natural sample studied contained only one FAPB phylotype, *Chloroflexus aurantiacus* (Fig. 3). The presence of a thermophilic FAPB species in mesophilic mats may probably be explained by its ability to grow at moderate temperatures while maintaining slow metabolism.

In agar stab cultures, FAPB produced brown-green spherical colonies 2–3 mm in diameter. Light microscopy revealed thin smooth filaments 0.5–0.7 μm thick and of indefinable length (Fig. 4a). The filaments were composed of rod-shaped slightly elongated cells. Bacterial cells contained bacteriochlorophyll *c* as the principal pigment (main absorption peak at 738 nm) and, in lower quantities, bacteriochlorophyll *a* (main absorption peak at 870 nm) (Fig. 4b). Cell morphology and pigment composition suggested that these bacteria belonged to the genus *Chloroflexus*. Molecular genetic analysis confirmed that all isolated FAPB cultures were belonged to the species *Cfl. aurantiacus* (Fig. 3).

Algobacterial mats of all temperature zones studied contained mesophilic purple non-sulfur bacteria (PNB). The isolated PNB cultures contained cells of three morphological types. The first type was represented by motile, budding, short rod-shaped bacteria of 0.7 × 1 μm with stalkless buds (Fig. 5a); they formed light green fuzzy-edged colonies. The absorption spectrum of intact cells had a maximum in the infrared region at 1030 nm, which suggested the presence of bacteriochlorophyll *b* (Fig. 5b). Morphologically, these bacteria corresponded to the genus *Blastochloris*, in agreement with the results of molecular genetic analysis of *puf*LM, which made it possible to identify them as *Blastochloris sulfoviridis* (Fig. 3). This species was present in the samples collected at all temperature zones. Another PNB morphotype was short oval rods (Fig. 6a) reproducing by budding and forming thin hyphae, which is typical for a known species, *Rhodomicrobium vannielii*. Identification of the isolated PNB species as *Rmi. vannielii* was confirmed by the results of molecular genetic analysis (Fig. 3). These bacteria produced wine-red colonies in agar media and contained bacteriochlorophyll *a* as the principal pigment (Fig. 6b). The third PNB species was phylogenetically close to the previously described *Rhodopseudomonas faecalis* (isolated at Gor-2 site) (Fig. 3). This species has not previously been isolated from a cyanobacterial mat of a thermal spring. In culture, its color varied from pink to brick-red. Rod-shaped bacteria reproduced by budding similarly to *Rhodopseudomonas palustris*, with a pronounced constriction between the maternal and the daughter cells (Fig. 7a). The main pigment in this strain was bacteriochlorophyll *a* (Fig. 7b).

It should be noted that microbial mats of the Goryachinsk spring also contained purple sulfur bacteria (PSB). Two cultures of purple sulfur bacteria were obtained from the mesophilic mat sample. The cell morphology suggested that these bacteria belonged to the genera *Allochromatium* (motile rod-shaped cells; Fig. 8a) and *Thiocapsa* (nonmotile spherical cells; Fig. 8b). Cells of both isolates contained internal sulfur granules. In agar stab cultures, they were growing as diffuse brick-red (*Allochromatium* sp.) or dense spherical dark red (*Thiocapsa* sp.) colonies. Figure 8c shows the absorption spectrum of live *Allochromatium* sp. cells. These bacteria contained

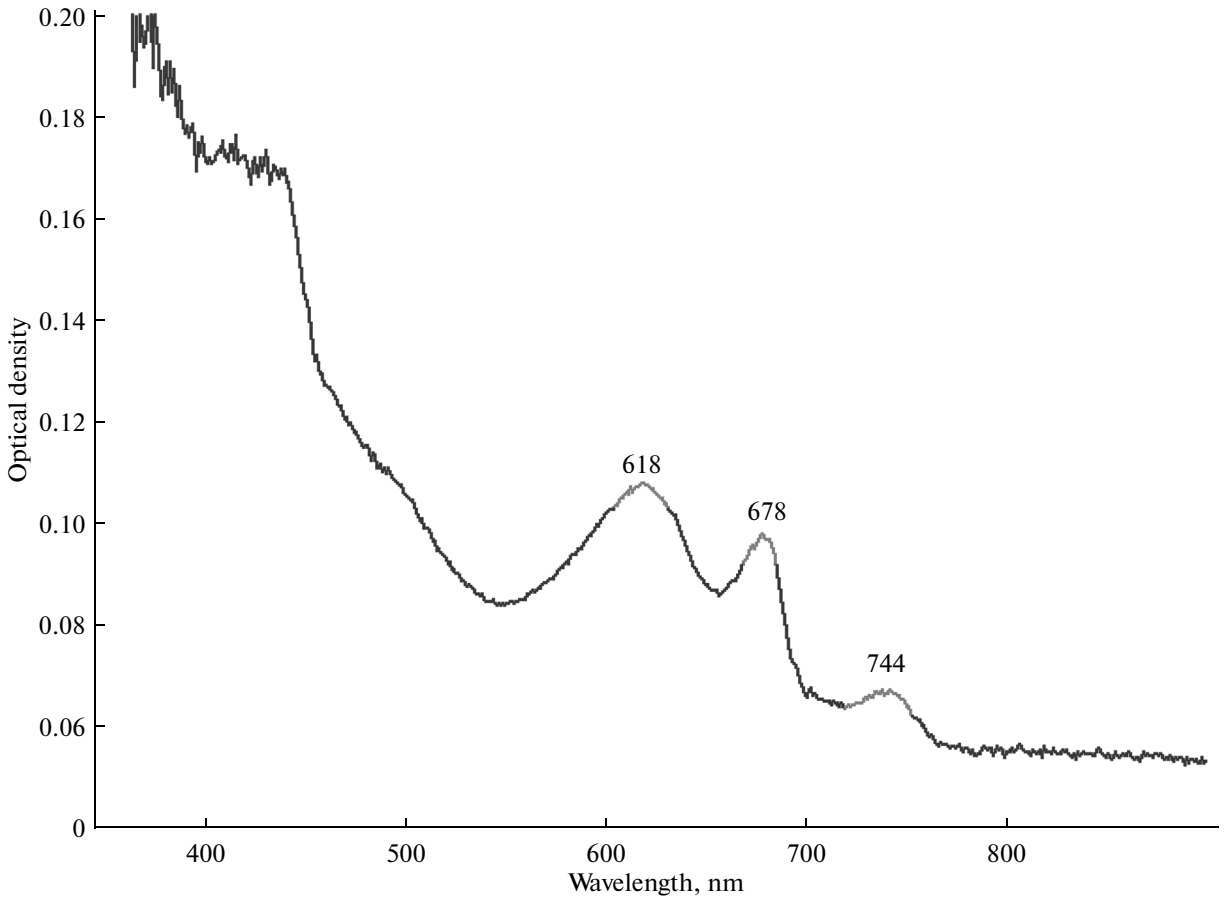


Fig. 2. Absorption spectrum of the microbial mat at Gor-1 site (50.1°C).

bacteriochlorophyll *a* with absorption peaks at 805 and 860 nm and carotenoids of the spirilloxanthin series with an absorption peak at 480 nm. The phenotypic traits suggest their identification as *Alc. vinosum*.

This work was the first study to detect the presence of green sulfur bacteria in a mesophilic microbial mat of a Baikal rift spring (site Gor-4). In agar stabs, green sulfur bacteria formed dark green lens-shaped colonies 1–2 mm in diameter. Individual rough-surfaced cells formed short branching chains (Fig. 9a). Sulfur granules resulting from sulfide oxidation were deposited outside the bacterial cells. The cell morphology corresponded to the characteristics of the genus *Chlorobium*. The principal pigment in the isolated green sulfur bacteria was bacteriochlorophyll *c* with an absorption peak at 749 nm (Fig. 9b). Comparison of the FmoA amino acid sequence obtained by *in silico* translation of the *fmo*A fragment amplified from the newly isolated culture to the reference sequences indicated that the isolated bacterium was most closely related to *Chlorobium limicola* (Fig. 10).

It should be pointed out that the mesophilic mat of the Goryachinsk spring contained aerobic bacteriochlorophyll *a*-containing bacteria, which were isolated as a pure culture. They grew well under aerobic heterotrophic conditions and formed pale yellow colonies. Cell morphology is shown on Fig. 11a. Based on a 1400 bp-long sequence fragment of the 16S rRNA gene, bacteria were identified as *Agrobacterium* (*Rhizobium*) *tumifaciens* (100% identity).

This is the first report on the presence of bacteriochlorophyll *a* with an absorption maximum at 870 nm (Fig. 11b) in live *Agrobacterium tumifaciens* cells. In phototrophic microorganisms, bacteriochlorophyll *a* is associated with the reaction center. Therefore, the isolated strain could be expected to carry the *puf*LM operon. Since PCR with the available primers to *puf*LM produced negative results, we designed a new set of primers to a *puf*M fragment (see Materials and Methods), which resulted in successful amplification. Sequencing of the PCR product showed that it indeed represented a *puf*M fragment; however, the obtained sequences were too short to construct a phylogenetic

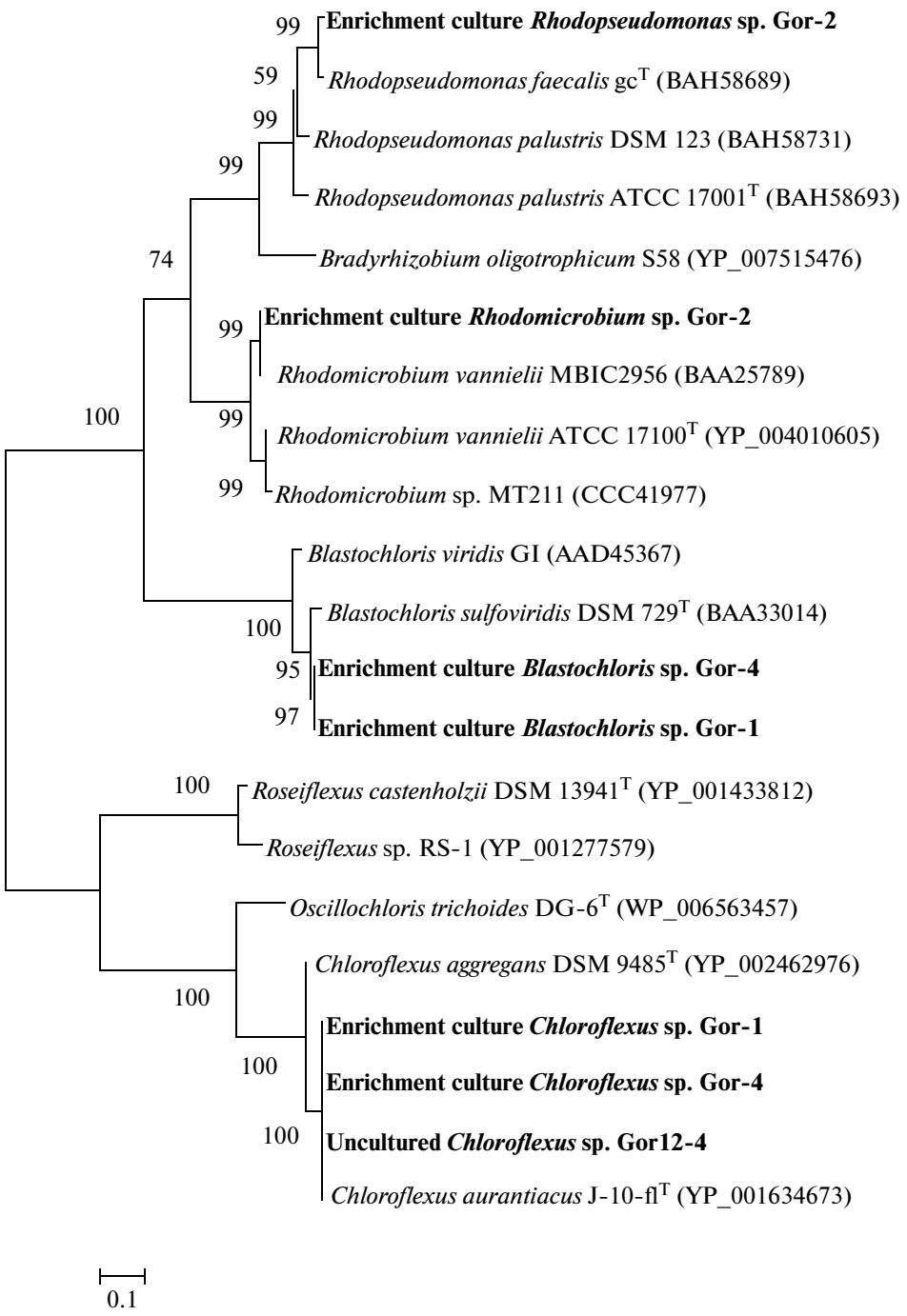
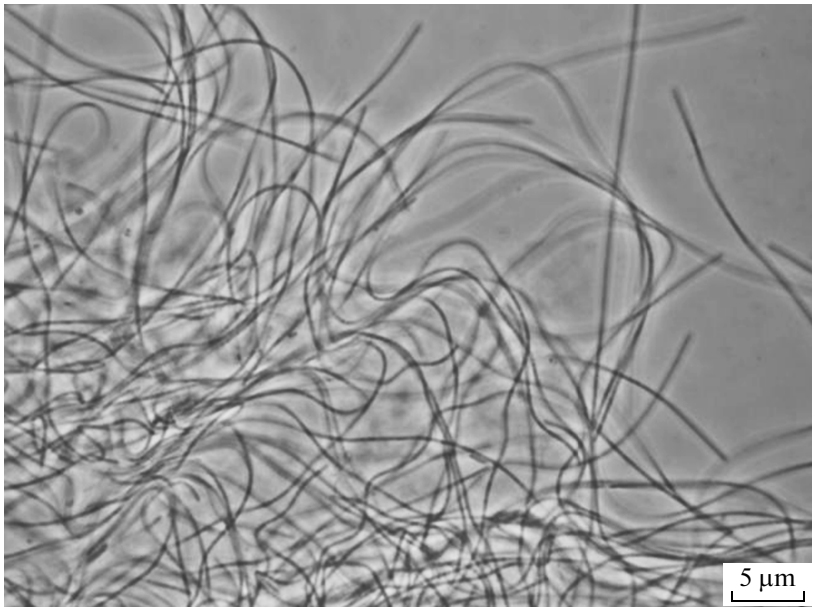


Fig. 3. Dendrogram of phylogenetic relationships between the cultures of anoxygenic phototrophic bacteria isolated from the Goryachinsk spring. The dendrogram was constructed based on the alignment of 202 residues of the PufL amino acid sequences using the maximum likelihood algorithm. The evolutionary distance scale corresponds to 10 substitutions per 100 amino acids of the sequence.

tree (240 bp). A BLASTp search showed that the translated sequence of the amplified fragment had 99% identity to the PufM protein of the reaction center of the PNB strain *Rhodocista* sp. W3 (AGK27891). The second most significant result was 97% identity to a PufM fragment of *Agrobacterium* (*Rhizobium*) *alberti-magni* AOL15 (WP_006724825). A search through the genomic sequence of *Agrobacterium* (*Rhizobium*)

(a)



(b)

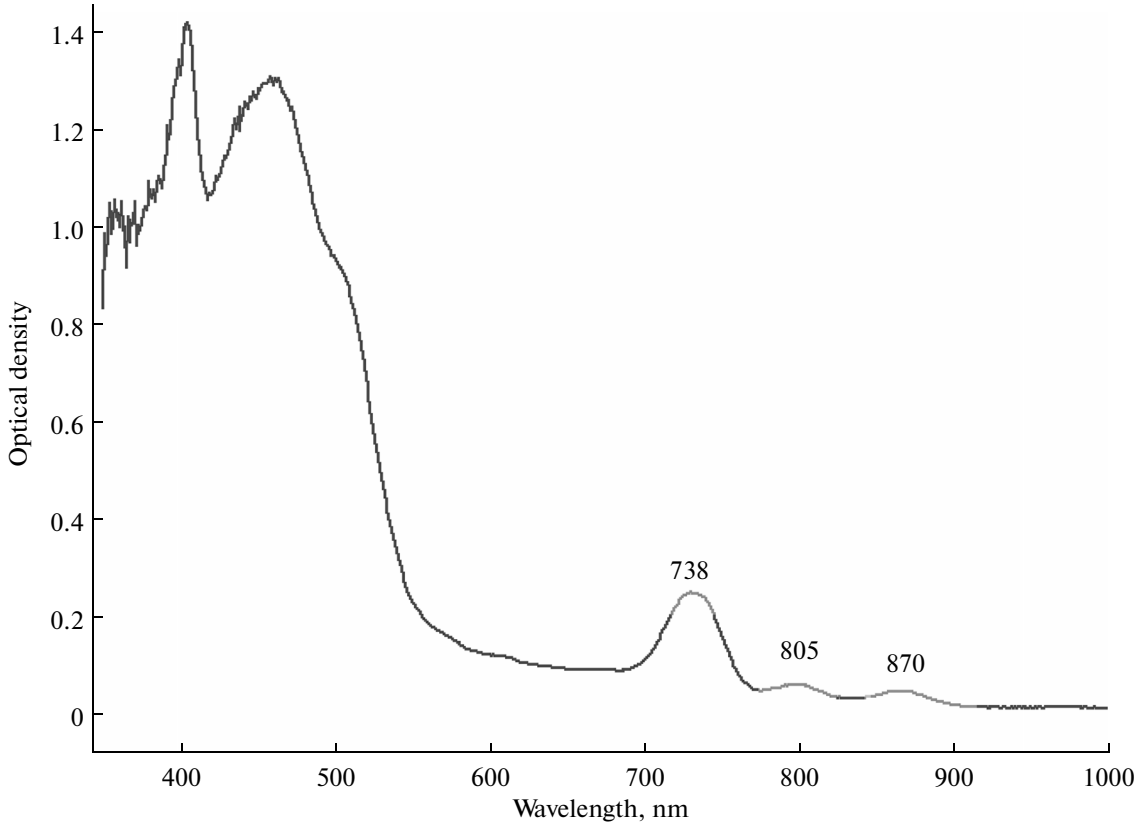


Fig. 4. Morphology (a) and absorption spectrum (b) of cultured *Chloroflexus aurantiacus* cells, Gor-1.

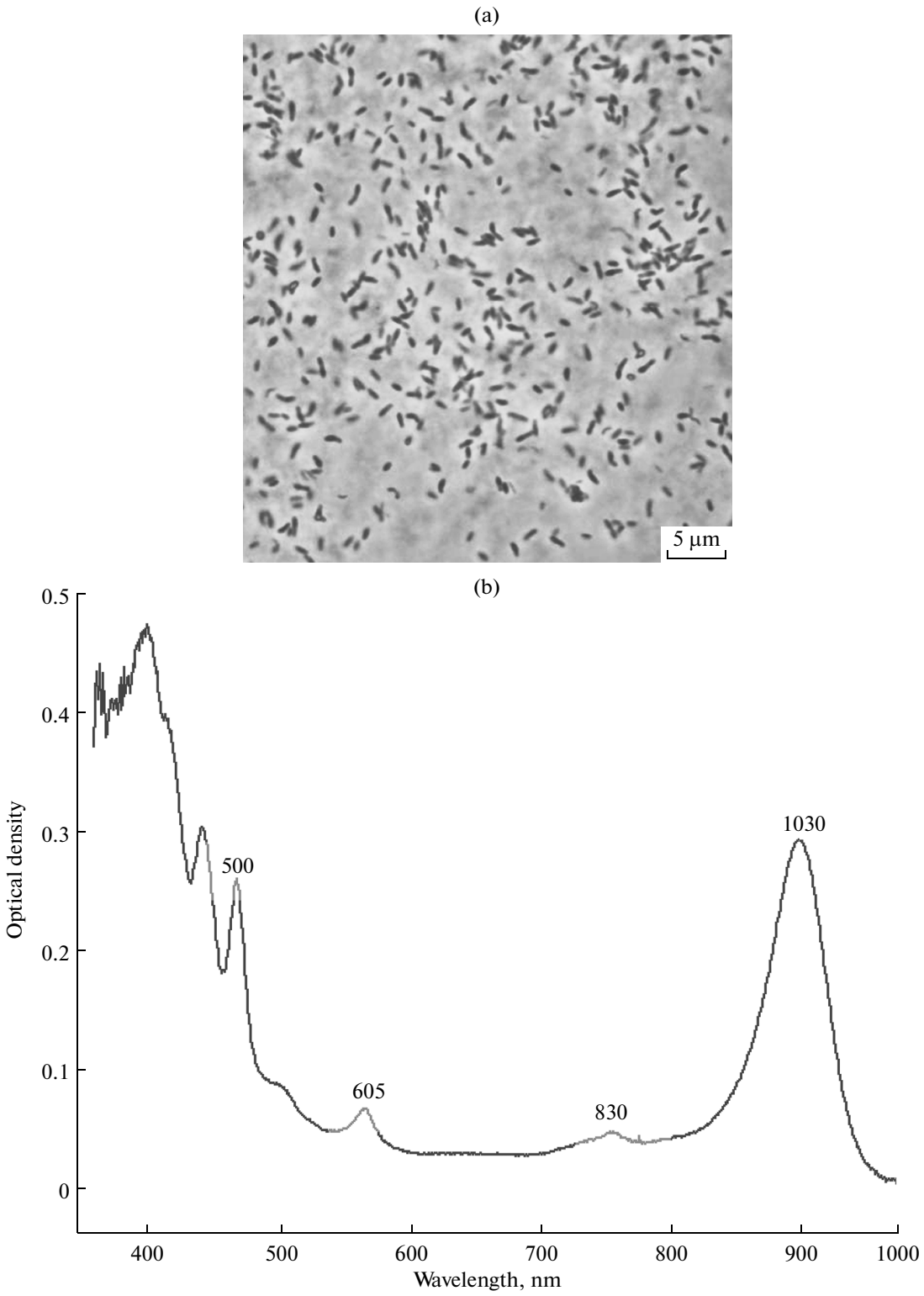


Fig. 5. Morphology (a) and absorption spectrum (b) of cultured *Blasrochloris sulfoviridis* cells, Gor-2.

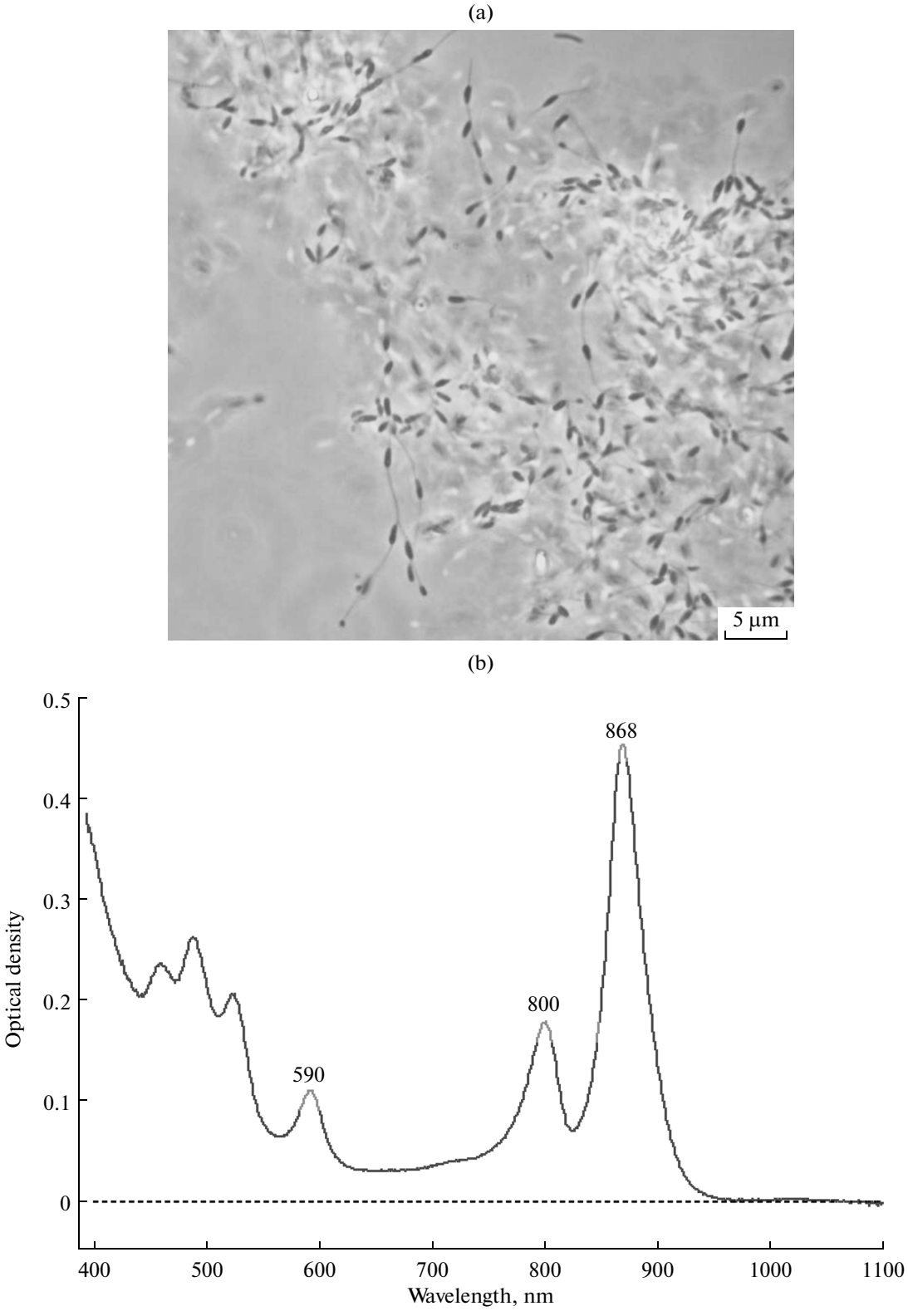
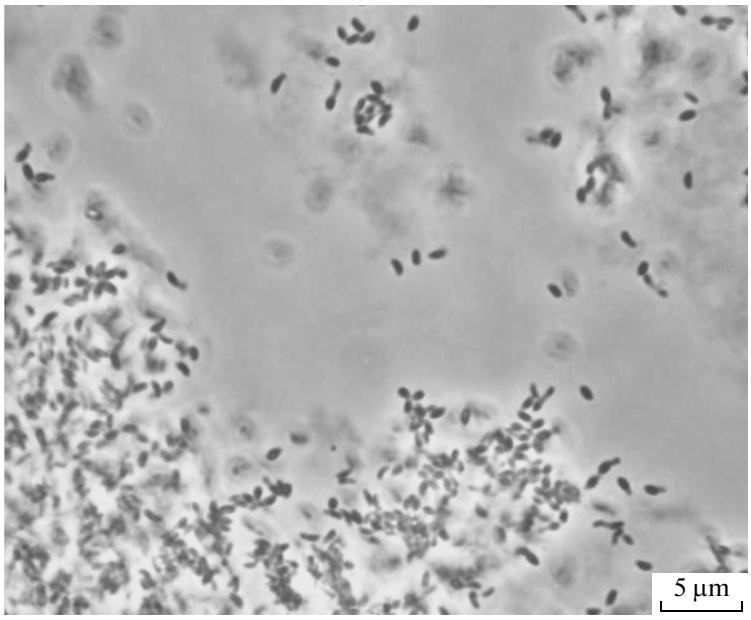


Fig. 6. Morphology (a) and absorption spectrum (b) of cultured *Rhodomicrobium vannielii* cells, Gor-2.

(a)



(b)

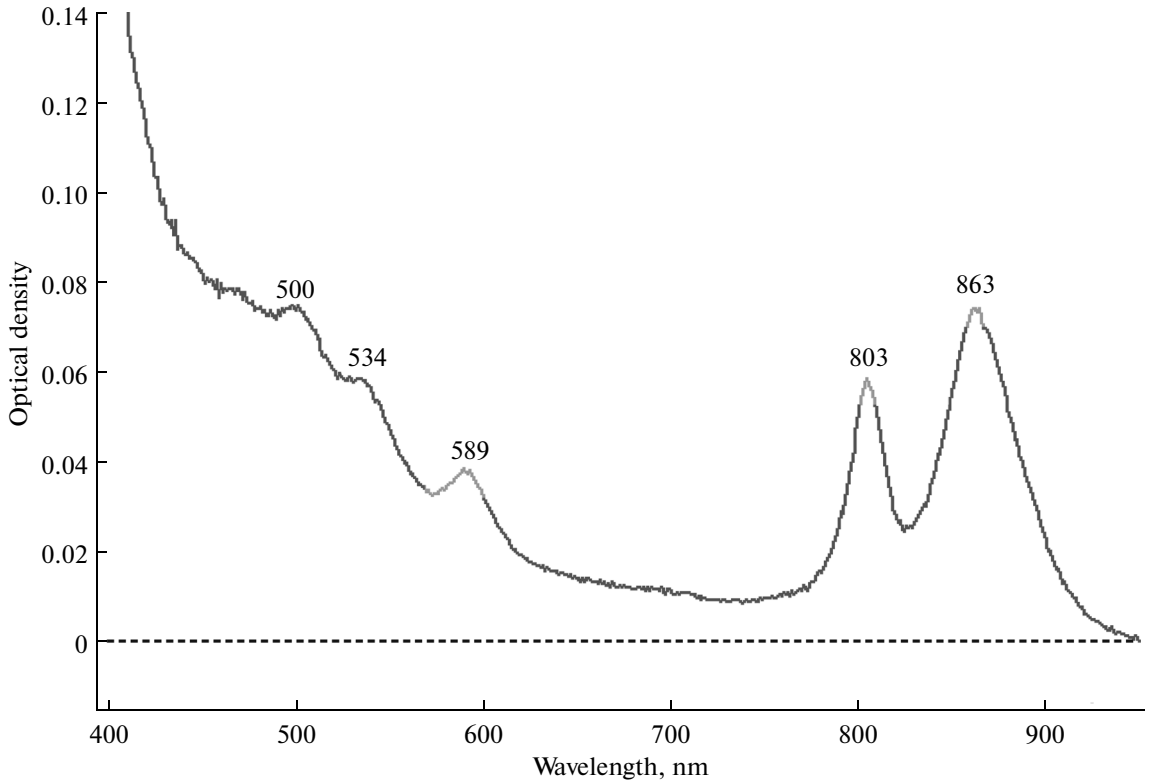


Fig. 7. Morphology (a) and absorption spectrum (b) of cultured *Rhodopseudomonas faecalis* cells, Gor-4.

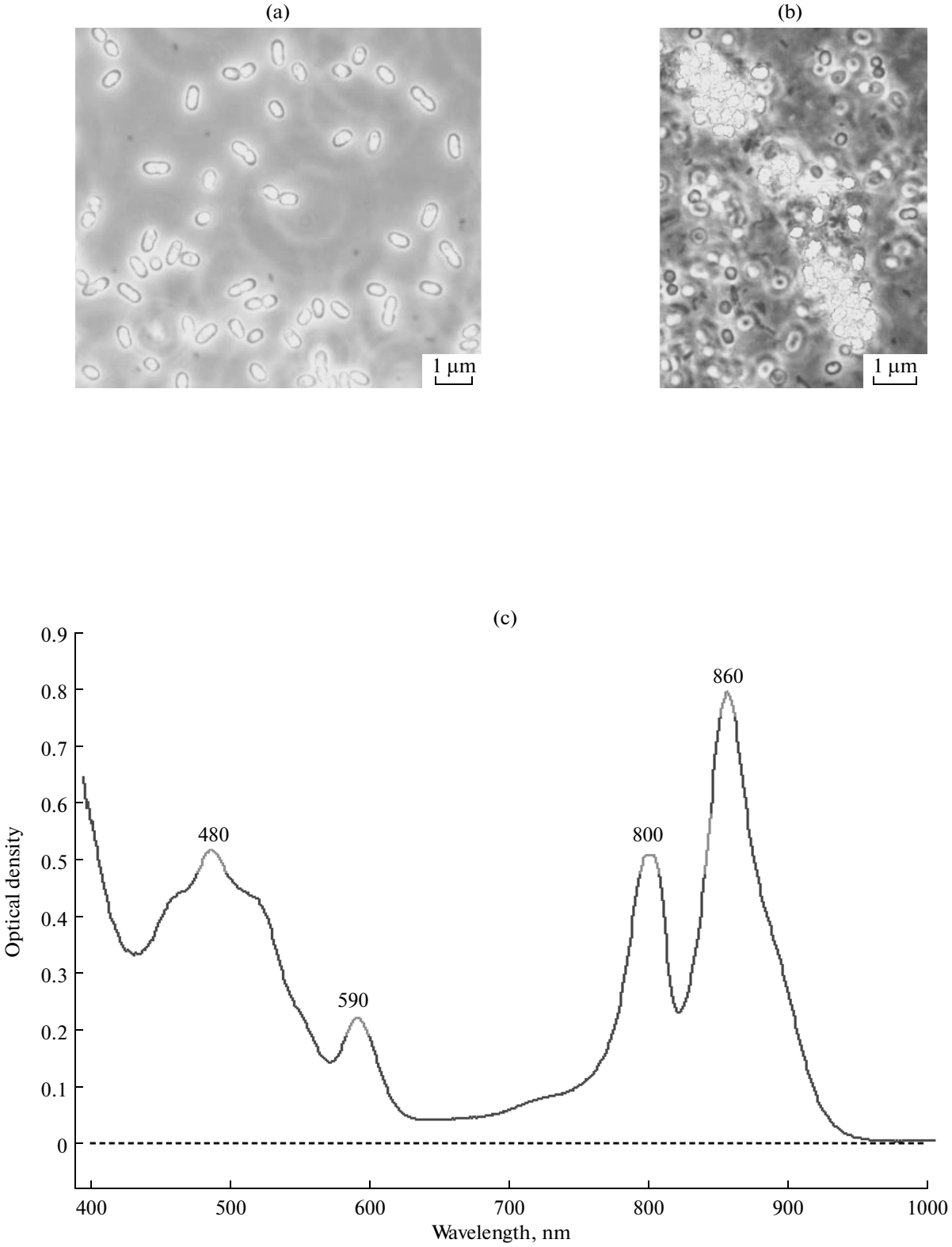


Fig. 8. Morphology of cultured *Allochromatium* sp. (a) and *Thiocapsa* sp. cells, (b) cells, both from Gor-3 site; absorption spectrum of *Allochromatium* sp., Gor-2 (c).

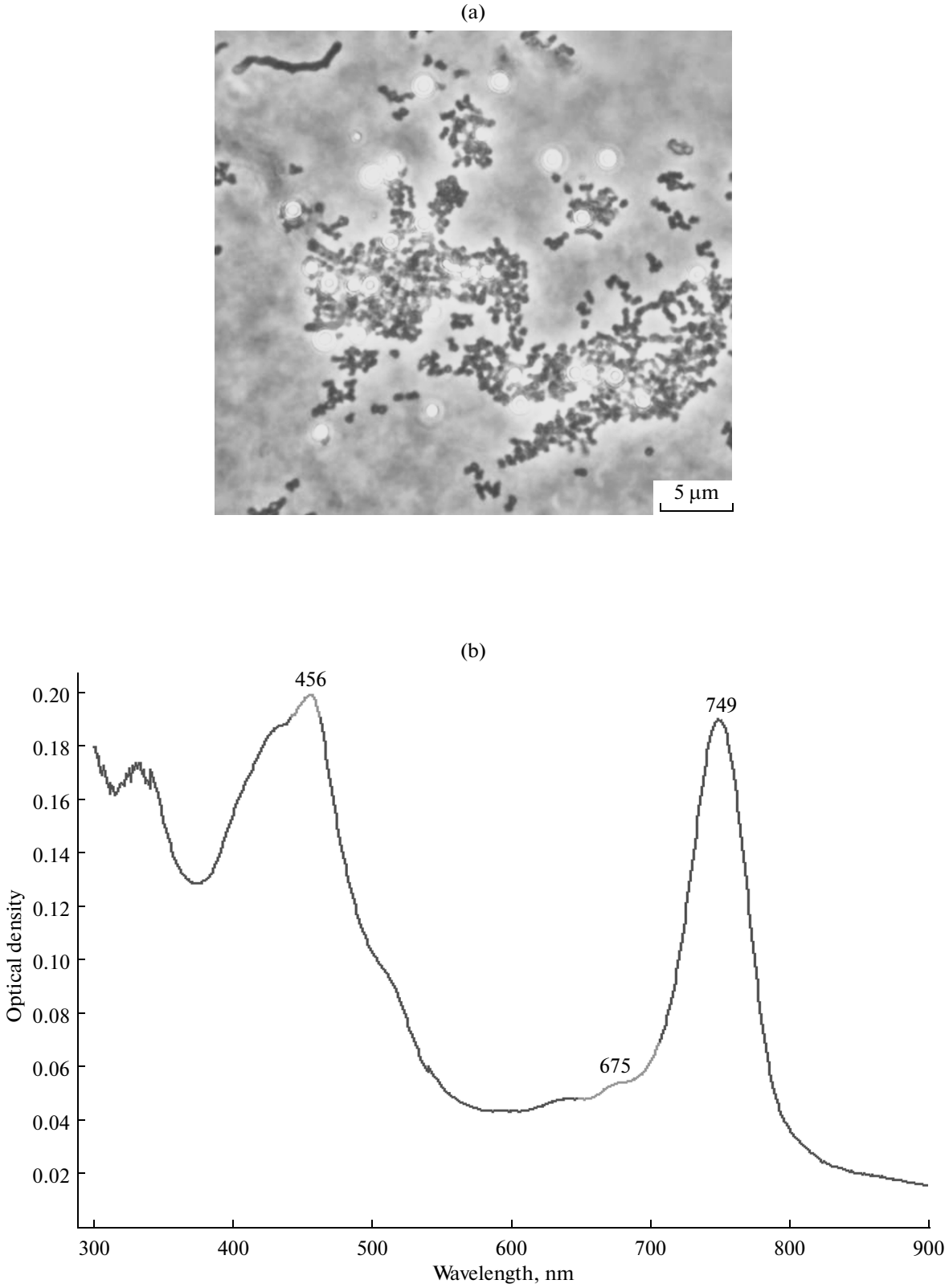


Fig. 9. Morphology (a) and absorption spectrum (b) of cultured *Chlorobium* sp. cells, Gor-4.

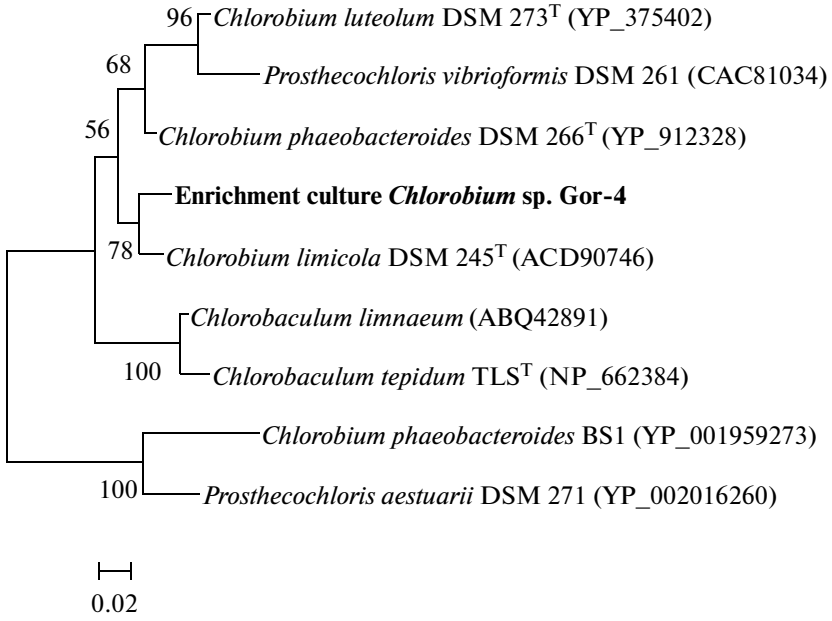


Fig. 10. Phylogenetic position of a green sulfur bacterium isolated from the Goryachinsk spring. The dendrogram was constructed based on alignment of 281 residues of the FmoA amino acid sequences using the maximum likelihood approach. The evolutionary distance scale corresponds to two substitutions per 100 amino acids of the sequence.

albertimagni AOL15 (PRJNA176603) isolated from a Californian thermal spring [16] showed that it possessed both genes of the type II reaction center, *puf*L and *puf*M. Thus, it was confirmed that bacteriochlorophyll *a*-containing agrobacteria (rhizobia) were present in microbial communities of thermal springs.

The Goryachinsk sodium sulfate spring belongs to the nitric thermal water province of the Baikal rift zone. This study was the first one to investigate the species composition of its phototrophic community using molecular genetic methods. Our data show that thermophilic microbial mats growing in the spring bed are dominated by filamentous cyanobacteria; at the same time, anoxygenic filamentous phototrophs are also present in considerable amounts. It is noteworthy that the thermophilic FAPB species *Chloroflexus aurantiacus* was present in both in the thermophilic and the stably mesophilic mat. Apparently, this species is highly adaptive to temperature conditions. *Cfl. aurantiacus* was the only thermophilic anoxygenic phototrophic species found in this spring. It should be noted that PNB species were always present in the mats with temperatures favorable for moderate thermophiles, as it was also observed in our previous work [5]. Most of the detected PNB could utilize sulfide as an electron donor. Mesophilic mats also contained two PSB species and green sulfur bacteria identified as members of the genus *Chlorobium*. Green sulfur bacteria have not previously been described in alkaline springs of the Baikal rift zone. Interestingly, while the water of the Goryachinsk spring contained only trace amounts of sulfides, bacteria of the sulfur cycle were an important part of the community. The fact that considerable amounts of sulfides are produced by bacterial sulfate reduction in the course of mat degradation probably explains why the microbial community of the Goryachinsk spring is dominated by sulfide-consuming APB. It was previously shown that sulfate reduction rates in thermal spring mats could be as high as 4.1 g S/m^2 per day, and the numbers of sulfate-reducing bacteria could exceed 10^7 cells/cm^3 [17]. Therefore, the detected APB species were typical for a sulfur cycle-dependent microbial community.

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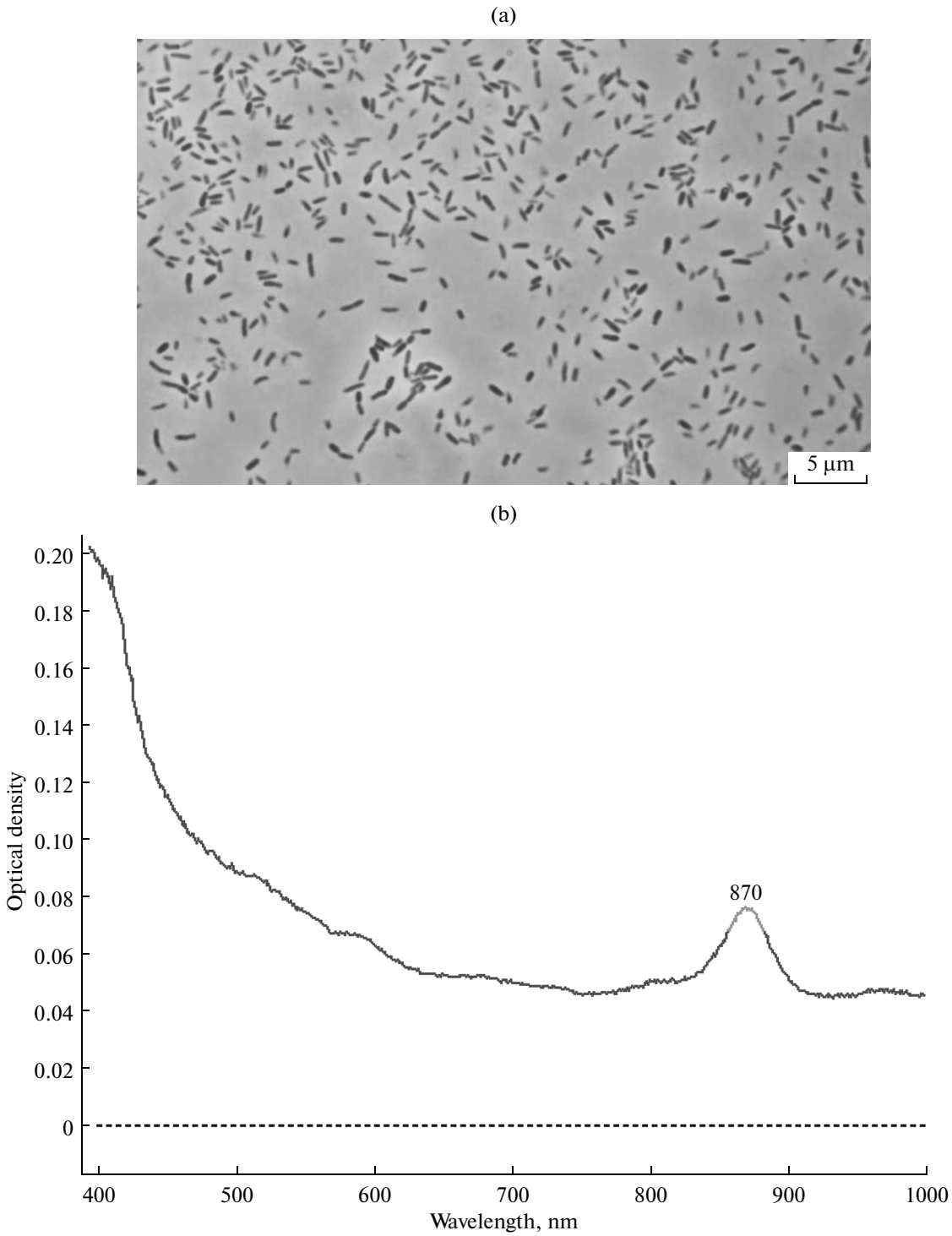

Fig. 11. Morphology (a) and absorption spectrum (b) of cultured *Agrobacterium tumifaciens* cells, Gor-4.

Presidium of the Siberian Branch of Russian Academy of Sciences (grant no. 94).

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