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EXPERIMENTAL ARTICLES

Anoxygenic Phototrophic Bacteria of the High-Altitude Meromictic Lake Gek-Gel, Azerbaijan

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Abstract—The anoxygenic phototrophic bacterial community of the high-altitude meromictic Lake Gek-Gel (Azerbaijan) was investigated in September 2003. The highest concentration of bacteriochlorophyll *e* (48 μg/l) was detected at a depth of 30 m; the peak of bacteriochlorophyll *a* (4.5 μg/l) occurred at 29 m. Phylogenetic analysis revealed that brown-colored green sulfur bacteria *Chlorobium phaeobacteroides* predominated in the lake. Nonsulfur purple bacteria phylogenetically close to *Blastochloris sulfoviridis* were found in insignificant amounts; these organisms have not been previously reported in Lake Gek-Gel.

Key words: meromictic lakes, anoxygenic phototrophic bacteria, *Chlorobium phaeobacteroides, Blastochloris sulfoviridis*, anoxygenic photosynthesis.

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The meromictic Lake Gek-Gel is located in the Caucasus Mountains in the Ag-Su river valley, 30 km south from Ganja (Azerbaijan).

The water in the lake is fresh, with a low content of sulfates, arriving with drain from the bedrock [1, 2]. The salinity gradient from surface to bottom is practically absent. However, the water is not mixed; below 29–30 m, sulfide is detected (a result of sulfate reduction) [2, 3].

A peak of CO_2 assimilation at the oxic–anoxic interface (in the redox zone) of Lake Gek-Gel has been reported earlier [1]. The first investigation of the community of anoxygenic phototrophic bacteria (APB) of the meromictic Lake Gek-Gel (1970–1971) revealed development of brownish-colored green sulfur bacteria *Chlorobium phaeobacteroides* immediately below the redox zone [2]

The goal of the present work was comparative investigation of the APB community in the upper part of the sulfide zone of Lake Gek-Gel, isolation of pure bacterial cultures, and their identification by microbiological and molecular techniques.

MATERIALS AND METHODS

The samples were collected with a 1-l glass bathometer in a deep hollow of the lake (40°24.816′N, 46°19.696′E, depth 72 m). Photosynthesis rates were determined by the radioisotope method with ^{14}C bicarbonate [4]. For this purpose, 0.2 ml of labeled bicarbonate solution was injected into 30-ml glass vials filled with lake water. The vials were then attached to a Nylon halyard and incubated at the horizons of sampling for 6–12 h. To determine dark CO_2 assimilation, the vials were wrapped in aluminum foil prior to sampling. After the incubation, the water and sediment samples were fixed with 0.5 ml of 10% orthophosphoric acid.

The concentrations of Cl^- and SO_4^{2-} were determined using a Biotronik ion chromatograph (Germany). The concentrations of oxygen and sulfide were determined immediately after sampling with Aquamerck test kits (Merck, Germany).

Total numbers of microorganisms were determined by the fluorescence method on polycarbonate membrane filters (0.2 μm pore diameter) with DAPI (diamidino-4',6'-phenyl-2-indoledichlorhydrate) [5]. Bacterial cells were counted under a Zeiss epifluorescence microscope (Germany).

For determination of bacteriochlorophylls (BChl), lake water samples (10–350 ml) were filtered through 0.2-μm nylon filters. The filters were then transported to the laboratory and treated with an acetone–methanol mixture (7 : 2). Absorption spectra in the wavelength range from 350 to 1100 nm were obtained on a SF-56 spectrophotometer (LOMO, Russia). The following

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equations were used to calculate the concentrations of the pigments:

$$C\ (\mu g\ BChl\ e/l) = 1/k((D_{654} - D_{850})\ (V\ extract\ (ml)/V\ sample\ (l))1000,$$

$$C\ (\mu g\ BChl\ e/l) = 1/k((D_{770} - D_{850})\ (V\ extract\ (ml)/V\ sample\ (l))1000,$$

where C is BChl concentration; k is an absorption coefficient; D is the optical density measured at the specified wavelength in a 1-cm cuvette; and V is the volume of an extract or sample. The absorption coefficients k used to calculate BChl *e* and BChl *a* concentrations were 98.0 and 46.1 l g^{-1} cm^{-1}, respectively [6, 7].

Phototrophic bacteria were isolated in medium containing the following (g/l): KH_2PO_4, 0.33; $MgSO_4 \cdot 7H_2O$, 0.33; NH_4Cl, 0.33; KCl, 0.33; $NaHCO_3$, 1.5; $CaCl_2 \cdot 6H_2O$, 0.15; $Na_2S \cdot 9H_2O$, 0.5; vitamin B_{12}, 20 μg/l; trace elements solution, 1 ml [8]; pH 6.8–7.2.

For the cultivation of green sulfur bacteria, this medium was supplemented with $CH_3COONa \cdot 3H_2O$ (0.5 g/l) and yeast extract (0.1 g/l); pH 6.8.

For the cultivation of green nonsulfur bacteria, medium was used containing the following (g/l): KH_2PO_4, 0.33; $MgSO_4 \cdot 7H_2O$, 0.33; NH_4Cl, 0.33; KCl, 0.33; $NaHCO_3$, 1.5; $CaCl_2 \cdot 6H_2O$, 0.15; Na_2SO_4, 0.5; $Na_2S \cdot 9H_2O$, 0.1; $CH_3COONa \cdot 3H_2O$, 0.5; yeast extract, 0.1; vitamin B_{12}, 20 μg/l; trace elements solution, 1 ml [8]; pH 7.0.

To obtain enrichment cultures of phototrophic bacteria, lake water samples (5 ml) were injected in the field into hermetically sealed penicillin vials with the medium (30 ml). The cultivation was carried out for three months under anaerobic conditions. For the first week, they were kept at room temperature, away from direct insolation (during transportation, they were stored in the dark). Subsequent incubation was carried out in a luminostat at 2000 lx and 20–25°C. Pure cultures were obtained by the terminal dilutions method using agarized (0.5%) medium of the same composition. The following characteristics were used for primary identification: size and shape of cells and microcolonies; colony color; the presence of gas vacuoles; formation and location of sulfur granules; and absorption spectra of whole cells in 50% glycerol and acetone–methanol extract (7 : 2).

Microphotographs were obtained by light microscopy with a 90× phase contrast immersion system (magnification 1200×).

For ultrathin sectioning, the material was prefixed in 1.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h at 4°C; washed twice with the same buffer; and fixed with 1% OsO_4 in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20°C. After dehydration in a series of alcohols, the material was embedded in Epon 812. Ultrathin sections were mounted on grids and contrasted with 3% uranyl acetate in 70% ethanol (30 min) and with lead citrate [9].

For whole-cell staining, bacterial suspensions were applied to Formvar-covered copper grids and stained with 0.2% uranyl acetate in water. Ultrathin sections and negatively stained preparations were examined with a JEM-100B electron microscope (JEOL, Japan) at 80-kV accelerating voltage.

DNA was isolated as described previously [10]. Amplification of 16S rRNA genes was carried out with the 27f and 1525r universal primers on GeneAmp PCR System 2700 (Applied Biosystems). The amplified 16S rDNA fragments were sequences on a CEQ2000XL automatic DNA sequencer (Beckman Coulter) with the Dye Terminator Cycle Sequencing kit (Beckman Coulter) according to the protocol provided with the kit.

The ClustalX software package [11] was used for alignment of 16S rDNA sequences. The rootless phylogenetic tree was constructed using algorithms realized in the TREECON software package [12].

RESULTS

Physicochemical characterization. The work on Lake Gek-Gel was carried out in September 12–19, 2003. During this period, oxygen in the deepest part of the lake (72 m) penetrated to a depth of 29 m; below was the sulfide zone (Fig. 1). Sulfide concentration at the bottom was 4.2 mg/l. The redox zone was located at a depth of 29–30 m. Sulfate content in the redox zone water was approximately 50 mg/l; in lower water layers, it decreased to 23 mg/l. The thermocline (temperature decrease from 17 to 5°C) was located at depths from 7 to 19 m. At the interface between oxidized and reduced water, the pH was 7.0.

APB activity. APB development was detected at the upper border of the sulfide zone (depths from 29 to 30 m). In the redox zone, the water was transparent; however, in the course of settling, small slimy brown aggregates precipitated which were easily disintegrated by shaking. A peak of microbial numbers (6.11×10^6 cells/ml) and a weak peak of light CO_2 assimilation (0.27 μg C/(l day)) were detected at 30 m (Fig. 2). Photosynthesis in the anaerobic zone constituted 16.4% of the total oxygenic photosynthesis.

Pigment analysis revealed the presence of BChl *e* and BChl *a* in the redox zone water (Fig. 3a). The highest BChl *e* content (48 μg/l) was detected at a 30-m depth. The highest BChl *a* content (4.5 μg/l) was detected somewhat higher, at a 29-m depth. No pigments were detected in lake water above 28 m (Fig.2).

Our results correlate well with the data obtained over 30 years ago [1, 2]. Low APB density (considering the data on total microbial numbers) and activity result from the significant depth of the sulfide layer, where light penetration is limited.

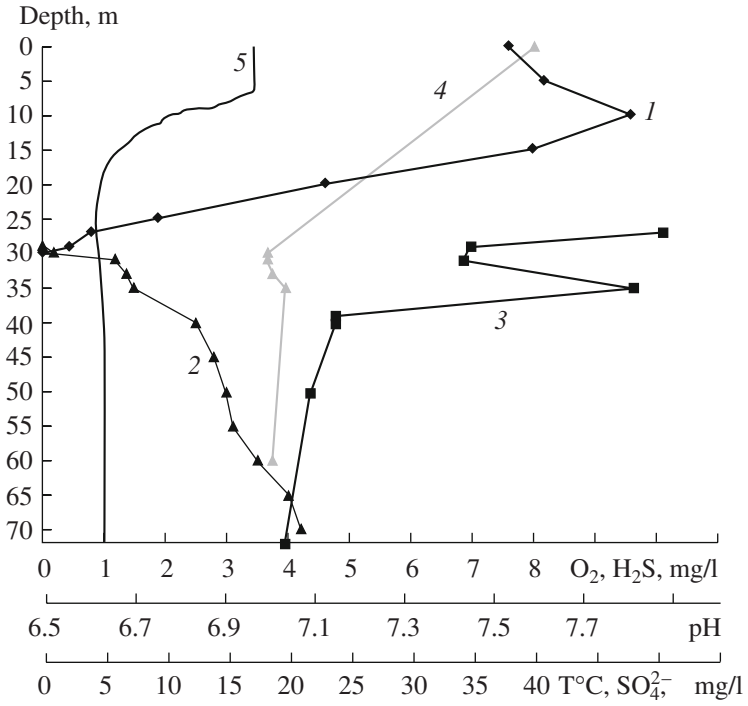

Fig. 1. Hydrochemical parameters of Lake Gek-Gel, September 2003: oxygen (*1*); sulfide (*2*); sulfate (*3*); pH (*4*); and temperature (*5*).

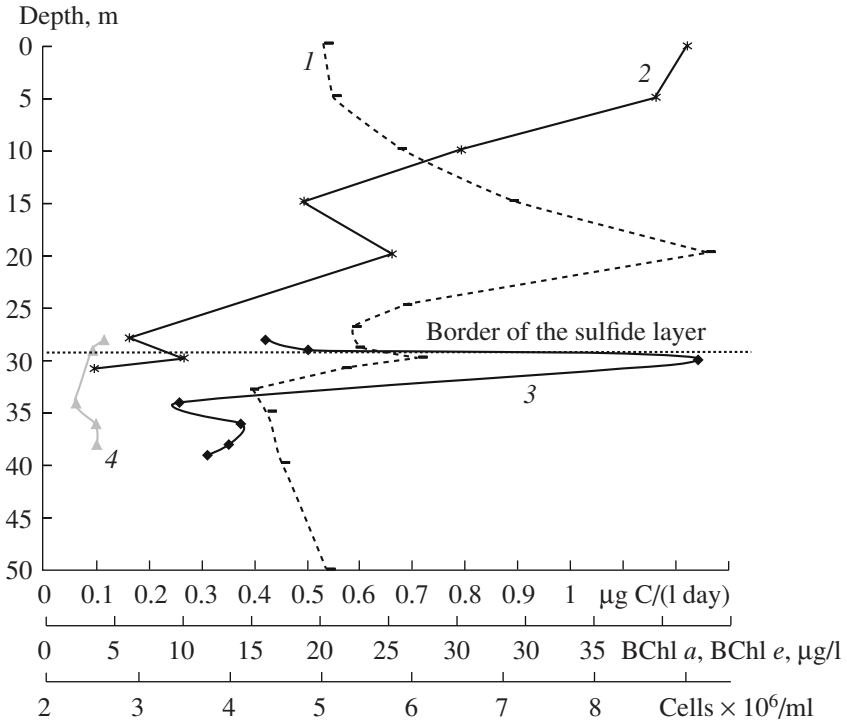

Fig. 2. Total microbial numbers, BChl content (in bacterial cells), and rate of light CO_2 assimilation in Lake Gek-Gel in September 2003: total microbial numbers (*1*); light CO_2 assimilation (*2*); BChl *e* content in bacterial cells (*3*); and BChl *a* content in bacterial cells (*4*).

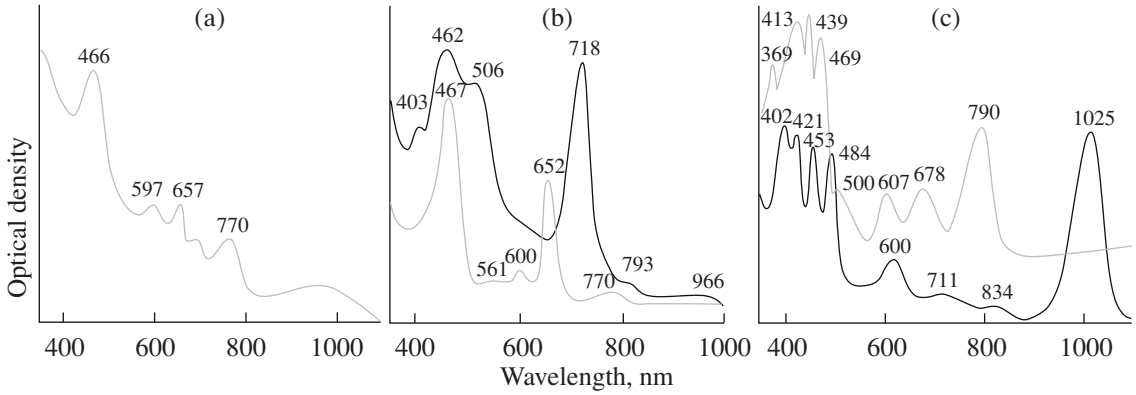

Fig. 3. Absorption spectrum of an acetone–methanol pigment extract of the water suspension from 28-m depth, Lake Gek-Gel, September 2003 (a); absorption spectra of the pigments of anoxygenic phototrophic bacteria isolated from Lake Gek-Gel: green sulfur bacterial strain G-g*Cphb*03 (b); and purple nonsulfur bacterial strain G-g*Blsv*03 (c). Solid lines indicate spectra of live cultures in glycerol; broken lines, spectra of acetone–methanol pigment extracts.

Isolation and identification of APB. Green sulfur bacteria of brown coloration were revealed in liquid enrichment culture obtained by inoculation of lake water from 31 m. Morphology and pigment composition of the isolate was similar to those of *Chl. phaeobacteroides* [13]. The cells were nonmotile rods (0.5 × 1–2 μm), mostly in chains (4–20 cells or longer), with the typical gram-negative cell wall structure. The cells did not contain gas vacuoles and sulfur inclusions. All the cytoplasm was filled with unusual granules surrounded by proteinaceous capsules (presumably polysaccharide inclusions) and polyphosphate granules (Figs 4a, 4b). The photosynthetic apparatus consisted of chlorosomes; BChl *e* and the carotenoid isorenieratine were the major pigments. Absorption spectra of the isolate coincided almost completely with the spectra of the water from 28–39 m (Figs 3a, 3b). The isolate of brown-colored green sulfur bacteria was assigned the strain name G-g*Cphb*03. Analysis of 16S rDNA sequence of strain G-g*Cphb*03 (GenBank accession no. EF654662) revealed 99.5% similarity with the type strain of *Chl. pheobacteroides* (Fig. 5).

Enrichment cultures of strain G-g*Cphb*03 contained small (0.15–0.8 μm) parasitic bacteria which have been observed to cause the lysis of this enrichment. The parasitic bacteria had vibrioid shape and gram-negative cell wall structure. Both single cells of parasitic bacteria and those attached to host cells similar to *Vampirovibrio* or *Micavibrio* were observed (Fig. 6) [14, 15].

Unlike earlier findings [2], minor numbers of purple nonsulfur bacteria (of green color) were revealed; their morphology and pigment composition resembled those of *Blastochloris sulfoviridis* (previously *Rhodopseudomonas sulfoviridis*) [16]. A single colony of these bacteria was found in the enrichment obtained by inoculation of a water sample from 30 m into agarized medium. The cells were relatively big (0.85 × 5–6.5 μm), budding, nonmotile, located in regular stacks parallel to each other. Subsequent cultivation resulted in sufficiently decreased cell size (0.7 × 2 μm). However, both small motile cells and larger budding nonmotile cells were present in young cultures (Figs. 4c, 4d). The cell wall was of a typical gram-negative type, with a characteristic outer membrane. No gas vacuoles or sulfur inclusions were present. Electron-transparent inclusions, probably of poly-β-hydroxybutyrate, were observed in the cytoplasm. The photosynthetic apparatus consisted of stacks of extensive membranes of lamellar type at the periphery of the cytoplasm (Figs. 4e–4h). Bacteria contained BChl *b*, which was responsible for a 1025-nm peak in the spectra of live cultures (Fig. 3c). Bacteria grew photoheterotrophically and utilized reduced sulfur compounds (sulfide or thiosulfate) as electron donors. The isolate was assigned the strain name G-g*Blsv*03. Analysis of the 16S rDNA sequence of strain G-g*Blsv*03 (GenBank accession no. EF654663) revealed 99.1% similarity with the type strain of *Bl. sulfoviridis* (Fig. 7).

DISCUSSION

Green sulfur bacteria of brown coloration develop in various types of meromictic water bodies. They include reservoirs with high water turbidity and those with great depths of the redox zone. *Chl. phaeobacteroides* are known to predominate in the fresh Green Lake (Fayetteville, New York) and in the saline Faro Lake (Sicily, Italy) [17]. In El Tobar lake (Cuenca, Spain), brown bacteria *Chl. phaeobacteroides* form an isolated layer [18]. Mass growth of *Chl. phaeobacteroides* in Lake Kinneret (northeastern Israel) was reported in [19]. *Chl. phaeobacteroides* of brown coloration often develop as components of APB communities. Oligotrophic meromictic Lake Maral-Gel (Azerbaijan), meromictic basins of the Banioles cavern lake (Catalonia, Spain), eutrophic meromictic Lake Vilar (Banioles,

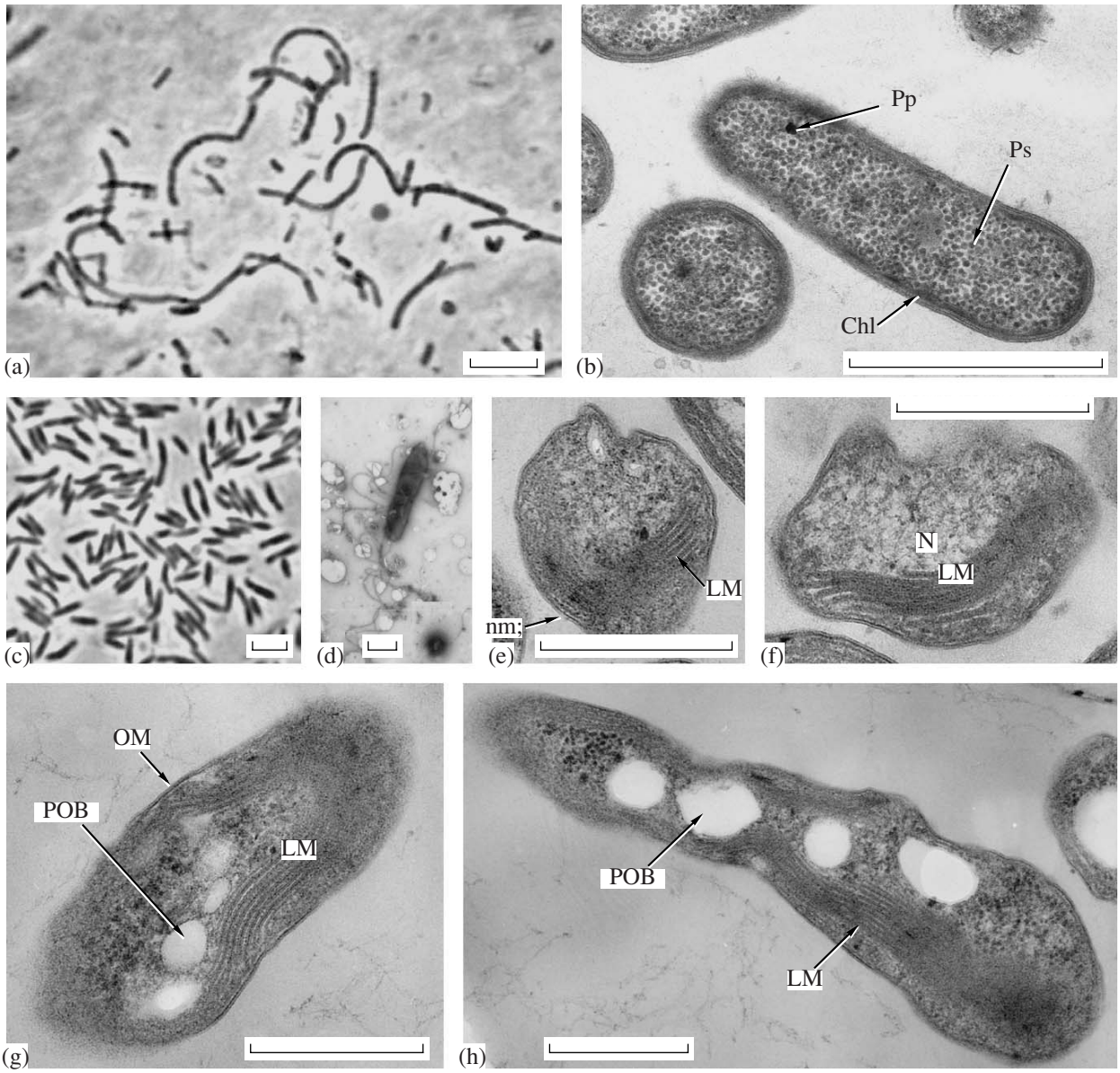

Fig. 4. Morphology and ultrastructure of strain G-g*Cphb*03 (a, b) and strain G-g*Blsv*03 (c–h). Phase contrast (a, c); section of green sulfur bacteria with chlorosomes located along the cell wall (b); negatively stained cell with peritrichous flagellation (d); transverse sections (e, f); longitudinal sections of the cells with the lamellar type of organization of intracytoplasmic photosynthetic membranes (g, h). Chl, chlorosomes; Pp, polyphosphate inclusions; Ps, unidentified inclusions surrounded by proteinaceous capsules (presumably polysaccharides); OM, outer membrane; LM, lamellar membranes; N, nucleoid, POB, poly-β-hydroxybutyrate. Scale bar is 5 μm (a); 1 μm (b, d); 2 μm (c), and 0.5 μm (e–h).

Spain), and the Cullera meromictic Mediterranean lagoon (Valencia, Spain) are some examples of this [17]. It has been previously noticed that the main characteristics of the Lake Gek-Gel regime and stratification of the water column are similar to those of the Black Sea [1]. An ecological niche occupied by phylogenetically related species of brown-colored green sulfur bacteria was therefore formed in the chemoclines of both water bodies [20].

While development of brown-colored sulfur bacteria at the depth of 30 m was to be expected, the presence of *Bl. sulfoviridis* in the chemocline of Lake Gek-Gel is highly unusual. These nonsulfur purple bacteria often occur in bog water, bogged soil, or wastewater, i.e., in the environments where dissolved sulfates are present, organic matter is abundant, and anaerobic or microaerobic conditions are created. Moreover, *Bl. sulfoviridis* are known to contain BChl *b* with the main absorption peak in the near infrared range (over 1000 nm). Longwave radiation does not penetrate deep into the water column; it is known to be quenched at a depth of several meters. Red and orange light is completely absorbed by

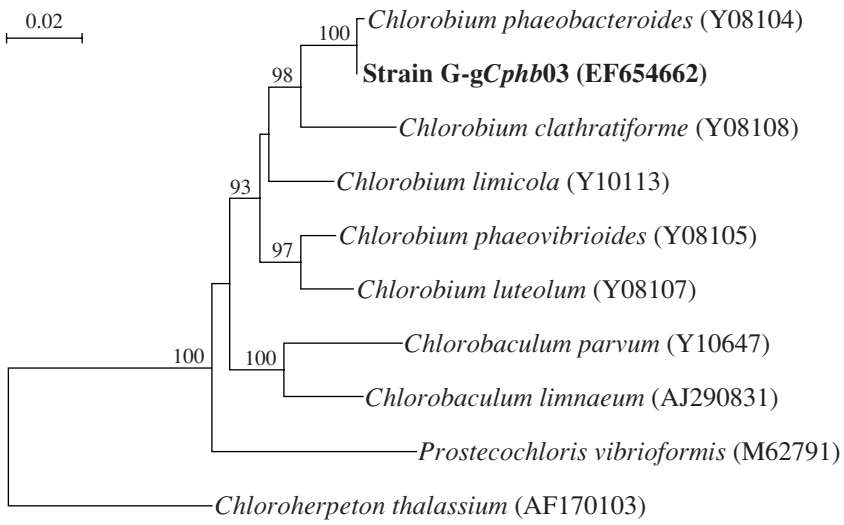

Fig. 5. Rootless phylogenetic tree of the family *Chlorobiaceae* demonstrating the position of strain G-g*Cphb*03. Numbers indicate bootstrap values.

a 5–8-m layer of water; only blue-green light penetrates deeper than 30 m into clear water [21].

Noticeable growth of *Bl. sulfoviridis* in Lake Gek-Gel chemocline is hardly probable. However, absorption spectra of live cultures of these bacteria include a peak in the blue range (450–460 nm). This is precisely the range penetrating to the greatest depth; the fact that brown-colored green sulfur bacteria absorb this wave range due to the presence of isorenieratine carotenoids enables their survival under conditions of almost complete absence of illumination [20].

Bl. sulfoviridis are freshwater bacteria with a pH optimum at 6.5–7 [15]. These parameters are relatively close both to the optimal values for *Chl. phaeobacteroides* (pH 6.5–7.3; salinity at least 1%) and to the hydrochemical state of the Lake Gek-Gel chemicline in September 2003 (Fig. 1) [13].

The presence of a weak BChl *a* maximum (at 29 m) suggests that low numbers of other as yet not isolated

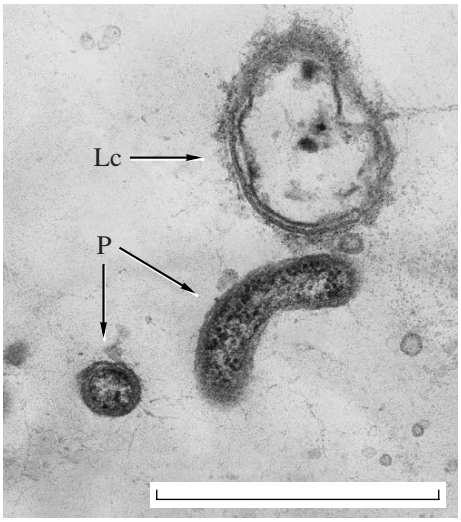

Fig. 6. Ultrathin section of the parasitic bacteria found in the enrichment culture of brown-colored sulfur bacteria G-g*Cphb*03. P, parasite cell; Lc, lysed host cell. Scale bar is 1 μm.

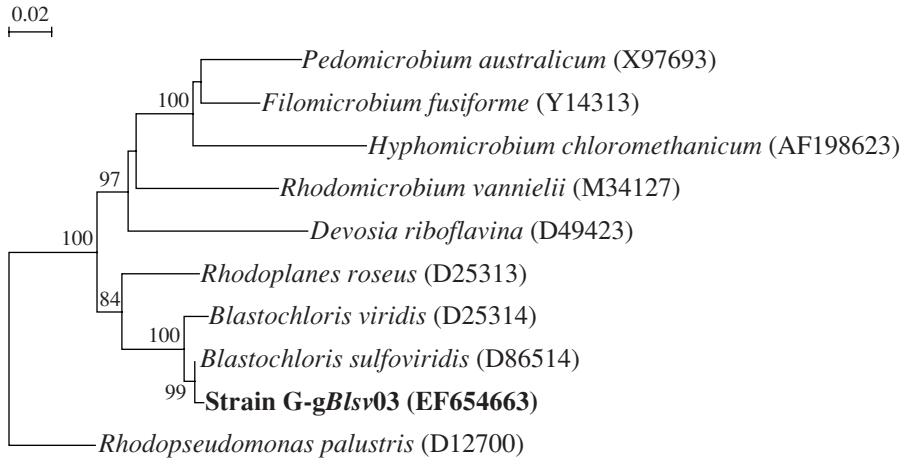

Fig. 7. Rootless phylogenetic tree of the family *Hyphomicrobiaceae* demonstrating the position of strain G-g*Blsv*03. Numbers indicate bootstrap values.

phototrophic bacteria adapted to low light intensity may be present at the Lake Gek-Gel oxic–anoxic interface, in spite of significant depth.

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