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EXPERIMENTAL ARTICLES

Chlorobaculum macestae sp. nov., a New Green Sulfur Bacterium

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Abstract—The investigated green sulfur bacterium, strain M, was isolated from a sulfidic spring on the Black Sea Coast of the Caucasus. The cells of strain M are straight or curved rods 0.6–0.9 × 1.8–4.2 μm in size. According to the cell wall structure, the bacteria are gram-negative. Chlorosomes are located along the cell periphery. Strain M is an obligate anaerobe capable of photoautotrophic growth on sulfide, thiosulfate, and H_2. Acetatate is utilized as an additional carbon source. It utilizes ammonium, urea, casein hydrolysate, and N_2 as nitrogen sources and sulfide, thiosulfate, and elemental sulfur as sulfur sources. Bacteriochlorophyll *c* and the carotenoid chlorobactene are the main pigments. The optimal growth temperature is 25–28°C; the optimal pH is 6.8. The strain does not require NaCl. Vitamin B_{12} stimulates growth. The content of the G+C base pairs in the DNA of strain M is 58.3 mol %. In the phylogenetic tree constructed on the basis of analysis of nucleotide sequences of 16S rRNA genes, strain M forms a separate branch, which occupies an intermediate position between the phylogenetic cluster containing representatives of the genus *Chlorobaculum* (94.9–96.8%) and the cluster containing species of the genus *Chlorobium* (94.1–96.5%). According to the results of analysis of the amino acid sequence corresponding to the *fmo* gene, strain M represents a branch which, unlike that in the "ribosomal" tree, falls into the cluster of the genus *Chlorobaculum* (95.8–97.2%). Phylogenetic analysis of the amino acid sequence corresponding to the *nifH* gene placed species of the genera *Chlorobaculum* and *Chlorobium* into a single cluster, whereas strain M formed a separate branch. The results obtained allow us to describe strain M as a new species of the genus *ChlorobacChlorobaculum – Chlorobaculum macestae* sp. nov.

Key words: green sulfur bacteria, phylogenetic analysis, 16S rRNA, *fmo, nifH, Chlorobaculum macestae.*

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Green sulfur bacteria are represented by the family *Chlorobiaceae*. They belong to a physiologically uniform group and form a separate phylogenetic branch (phylum *Chlorobi*) [1, 2]. All of them are obligate anaerobes and phototrophs. Their photosynthetic apparatus is located in chlorosomes. The cells contain bacteriochlorophyll *a*, although in trace concentrations. The main bacteriochlorophyll (bchl) pigments in green sulfur bacteria are bchl *c*, *d*, or *e*. Brown-colored species contain bchl *e* and, as a rule, the carotenoids isorenieratene and β-isorenieratene as the major light-harvesting pigments, whereas green-colored species contain bchl *c* or *d* and the carotenoids chlorobactene and OH-chlorobactene.

When grown autotrophically, green sulfur bacteria utilize various reduced sulfur compounds and molecular hydrogen as electron donors. Autotrophic assimilation of carbon dioxide occurs via operation the reductive tricarboxylic acid cycle. Only a few organic compounds (including acetate, pyruvate, and propionate) can be utilized as carbon sources supplementary to CO_2.

The traditional taxonomy of this group of bacteria is based on such phenotypic properties as cell morphology, pigment composition, and physiological and biochemical properties [1, 2]. The formation of gas vacuoles was considered an important trait to differentiate genera within this group. At the species level, strong emphasis was placed on the culture coloration (green/brown); on the basis of thiosulfate utilization capacity, subspecies were differentiated.

The recent reorganization of the taxonomy of green sulfur bacteria [3] was based on comparative analysis of the nucleotide sequences of the 16S rRNA genes, as well as of the *fmo* gene, encoding the Fenna–Matthews–Olson (FMO) protein, which occurs only in representatives of this bacterial group. Only those species whose type strains are not present in culture collections

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escaped reclassification, as well as *Chloroherpeton thalassium*, which forms a separate branch in the phylogenetic tree and exhibits clear-cut phenotypic distinctions from other members of the family *Chlorobiaceae*. The properties which were previously taken into account when dealing with the taxonomy of green sulfur bacteria have no taxonomic significance any more. Although the cell size, DNA G+C content, and requirement for NaCl are still taken into consideration, the phylogenetic positions of the bacteria under question in the relevant trees are now the focus of attention [3]. Consequently, strains that had been previously affiliated to the same species were distributed, in a number of cases, among several species and even genera.

The purpose of this work was to describe a new green sulfur bacterium isolated from a freshwater sulfidic spring at Matsesta Spa Resort (Russia).

MATERIALS AND METHODS

Isolation and cultivation. Strain M was isolated and cultivated on Larsen medium [4] supplemented with $NaHCO_3$, 2 g/l; $Na_2S \cdot 9H_2O$, 1 g/l; and $Na_2S_2O_3$, 2 g/l. Isolation of pure cultures was carried out using agarized (0.7%) medium of the same composition. The obtained culture was tested for purity by microscopic examination, as well as by inoculation of agarized media for sulfate-reducing bacteria and nutrient broth with 1% glucose.

The culture was grown under anaerobic conditions at 25–28°C in 50-ml glass vials with ground glass stoppers and 30-ml tubes completely filled with medium and placed in a luminostat (2000 lx).

Cell morphology and ultrastructure. The cell morphology of the isolate was studied under a Laboval-4 phase-contrast microscope equipped with a digital photographic camera. The cell ultrastructure was studied under a Hitachi-12 transmission electron microscope at an accelerating potential of 75 kV as previously described [5].

Cell pigment composition. The pigment composition of the bacterium was studied in extracts of ultrasonically disrupted cells after sedimentation of debris and undestroyed cells by centrifugation (16000 *g*, 4°C, 20 min), as well as in acetone–methanol (7 : 2) extracts. The absorption spectra were recorded using a Hitachi 200-20 spectrophotometer (wavelength range, 350 to 900 nm). The carotenoid composition was analyzed by thin-layer chromatography [6].

The fatty acid composition was determined using a Hewlett Packard HP-5973 mass spectrometer [7]. Cells grown under autotrophic conditions and harvested at the late exponential phase were used for this analysis.

Physiological and biochemical properties. To determine the ability of the isolate to utilize organic compounds as the only or additional carbon source, the medium was supplemented with the following compounds in a concentration of 0.1%: glucose, maltose, lactose, acetate, pyruvate, propionate, lactate, malate, succinate, fumarate, butyrate, ethanol, methanol, or glycerol.

To determine the ability of the isolate to utilize various nitrogen sources, the following compounds were added to the medium in a concentration of 0.1%: ammonium chloride, potassium or sodium nitrates, urea, amino acids (proline, asparagine, aspartate, glutamine, glutamate, or arginine), yeast extract, or casein hydrolysate. Larsen medium that did not contain any nitrogen source was used as a control. The ability to assimilate molecular nitrogen was tested by cultivating the bacterium in 500-ml vials filled to 1/3 of the volume with Larsen medium devoid of bound nitrogen, gassed with N_2 under sterile conditions and sealed with rubber stoppers.

The following inorganic sulfur-containing compounds were tested as sulfur sources: molecular sulfur, sulfide, thiosulfate, and sulfite (0.05–0.1%).

The growth of the bacteria was assessed by measuring optical density of the medium at 750 nm with a Hitachi-200-20 spectrophotometer.

The content of G+C base pairs in the DNA was determined as previously described in [8]; DNA was extracted from the biomass by the Marmur method [9].

DNA isolation and PCR. The DNA for PCR analysis were obtained according to the previously described technique [10]. Amplification of the 16S rRNA genes was performed with standard bacterial primers [11]; the fragments of the *nifH* and *fmo* genes were amplified as described in [10] and [12], respectively.

Sequencing of PCR products was performed on an automatic ABI 3700 sequencer (Applied Biosystems, United States) using a Big Dye Terminator sequencing kit (version 3.1).

Phylogenetic analyses. Primary comparison of the de novo determined sequences with sequences within the GenBank database was performed using the NCBI BLAST software package (http://www.ncbi.nlm.nih.gov/blast). The nucleotide and corresponding amino acid sequences were aligned with sequences retrieved from the GenBank database using the CLUSTAL W software package. The phylogenetic trees were constructed with the use of various algorithms implemented in the TREECONW software package [13].

Deposition of nucleotide sequences. The obtained nucleotide sequences of the 16S rRNA, *fmo*, and *nifH* genes have been deposited in the GenBank under the accession numbers EF560696, EF560697, and EF560698, respectively.

RESULTS AND DISCUSSION

Isolation of the new green sulfur bacterium. The studied green sulfur bacterium, strain M, was isolated

from a cyanobacterial mat from a sulfidic spring at Matsesta Spa Resort on the coast of the Black Sea (Krasnodar Krai, Russia). The total water mineralization in the spring was 11 g/l; the hydrogen sulfide concentration was 200 mg/l; the total CO_2 content was 200 mg/l; the pH of the water was 6.8; and the temperature at the moment of sampling was 25°C.

Cultural properties of strain M. When grown in liquid medium with sulfide, the exponential phase culture turns turbid due to the production of elemental sulfur (which accumulates outside the cells) and light green in color. Later on, upon the exhaustion of sulfide, the accumulated sulfur is consumed, and the culture turns dark green in color. The stationary phase culture gets slimy and, with time, turns yellow. On agarized medium, small spherical colonies are formed.

Morphological properties of strain M. The cells of the strain M are straight or curved rods 0.6–0.9 × 1.8–4.2 μm in size, multiplying by binary fission and occurring singly or in pairs (Fig. 1). The ultrastructure of the cell wall is of the gram-negative type. Along the cell periphery, chlorosomes are located. The cytoplasm contains electron-dense inclusions resembling polyphosphate granules. No gas vacuoles were detected in strain M cells.

Pigments. In the absorption spectra of sonicated strain M cells, the absorption maximum was observed in the infrared spectral region at 736 nm; in the methanol–acetone extract, the maximum occurred at 660 nm. These facts suggest the presence of bchl *c* in the cells (Fig. 2). Chlorobactene is the main carotenoid, with absorption maximums at 435, 460, and 489 nm in acetone.

Physiological properties of strain M. Strain M is an obligate anaerobe able to grow photoautotrophically and to utilize sulfide, thiosulfate, and H_2 as electron donors. The optimal concentration of sulfide (in the form of $Na_2S \cdot 9H_2O$) for strain M is 0.25–1.00 g/l; that of sodium bicarbonate, 1–2 g/l. Sulfide and thiosulfate are oxidized to sulfates with the resulting formation of elemental sulfur. The best growth of strain M was noted under autotrophic conditions in the presence of sulfide and bicarbonate. Acetate is utilized as an additional carbon source.

Ammonium chloride and casein hydrolysate were the best nitrogen sources for strain M. Urea could be the nitrogen source as well. The presence of nitrates did not stimulate the culture growth; growth on amino acids was poor. Strain M exhibited the capacity for nitrogen fixation.

The bacterium utilized sulfide, thiosulfate, and elemental sulfur as sources of sulfur. Sulfite, sulfates, methionine, and cysteine did not support growth.

Vitamin B_{12} produced a stimulatory effect on the growth of the studied bacterium. The optimal growth temperature was 25 to 28°C. The strain grew in a pH range of 6.5–7.5 with an optimum at 6.8.

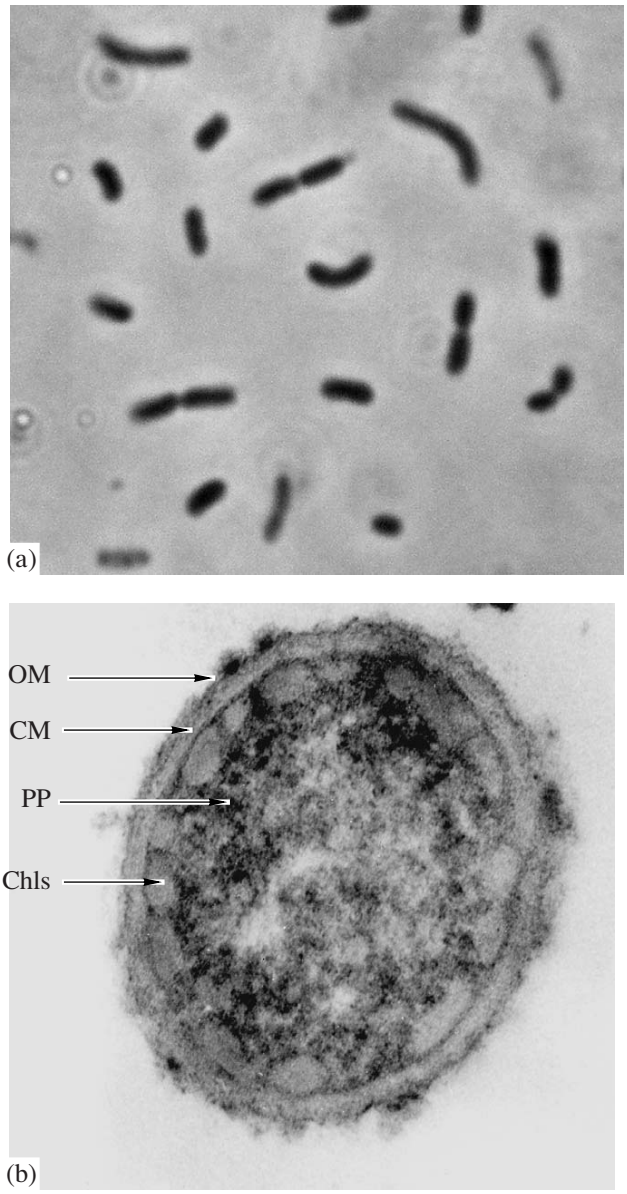

Fig. 1. Morphology and ultrastructure of strain M cells: (a) phase contrast micrograph (bar, 10 μm); (b) electron micrograph of an ultrathin section of a cell, showing chlorosome (Chls), outer (OM) and cytoplasmic (CM) membranes, and polyphosphate granules (PP).

Strain M does not require NaCl. Its addition to the medium in a concentration up to 2% did not exert a pronounced effect on the growth. A further increase in the NaCl concentration decreased growth rate; growth ceased at a NaCl concentration of 5%.

Phylogenetic position. The nearly complete 16S rRNA gene sequence (1394 nucleotides) of strain M has been determined. Preliminary screening in the GenBank database using BLAST software revealed that the affiliation of the strain in question with green sulfur bacteria. A more detailed phylogenetic analysis showed that strain M is most closely related to strain 1 of the

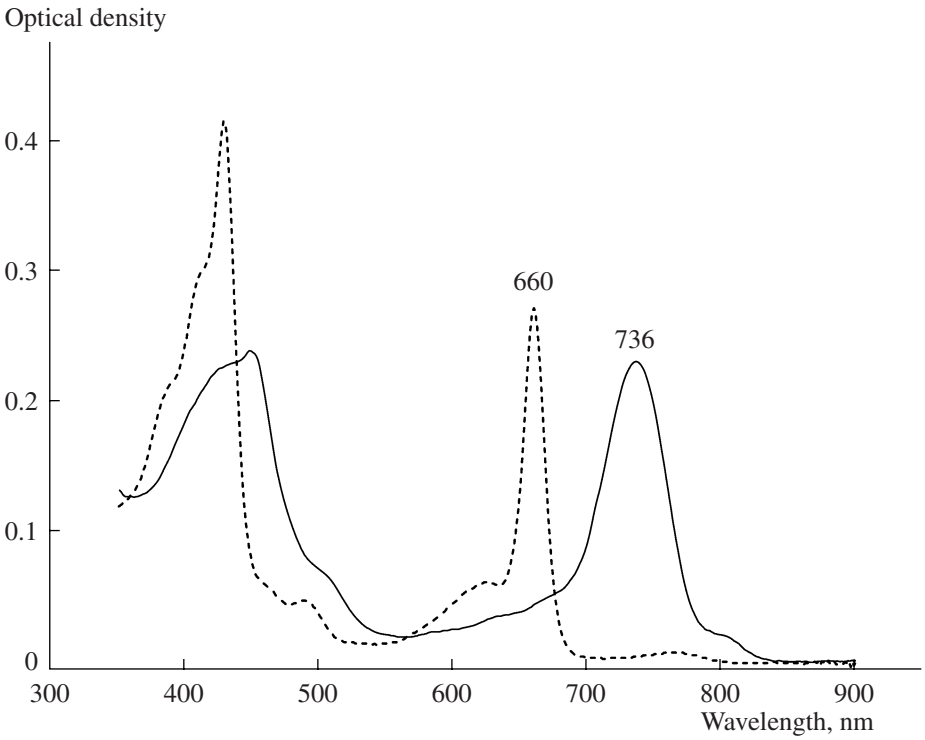

Fig. 2. Absorption spectra of whole cells (continuous line) and acetone–methanol extracts (discontinuous line) of strain M cells.

species 'Clathrochloris sulfurica' (98.1% 16S rDNA similarity) [14]; however, this species has been excluded from the taxonomy of *Chlorobiaceae* due to the loss of the type strain and lack of effective publication [3]. At the same time, noteworthy is the fact that the phenotypic properties of 'Ctl. sulfurica' (pigment composition, ability to form gas vacuoles, and peculiar net-like microcolonies) [15] coincide with those of *Chlorobium luteolum* (formerly, *Pelodictyon luteolum*) and differ from those of representatives of the genus *Chlorobaculum*, as well as from the properties of strain M.

In the constructed phylogenetic tree (Fig. 3), strain M and 'Ctl. sulfurica' form a separate branch which occupies an intermediate position between the phylogenetic cluster which has been recently classified as a new genus, *Chlorobaculum*, and that the cluster comprising representatives of the genus *Chlorobium* [3]. The levels of 16S rRNA similarity between strain M and representatives of the genera *Chlorobaculum* and *Chlorobium* were almost the same (94.9–96.8 and 94.1–96.5%, respectively). The levels of similarity between the studied strain and species of the genera *Prosthecochloris* and *Chloroherpeton* were noticeably lower (91.0–95.4 and 88.2%, respectively).

To determine more precisely the phylogenetic position of strain M, the nucleotide sequence of a *fmo* gene fragment was determined. From the obtained nucleotide sequence, and the amino acid sequence (283 amino acid residues) was deduced and aligned with the corresponding sequences of *Chlorobiaceae* representatives. In the obtained phylogenetic tree (Fig. 4), strain M occupies a separate branch which, unlike that on the "ribosomal" tree, falls into the cluster formed by the genus *Chlorobaculum.* The level of FMO amino acid identity between strain M and representatives of the genus *Chlorobaculum* (95.8–97.2%) was considerably higher than that between this strain and members of other genera of green sulfur bacteria (76.0–89.4%).

The *nifH* gene, a component of the nitrogenase complex, has been analyzed as well. From the determined nucleotide sequence, the amino acid sequence (149 amino acid residues) was deduced and aligned with the corresponding sequences of *Chlorobiaceae* representatives. In the obtained phylogenetic tree (Fig. 5), the species of the genera *Chlorobaculum* and *Chlorobium* form a single cluster, and strain M occupies a separate phylogenetic branch specifically related to this cluster with a bootstrap support of 86%.

The content of G+C base pairs in the DNA of strain M is 58.3 mol % (Tm).

Fatty acid (FA) composition. Analysis of the fatty acid composition of strain M cells indicated that myristic (14:0), palmitic (16:0), and hexadecenoic (16:1) fatty acids prevailed, which is typical of *Chlorobiaceae* [2, 3]. However, the fatty acid composition of strain M differed from those of other members of the genus *Chlorobaculum*. In this strain, 14:0, 16:0, and 16:1 fatty acids make up 16, 25, and 50%, respectively, whereas in *Cba. thiosulfatiphilum*, these values comprise 21, 10,

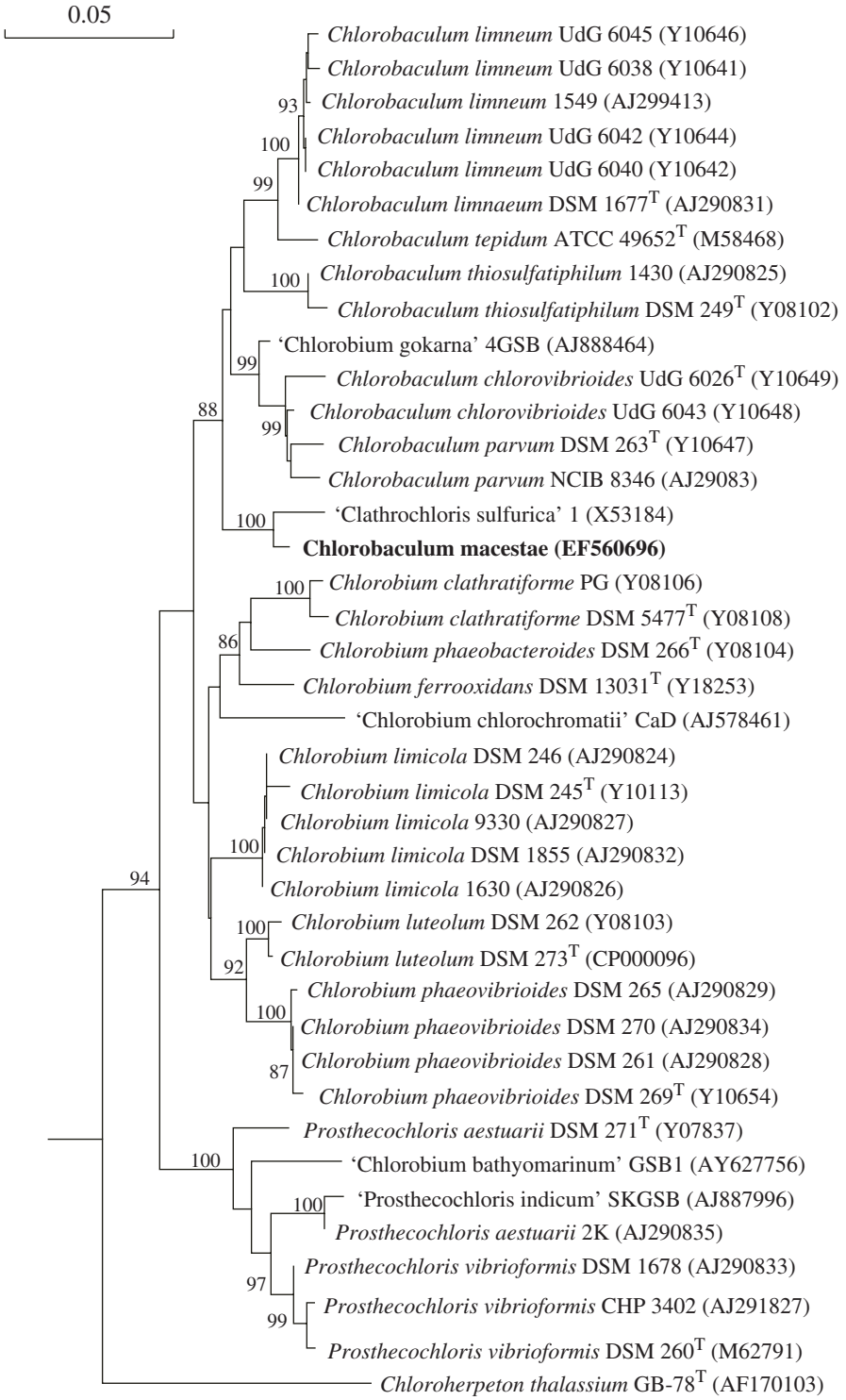

Fig. 3. Phylogenetic tree of the family *Chlorobiaceae* constructed on the basis of the 16S rRNA gene sequences.

and 43%, and in *Cba. parvum*, 24, 23, and 43% [3] (see Tables 1 and 2). At the same time, caution is required in the interpretation of the results obtained during the analysis of the fatty acid composition, since this parameter has been assessed in only a half of the known species of green sulfur bacteria. Moreover, it is well-known that the composition of fatty acids may vary depending on the culture age and growth conditions.

Conclusion. The levels of 16S rRNA similarity between strain M and the known representatives of the

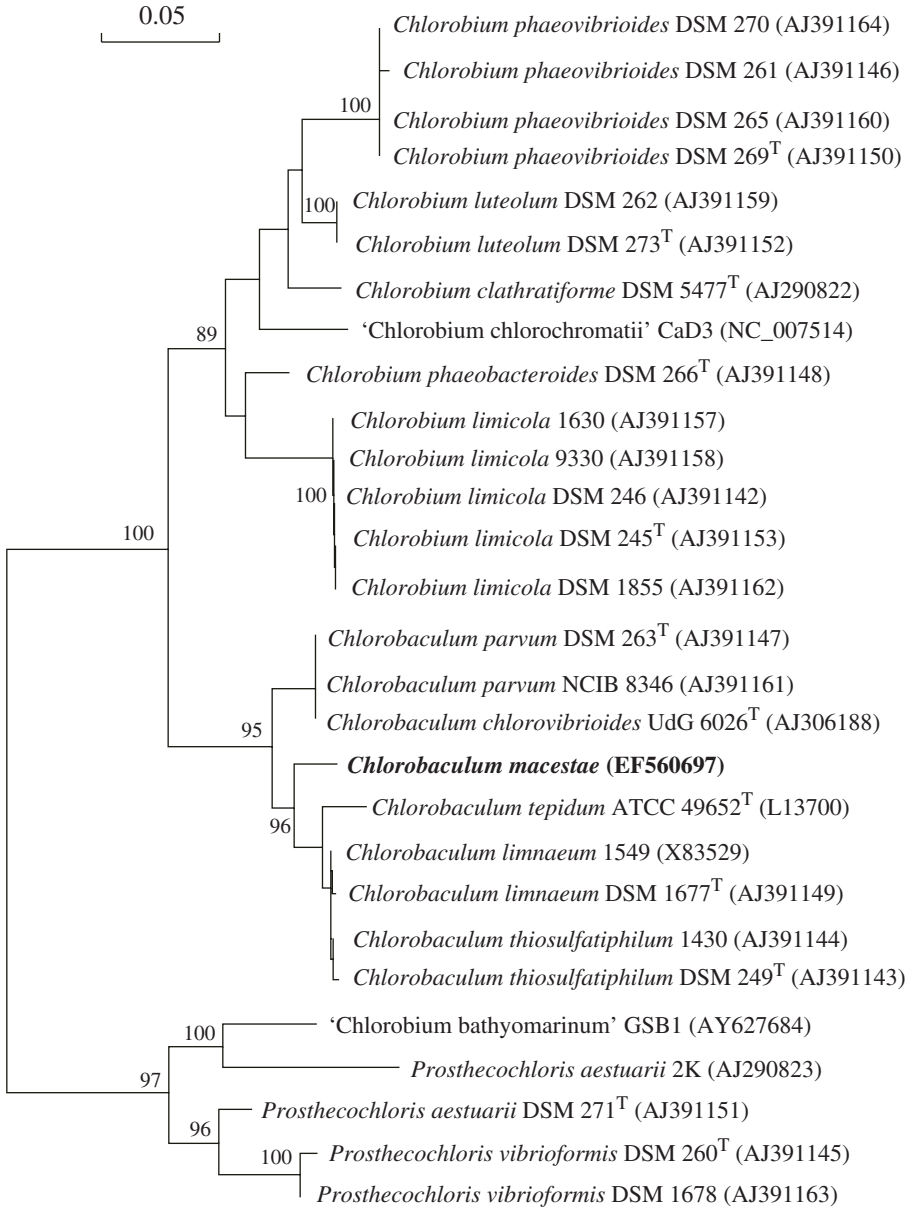

Fig. 4. Phylogenetic tree of the family *Chlorobiaceae* constructed on the basis of the analysis of the amino acid sequences of FMO proteins.

genera *Chlorobaculum* and *Chlorobium*, the closest relatives of the strain under study, do not differ significantly and, at the same time, do not exceed 96.8%. This fact prevented us form affiliating it with any previously described species; moreover, it hindered its generic affiliation. Analogous results were obtained in the analysis of the amino acid sequences of the *nifH* gene. At the same time, the results of the analysis of the gene encoding FMO protein, specific to green sulfur bacteria, indicated that strain M belongs to the genus *Chlorobaculum*. According to the data obtained, among the known representatives of the genus *Chlorobaculum*, strain M is phylogenetically closest to *Cba. parvum* and *Cba. chlorovibrioides*; however, it exhibits clear-cut phenotypic distinctions from these species (Table 2). The content of G+C base pairs in the DNA of strain M is 58.3%, whereas it is 53.5% and 54.0% in *Cba. parvum* and *Cba. chlorovibrioides*, respectively. Unlike in *Cba. parvum* and *Cba. chlorovibrioides*, bchl *c* rather than bchl *d* is the main bacteriochlorophyll in strain M. In addition, these microorganisms differ in their fatty acid compositions. The phenotypic properties of strain M are closest to those of *Cba. thiosulfatiphilum*; however, these bacteria differ significantly in their 16S rRNA and *fmo* gene sequences, as well as in the fatty acid composition. Thus, the results obtained indicate that the taxonomic status of strain M conforms to at least a species level.

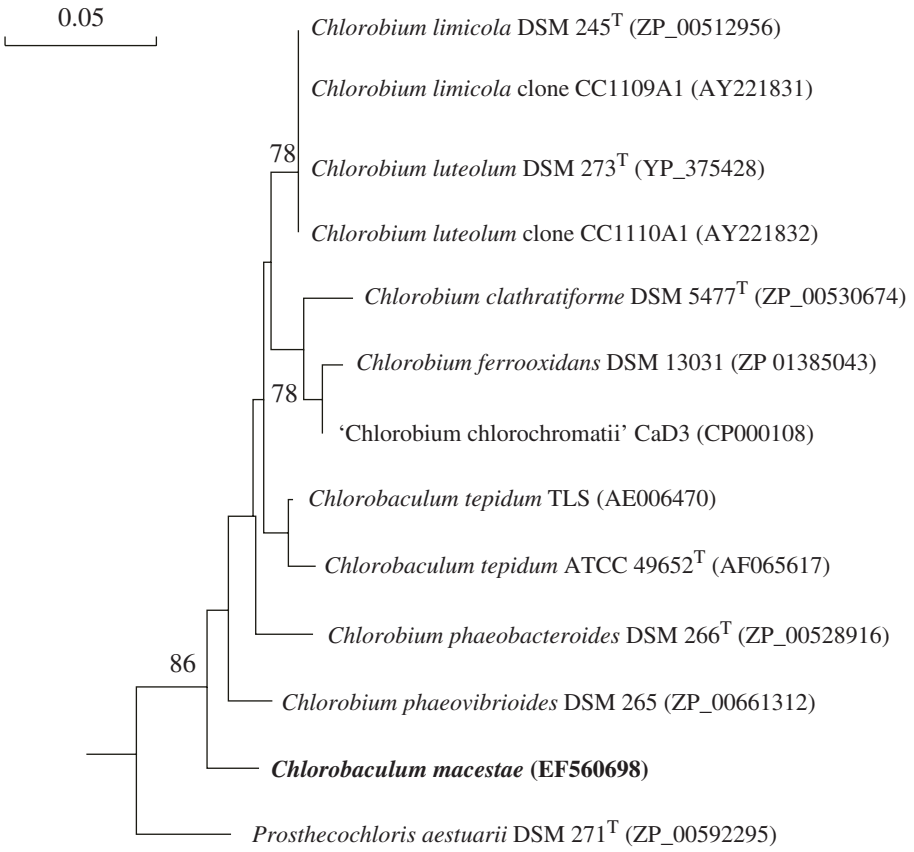

Fig. 5. Phylogenetic tree constructed on the basis of the analysis of the amino acid sequences corresponding to the *nifH* genes.

Description of *Chlorobaculum macestae* sp. nov. *macestae* (ma'ces.tae, from Matsesta).

Cells are rods, 0.6–0.9 × 1.8–4.2 μm, occurring singly or in pairs and multiplying by binary fission. The ultrastructure of the cell wall is of the gram-negative type. Chlorosomes are located along the cell periphery. When grown in liquid medium with sulfide, the culture is green-colored; bchl *c* and chlorobactene (the main carotenoid) are the major photosynthetic pigments. The bacterium is an obligate phototroph and anaerobe incapable of assimilatory sulfate reduction. Photolithoautotrophic growth occurs in the presence of sulfide, thiosulfate, sulfur, or H_2 as electron donors. Sulfide and thiosulfate are oxidized to sulfates with the formation of elemental sulfur as an intermediate product. Acetate is utilized as an additional carbon source. The optimal growth temperature is 25–28°C; the optimal pH is 6.8. NaCl is not required; however, active growth occurs at NaCl concentrations of up to 20 g/l. Vitamin B_{12} stimulates growth. Ammonium chloride and casein hydrolysate are the best nitrogen sources. The strain is capable of nitrogen fixation. Myristic (14:0), palmitic (16:0), and hexadecenoic (16:1) are the major fatty acids (16, 25, and 50%, respectively). The DNA G+C content is 58.3 mol %.

The levels of 16S rRNA similarity with representatives of the genera *Chlorobaculum* and *Chlorobium* are 94.9–96.8% and 94.1–96.5%, respectively.

The type strain is M (KM MGU-466, Collection of Microorganisms, Moscow State University).

Table 1. Fatty acid composition of the green sulfur bacterium strain M

Fatty acids		%
Lauric	12:0	2.8
Tetradecenoic	14:1	1.7
Myristic	14:0	16.1
Pentadecanoic	15:0	0.5
ω5-Hexadecenoic	16:1ω5	50.8
ω7-Hexadecenoic	16:1ω7	1.1
Palmitic	16:0	25.2
Oleic	18:1w9	0.9
cis-Vaccenic	18:1w7	0.5
Stearic	18:0	0.45

Table 2. Properties of green sulfur bacteria (data from [2, 3, and 16] and this work)

Organism name	Utilization of $S_2O_3^{2-}$	Cell width, μm	NaCl requirement	Vitamin requirements	Major bchl	Main carotenoids	G+C, mol. %	Gas vacuoles	Major fatty acids , %		
									14:0	16:0	16:1
Chlorobium											
Chlorobium limicola	+/–	0.7–1.1	No	B_{12}	*c* or *e*	chlb, iso	51.0–52.5	–	13	17	57
Chlorobium phaeobacteroides	–	0.6–0.8	No	B_{12}	*e*	iso	49.0–50.0	–	16	15	64
Chlorobium clathratiforme	+/–	0.7–1.2	No	B_{12}	*c* or *e*	chlb, iso	47.9–49.0	+			
'Chlorobium ferrooxidans'		0.5	No		*c*	chlb		–			
Chlorobium luteolum	–	0.6–0.9	>1%	B_{12}	*c*, *d*	chlb	57.1–58.1	+/–	14	21	47
Chlorobium phaeovibrioides	+/–	0.3–0.7	>1%	B_{12}	*c*, *d*, *e*	chlb, iso	52.0–53.5	–	10–12	23–29	51–52
Chlorobium chlorochromatii	–	0.4–0.6	No	B_{12}	*c*	γ-carotene	46.7	–			
Prosthecochloris											
Prosthecochloris aestuarii	–	0.5–0.7	2–5%	B_{12}	*c*	chlb	52–56.1	–			
Prosthecochloris vibrioformis	–	0.5–0.7	>1%	B_{12}	*c*, *d*, *e*	chlb, iso	53.5	+/–			
*Prosthecochloris phaeoasteroidea**	–	0.5–0.6	0.5–2%	–	*e*	iso	52.2 ± 0.8	–			
Chlorobaculum											
Chlorobaculum tepidum	+	0.6–0.8	No	B_{12}	*c*	chlb	56.5	–			
Chlorobaculum limnaeum	–	0.6–0.8	No	B_{12}	*e*	iso		–			
Chlorobaculum thiosulfatiphilum	+	0.7–1.1	No	B_{12}	*c*	chlb	58.1	–	21	10	43
Chlorobaculum parvum	+	0.7–1.1	>1%		*d*	chlb	56.1–56.6	–	24	23	42.8
'Chlorobaculum chlorovibrioides'	–	0.3–0.4	2–3%		*d*	chlb	54.0	–			
Chlorobaculum macestae	+	**0.6–0.9**	No	**B_{12}**	***c***	**chlb**	**58.3**	–	**16**	**25**	**50**
'*Pelodictyon*'**											
*'Pelodictyon phaeum'**		0.6–0.9	3%	B_{12}	*e*	iso	ND	+			
*Ancalochloris**											
*Ancalochloris perfilievii**	ND	0.5–1.0	ND	B_{12}	ND	ND	ND	+			
Chloroherpethon											
Chloroherpethon thalassium	ND	1	1–2%	B_{12}	*c*	γ-carotene	47.8	+			

Notes: Chlb stands for chlorobactene; iso stands for isorenieratene; ND stands for "no data".

* The type strains have been lost.

** The future of these species and genus is uncertain, since the type strain of the type species *Pelodictyon clathratiforme* has been lost, and the only known strain of *Pld. clathratiforme* has been merged with *Pld. phaeoclathratiforme* and transferred to *Chlorobium clathratiforme*.

Deposited with VKM (VKM-2470).

The 16S rRNA gene sequence of the type strain has been deposited with GenBank under accession number EF560696.

The habitat is a sulfidic spring at Matsesta Spa Resort, on the Coast of the Black Sea in the Caucasus. The total water mineralization in the spring is 11 g/l, the hydrogen sulfide concentration is 200 mg/l, the total content of carbon dioxide is 200 mg/l, the pH is 6.8, and the temperature at the moment of sampling was 25°C.

The strain belongs to the phylum *Chlorobi*, class *Chlorobia*, order *Chlorobiales*, family *Chlorobiaceae.*

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