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EXPERIMENTAL ARTICLES

Rubribacterium polymorphum gen. nov., sp. nov., a Novel Alkaliphilic Nonsulfur Purple Bacterium from an Eastern Siberian Soda Lake[1]

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Abstract—An alkaliphilic nonsulfur purple bacterial (NPB) strain "Green" was isolated from sediments of the littoral zone of the soda lake (mineralization 22 g/l, pH 9.5) in the Barguzin River valley (Eastern Siberia). The cells of the new isolate are ovoid or polymorphic at latter stages. The photosynthetic membrane structures are of vesicular type. Bacteriochlorophyll *a* and carotenoids of both spheroidene and spirilloxanthin type are the photosynthetic pigments. Two light-harvesting systems (LH1 and LH2) are present. The new isolate is a photoheterotroph and a facultative aerobe. It grows well in the dark on organic substrates; anaerobic phototrophic growth is poor. The isolate is alkaliphilic with pH optimum of 8.5–9.5. The most abundant cell growth occurred at 5–40 g/l NaCl (optimum at 10 g/l) and 30°C. The DNA G+C base content was 69.9 mol %. Analysis of 16S rRNA gene sequences revealed a 10% difference with the most closely related NPB (*Rhodobacter* species). *Rubrimonas cliftoensis*, a bacteriochlorophyll *a*-containing bacterium, is the closest relative (93.3% similarity). It is proposed that strain "Green" should be placed in the new genus and new species *Rubribacterium polymorphum* gen. nov., sp. nov. GenBank accession number: 16S rRNA–EU857676.

Key words alkaliphilic anoxygenic phototrophs, nonsulfur purple bacteria, *Rubribacterium polymorphum* gen. nov., sp. nov.

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During last years, several new representatives of the alkaliphilic purple sulfur bacteria of *Ectothiorhodospiraceae* (*Gammaproteobacteria*) were described, which have characteristic new propertiescompared to previously known members of this family [1–4]. Purple nonsulfur bacteria (NPB) are shown to be widespread in weakly and moderately mineralized soda lakes [5–8]. Nevertheless, only two alkaliphilic species are known; they belong to the genus *Rhodobaca* [9–11]. In the present work, morphophysiological characterization and taxonomic description of a new alkaliphilic nonsulfur purple bacterium, *Rubribacterium polymorphum* gen. nov., sp. nov., isolated from a low mineralized soda lake in the Barguzin Valley (Eastern Siberia) is given.

MATERIALS AND METHODS

Sample collection and enrichment. The new strain "Green" was isolated from the samples of sediments of the littoral zone of a small nameless soda lake in the Barguzin Valley of Eastern Siberia (54°11'07" N, 110°29'35" E). Soda deposits are present along the coast. At the time of sampling, mineralization of the water was 22 g/l; pH 9.5. Due to intense growth of plankton cyanobacteria, the water had green coloration.

Culture medium and enrichment. For isolation and cultivation of phototrophic bacteria, the medium was used containing the following (g/l): NH_4Cl, 0.4; KH_2PO_4, 0.5; $MgCl_2$, 0.2; Na_2SO_4, 0.5; $Na_2S_2O_3 \cdot 5H_2O$, 1.0; NaCl, 10.0; KCl, 0.5; $NaHCO_3$, 5.0, Na_2CO_3 2.0; yeast extract, 1.0; peptone, 1.0; soytone, 1.0; sucrose, 2.0; sodium pyruvate, 1.0; vitamin B_{12}, 10 μg; trace element solution, 1 ml [12].

Solutions of $NaHCO_3$ (10%) and Na_2CO_3 (10%) were prepared and sterilized separately and added to the medium immediately before inoculation. In complete medium, pH was 8.5–9.0. Bacteria were cultivated anaerobically in the light (2000 lx) in 30 ml screw-capped vials and aerobically in the dark in 150 ml of the medium in 500-ml conical flasks. Pure cultures were obtained by plating on Petri dishes with

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agarized media (2%) and subsequent transfers of individual pigmented colonies.

Morphology and thin structure. Morphology and ultrathin structure of strain "Green" was studied using an Olympus phase contrast microscope and a Jeol JEM-100C electron microscope (Japan). Total preparations for electron microscopy were contrasted with 0.2% aqueous uranyl acetate solution. Ultrathin sectioning were prepared form the cells collected by centrifugation, fixed according to Kellenberger, dehydrated, and embedded in Epon; an LKB ultramicrotome (Sweden) was used. The sections were placed on Formvar-coated copper grids and contrasted according to Reynolds [13].

Pigment composition. The pigment composition was studied in cell suspensions treated in an UZDN-A sonicator (5 min) and in acetone–methanol extracts (7 : 2). Absorption spectra were obtained on a Cary 50 (Shimadzu, Japan). Carotenoids were separated by high-performance liquid chromatography (HPLC) as described earlier [14, 15].

Growth parameters. The optimal values of pH and NaCl concentration for growth were determined in liquid medium under anaerobic conditions in the light as described earlier [11]. The optimal growth temperature was determined using a gradient thermostat within the 10–50°C temperature range.

Utilization of organic substrates was studied in the growth medium with vitamin B_{12} (10 μg/l) and yeast extract (0.1 g/l) as vitamin sources, without other organic compounds. The substrates (1 g/l) were added to the medium and pH was adjusted to 8.5 – 9.0.

Bubble formation in the culture after addition of 3% hydrogen peroxide to the colonies on agarized media [16] was used as an indicator of catalase activity.

In order to determine the capacity of the strain for anaerobic growth in the dark by nitrate reduction, microbial biomass and products of nitrate reduction were monitored. N_2 production was assayed as gas formation; NO_2^- was determined using the Griess reagent [16].

In all experiments, the biomass was assayed as OD_{650} on a KFK-3 spectrophotometer (Russia, Zagorsk Optic Mechanical Factory).

Fatty acid analysis. Cellular fatty acids were analyzed by gas chromatography and chromato-mass spectrometry. Dry biomass samples (5 mg) were treated for 1 h with 0.4 ml of 1 N HCl in methanol at 80°C (acid methanolysis). The resulting methyl ethers of fatty acids were extracted with hexane and injected into a Sherlock gas chromatograph (Microbial identification system, MIDI Inc., United States) [17].

Antibiotic sensitivity. Sensitivity to antibiotics was determined on lawns of aerobically grown cells as formation of growth inhibition zones around the disks containing antibiotics under study (Difco) [16].

Genetic properties. DNA was isolated according to Marmur [18]. The DNA G+C base content was determined according to Owen et al. [19].

Amplification and sequencing of the 16S rRNA gene was carried out with universal primers [20]. Obtained nucleotide sequences were aligned with the relevant sequences of related bacterial species using the CLUSTALX software package. The rootless phylogenetic trees were constructed using the TREECON software package [21]. The 16S rRNA gene sequences were deposited in GenBank; accession no. EU857676. The presence of RuBisCO and *nif* genes was determined as described previously [22, 23].

RESULTS

Cultural characteristics. On a surface of agarized media, bacteria of the only isolated strain "Green" form convex colonies 1–2 mm in diameter, with even edges. When grown aerobically in the dark, young colonies are colorless; subsequently they become wine red in color. Under anaerobic conditions, cell suspensions are homogeneous, pink in color. The suspension does not change color when exposed to oxygen.

Morphology and thin structure. Young cells are oval, later polymorphic. When intracellular storage compounds are accumulated, the cells become spindle-shaped with an irregularly swollen central part (Fig. 1a). The cells are 0.7–1.0 μm; with accumulation of storage compounds, the size increases to 3 μm. Cell division is binary fission, often nonuniform, by constriction (Figs. 1a, 1c). Some cells are motile, with a polar flagellum (Fig. 1b). Ultrathin sections reveal Gram-negative structure of the cell wall (Fig. 1c). The cells grown in the light under anaerobic conditions contain photosynthetic membrane structures of vesicular type; these structures fill most of the cell volume (Fig. 1c). Although aerobically grown cultures also contain vesicular structures, these occupy only a fraction of the cytoplasm volume. Storage compounds are present as spherical electron-transparent inclusions, sometimes of considerable size. Such inclusions are characteristic of poly-p-hydroxybutyrate (Fig. 1c).

Pigments. Among photosynthetic pigments, bacteriochlorophyll *a* and carotenoids were detected (Fig. 2). In absorption spectra of sonicated cells, the peaks were registered at 477, 507, 549, 795, 837, and 875 nm (Fig. 2). This is an indication of the presence of two light-harvesting systems, LH1 (795 nm) and LH2 (837 and 875 nm). Absorption spectra of the membranes reveal low content of the LH1 Iight- harvesting complex (Fig. 2).

The carotenoids revealed were spheroidene, hydroxyspheroidene, dimethylspheroidene, spirilloxanthin, diketomonodimethylspirilloxanthin, diketospirilloxanthin, anhydrorhodovibrin, neurosporene and lycopene (Table 1). Thus, bacteria of strain "Green" contained carotenoids both of the spheroidene (62.2%)

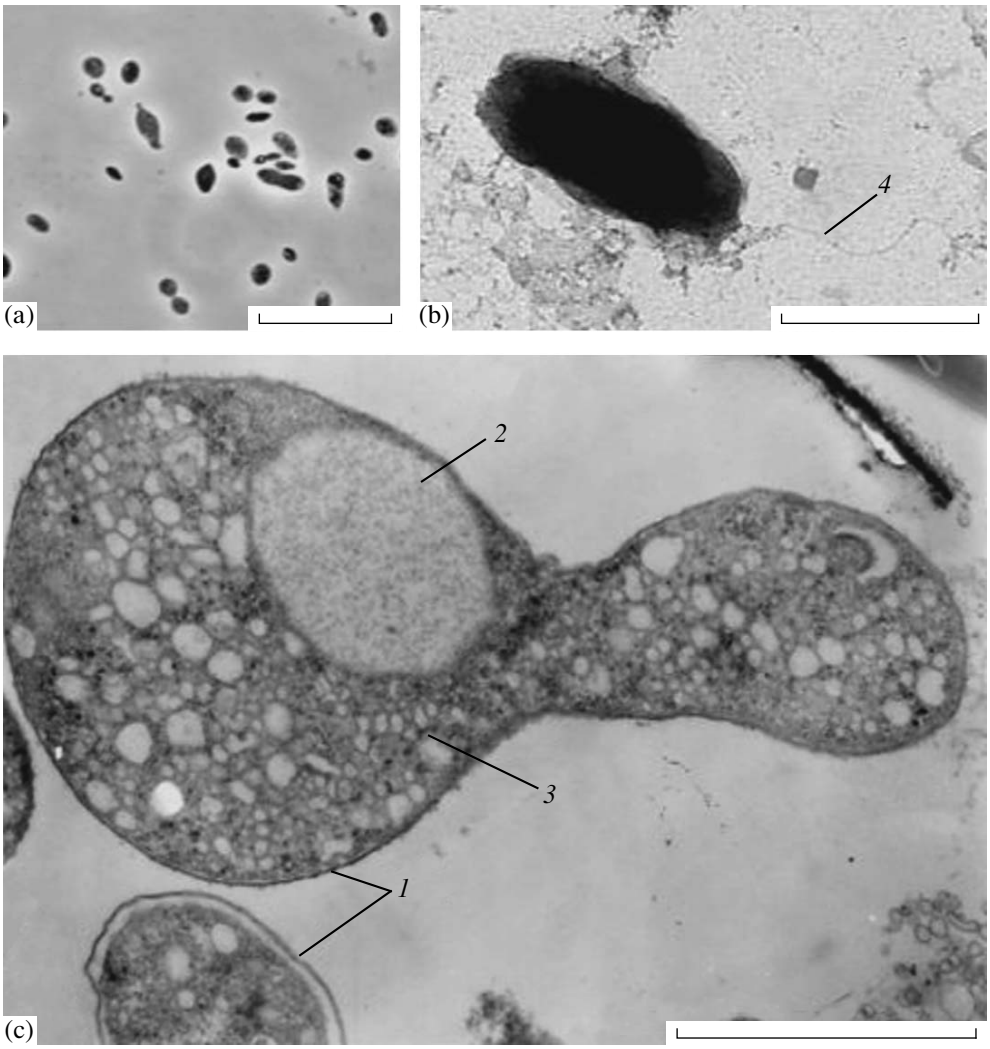

Fig. 1. Cell morphology and ultrastructure of strain "Green" grown anaerobically in the light: a, light microscopy (scale bar, 10 μm); b, total cells (scale bar, 1 μm); c, ultrathin sections (scale bar, 0.5 μm). *1*, outer membrane; *2*, storage compounds; *3*, vesicular-type intracytoplasmic membrane; *4*, flagellum.

and spirilloxanthin group (37.8%). The presence of both spheroidene and spirilloxanthin carotenoids has previously been reported only for one alphaproteobacterium, *Roseobacter denitrificans*, and for betaproteobacteria *Rhodoferax fermentans* and *Rubrivivax gelatinosus* [24]. The presence of dimethylspheroidene is another characteristic feature of strain "Green". This carotenoid has been previously reported only for the members of an alkaliphilic genus *Rhodobaca* [9, 11] and for *Rhodovulum sulfidophilum* [24].

Physiological characteristics. Bacteria of strain "Green" are anaerobic phototrophs, organotropics, and facultative aerobes. Physiologically, they are nonsulfur purple bacteria. The key enzyme of CO_2 assimilation, ribulose bisphosphate carboxylase (RuBisCO), is absent. Unlike most other nonsulphur purple bacteria, under aerobic conditions bacteria grow much better than anaerobically under illumination. The strain was also capable of nitrate reduction; nitrites were produced as the terminal reduction product. However, nitrate reduction did not result in cell growth.

Under anaerobic conditions in the light and aerobically in the dark, the new nonsulphur purple bacteria strain utilized the following carbon sources: glucose, fructose, sucrose, pyruvate, malate, casein hydrolysate, soytone, and yeast extract. Ribose, maltose, arabinose, acetate, butyrate, citrate, succinate, lactate, propionate, formate, fumarate, benzoate, tartrate, ethanol, methanol, mannitol, glycerol, and glutamate were not utilized (Table 2).

Ammonium salts were used as a nitrogen source. It is not capable of nitrogen fixation and does not contain the *nifH* gene.

The new isolate is mesophilic, growing best within the temperature range from 20 to 35°C. Good growth occurred at NaCl concentrations from 5 to 40 g/l with

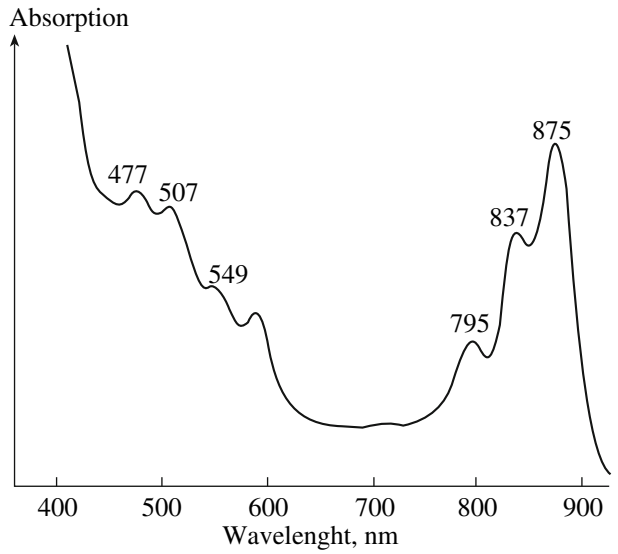

Fig. 2. Absorption spectrum of strain "Green" in vivo.

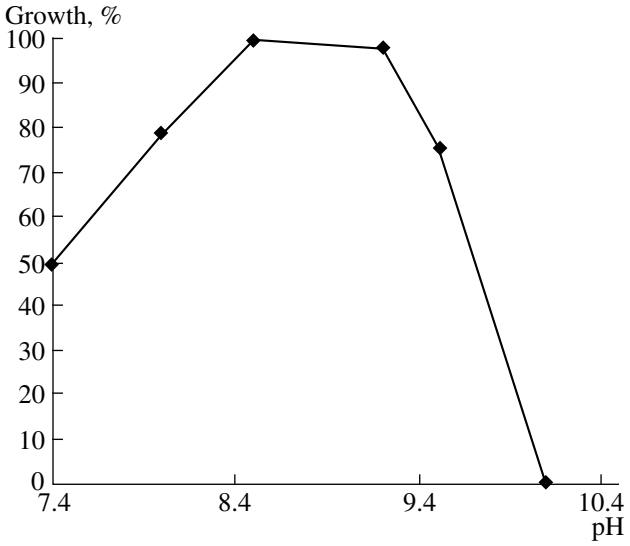

Fig. 3. Effect of pH on strain "Green" growth under anaerobic conditions (maximal growth, 100%).

an optimum at 10 g/l. Optimal growth was detected at pH 8.5–9.5 (Fig. 3). No growth occurred at neutral pH. Thus, the organism is alkaliphilic and moderately halophilic.

New isolate was resistant to amikacin, ampicillin, vancomycin, benzylpenicillin, gentamycin, kanamycin, lincomycin, nalidixic acid, neomycin, novobiocin, polymyxin, and rifampicin. The organism was sensitive to streptomycin, tetracycline, and erythromycin.

Fatty acids profile. The fatty acids composition (Table 3) is similar to that of the members of the family *Rhodobacteraceae.* An isomer of the monounsaturated C18:1ω7 acid was predominant (74.28% of the total fatty acids), significant amounts of hexadecanoic (16:0) and octadecanoic (18:0) acids were also found (9.00 and 6.84%, respectively).

Phylogenetic position of the new strain. The DNA G+C base content of strain "Green" was 69.9 mol %. In order to determine the phylogenetic position of the strain, an almost complete 16S rRNA gene sequence was determined (1380 nucleotides; *E. coli* positions 31–1495). The phylogenetic analysis placed the strain within the family *Rhodobacteraceae* of the order *Rhodobacterales*, class *Alphaproteobacteria*. On the phylogenetic tree, the type strain of aerobic, bacteriochlorophyll *a*-containing bacteria *Rubrimonas cliftoensis* [25] was the closest to the new strain (Fig. 4); the similarity level, however, was only 93.3%. These two organisms formed a cluster with the highest level of bootstrap support (100%). The similarity with other members of *Rhodobacteraceae* was significantly lower, not exceeding 90%. Thus, phylogenetic analysis suggested an isolated position of the new nonsulfur purple bacterium within the family *Rhodobacteraceae*; the isolate therefore can not be assigned to any of the known species of this family. Ability to grow anaerobically in the light differentiates it from the aerobic anoxygenic phototrophic bacterium *Rubrimonas cliftoensis.* The new isolate of nonsulphur purple bacteria has a number of specific phenotypic characteristics (Table 4). Strain "Green" is proposed as the type strain of the new species of the new genus, *Rubribacterium polymorphum* gen. nov., sp. nov.

Description of Rubribacterium gen. nov.

Rubribacterium gen. nov. *Ru.bribac.terium.* Lat. adj. *rubber bra brum*, red; *bacterium*, a rod; N.L. neut. n. *Rubribacterium* red rod.

Table 1. Carotenoid composition of strain "Green" cells grown anaerobically in the light

no.	Carotenoid	%
1	Diketomonodimethylspirilloxanthin	2.5
2	Diketospirilloxanthin	10.9
3	Hydroxyspheroidene	23.4
4	Spirilloxanthin	23.7
5	Dimethylspheroidene	25.3
6	Anhydrorhodovibrin	0.4
7	Spheroidene	12.7
8	Lycopene	0.8
9	Neurosporene	0.3

Table 2. Organic compounds utilized as carbon sources by strain "Green" and closely related NPB *Rhodobaca bogoriensis*[1], *Rhodobacter sphaeroides*[2] and AAP bacterium *Rubrimonas cliftonensis*[3]

Substrates	*Rhodobaca bogoriensis*[1]	*Rhodobacter sphaeroides*[2]	*Rubrimonas cliftonensis*[3]	Strain "Green"	
				Aerobically	Anaerobically
Glucose	++	+	+	+	+
Fructose	++	+	+	+	+
Sucrose	++	ND	ND	+	+
Ribose	+–	ND	+	–	–
Maltose	ND	ND	+	–	–
Arabinose	ND	–	ND	–	–
Acetate	++	+	–	–	–
Pyruvate	++	ND	ND	+	+
Glutamate	ND	+	–	–	–
Butyrate	++	+	+–	–	–
Malate	++	+	–	+	+
Citrate	–	+	ND	–	–
Succinate	++	ND	–	–	–
Lactate	+	+	–	–	–
Formate	–	–	ND	–	–
Fumarate	++	+	–	–	–
Propionate	++	+	+	–	–
Benzoate	–	ND	ND	–	–
Tartrate	ND	+	ND	–	–
Ethanol	–	+	+	–	–
Methanol	–	+–	ND	–	–
Glycerol	ND	+	ND	–	–
Mannitol	++	+	ND	–	–
Casein hydrolysate	+	ND	ND	+	+
Yeast extract	+	ND	ND	++	++
Soytone	+	ND	ND	++	++

Notes: "++", very good growth; "+", good growth; "–", no growth; "+–", weak growth; ND, no data; [1], Milford et al. 2000; [2], Bergey's manual of systematic bacteriology 2005; [3], Suzuki et al. 1999.

The cells are oval or polymorphic, 0.7 × 1.0–1.2 μm. Under certain conditions, polymorphic cells may increase in size to 3.0 μm. Cell division is binary fission, often irregulars, by constriction. The cells may be motile due to polar flagella. Bacteria are gram-negative. Vesicular type of photosynthetic membranes distributed throughout the cell volume. Bacteriochlorophyll *a* and carotenoids of both spirilloxanthin and spheroidene groups are the photosynthetic pigments. The organisms are facultative aerobes and photoheterotrophs. Aerobic growth by respiration is possible. Ammonium is utilized as a nitrogen source for biosynthesis. A wide range of organic substrates is utilized, including organic acids, sugars, and polypeptides. DNA G+C base content of the type species is 69.9 mol %.

Type species *Rubribacterium polymorphum* sp. nov.

Description of Rubribacterium polymorphum gen. nov., sp. nov.

Rubribacterium polymorphum gen. nov., sp. nov. *po.ly.mor'phus*—Gr. adj. *poly* many; Gr. n. *morphus* shape, body; M.L. adj. *polymorphum* many shapes.

Young cells are oval, later polymorphic. When intracellular storage compounds are accumulated, the cells become spindle-shaped with an irregularly swollen central part. Cell size is 0.7 × 1.0–1.2 μm. Polymorphic cells may increase in size to 3.0 μm. Cell division is binary fission, nonuniform, by constriction. Some cells are motile, with a polar flagellum. The cell wall is of gram-negative type. Intracytoplasmic membranes of vesicular type are present, spreading throughout the cytoplasm. Intracellular storage compounds form electron-transparent inclusions, characteristic of poly-β-hydroxybutyrate. Photosynthetic pigments have in vivo absorption maxima at 477, 507, 549 795, 837, and 875 nm, indicating the presence of bacteriochlorophyll *a* and carotenoids. Two light-harvesting systems are present, LH1 and LH2. Spheroidene and spirilloxanthin, as well as their derivatives (spheroidenone, hydroxyspheroidene, dimethylspheroidene, spirilloxanthin, diketomonodimethylspirilloxanthin, diketospirilloxanthin, anhydrorhodovibrine, and lycopene) are the major carotenoids. The organism is an anaerobic phototroph, heterotroph, and facultative aerobe. The key enzyme of CO_2 assimilation, ribulose bisphosphate carboxylase (RuBisCO), is absent. Aerobic growth in the dark is significantly better than anaerobic growth in the light. Nitrate reduction is carried out with nitrite as the terminal reduction product. The cells do not grow by nitrate reduction. The microorganism is mesophilic (best growth occurs at 20–35°C), weakly halophilic (grows well at 5–40 g/l NaCl with the optimum at 10 g/l), and alkaliphilic (optimal pH 8.5–9.5). No growth occurs at neutral pH. Glucose, fructose, sucrose, pyruvate, malate, casein hydrolysate, yeast extract, and soytone are used as substrates for photosynthesis and for aerobic growth in the dark. Ribose, maltose, acetate, arabinose, glutamate, butyrate, citrate,

Table 3. Fatty acid composition of strain "Green"

Symbol	*Rhodobaca bogoriensis*,[1] AF248638	*Rhodobacter capsulatus*,[2] DSM 1710	Strain "Green"
12:0	0.19	–	–
14:0	1.03	0.2	–
14:1	2.12	0.5	–
2h14	0.75	–	–
i15	0.15	–	–
a15	0.34	–	–
h15	0.57	–	0.34
16:0	18.66	4.2	9.0
16:1	2.41	7.4	2.01
17:0	–	0.6	–
18:0	2.4	3.8	6.84
18:1	67.49	81.4	74.86
11Me18:1	3.89	–	2.04
i19	–	–	1.82
19:1	–	ND	–
19 cyc	–	ND	0.82
20:1	–	0.5	–
20:3ω6	–	–	1.89
20:2ω6	–	–	0.38

Notes: ND, no data; [1], Milford et al. 2000; [2], Bergey's manual of systematic bacteriology 2005..

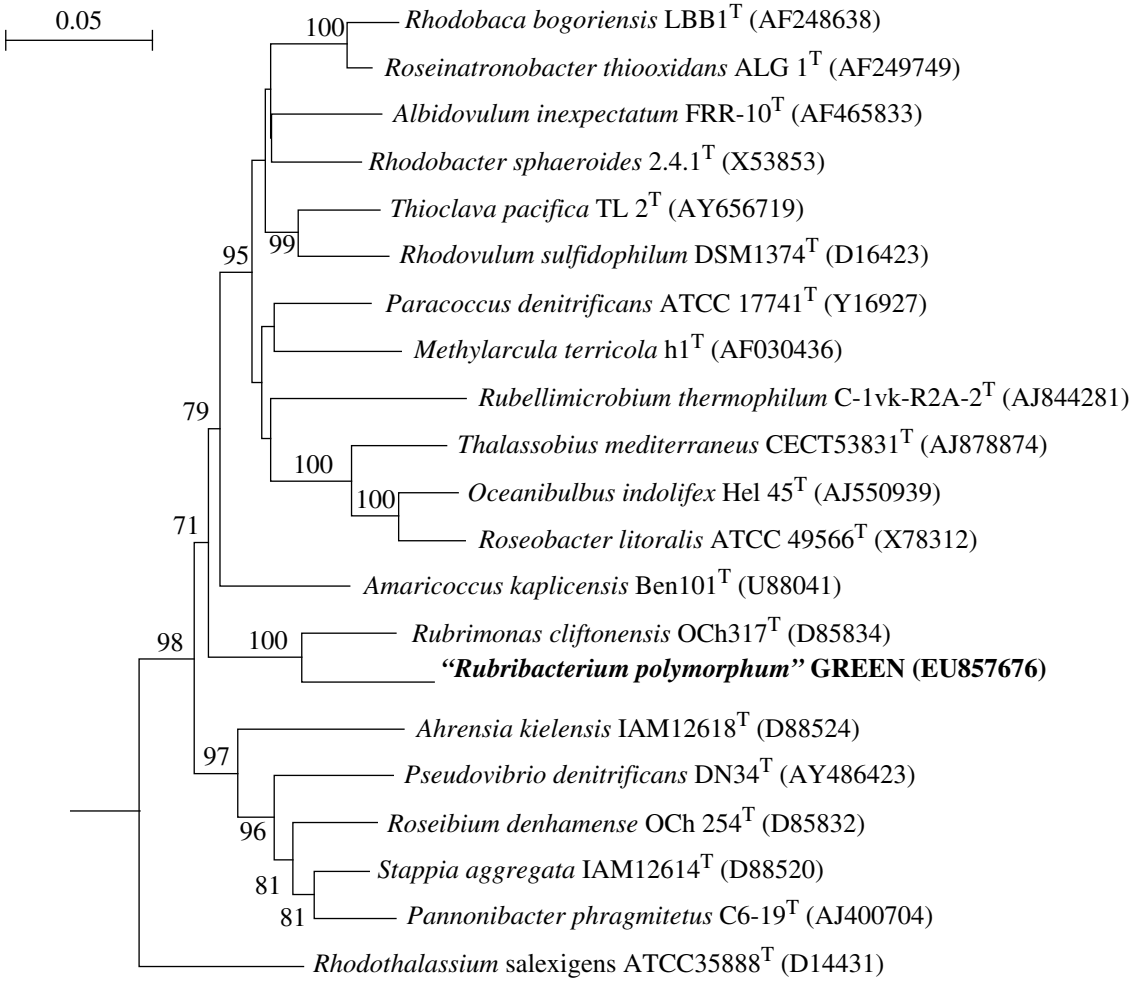

Fig. 4. 16S rDNA sequence-based phylogenetic tree showing the phylogenetic position of strain "Green" among the genera of *Rhodobacteraceae*. Bootstrap values are shown at the tree nodes. The marker indicates the number of nucleotide substitutions at the homologous site of compared sequences.

succinate, lactate, propionate, fumarate, benzoate, tartrate, ethanol, methanol, glycerol, and mannitol are not utilized. Ammonium salts were used as a nitrogen source. It is not capable of nitrogen fixation and does not contain the *nifH* gene. The fatty acid composition is similar to that of the members of the family Rhodobacteraceae. An isomer of the monounsaturated C18:1ω7 acid was predominant (74.28% of the total fatty acids), significant amounts of hexadecanoic (16:0) and octadecanoic (18:0) acids were also found (9.00 and 6.84%, respectively). The strain is resistant to amikacin, ampicillin, vancomycin, benzylpenicillin, gentamycin, kanamycin, lincomycin, nalidixic acid, neomycin, novobiocin, polymyxin, and rifampicin. The organism was sensitive to streptomycin, tetracycline, and erythromycin.

DNA G+C base content is 69.9 mol%.

Type strain: "Green", VCM B-2481

GenBank accession number: 16S rRNA–EU857676.

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Table 4. Comparative properties of strain "Green" and the related anoxygenic phototrophic bacteria

Comparative characterization of strain "Green" and members of the *Rhodobacter–Rhodovulum* cluster characteristics	*Rhodobaca bogoriensis*[1]	*Rhodobacter capsulatus*[2]	*Rubrimonas cliftonensis*[3]	strain "Green"
Habitat	Alkaline soda lakes	Eutrophic lakes and other environment	Saline lake	Steppe soda lake
Cell shape and size, μm	Oval rods, 0.8–1.0 × 0.8–1.5	Oval rods, 0.5–1.2 × 2.0–2.5	Oval rods, 1.0–1.5 × 1.2–2.0	Oval rods, 0.7 × 1.0
Pigment in vivo, nm; carotenoid type	450, 485, 525, 870, spheroidene	450, 478, 508, 590, 805, 863, spheroidene	806, 871	477, 507, 549, 590, 795, 837, 875, spheroidene + spirilloxanthine
Type of photosynthetic structures	Vesicles in the cell periphery	Vesicles spread throughout the cell volume	Absent	Vesicles spread throughout the cell volume
LH1, LH2 content	LH1	LH1, LH2	LH1, LH2	LH1, LH2
Relation to oxygen	Facultative aerobe	Facultative aerobe	Obligate aerobe	Facultative aerobe
Phototrophic growth under anaerobic conditions	+	+	–	+
NaCl (optimum), %	1–2	NaCl not required	0.5–7.5	1
pH (optimum)	9.0	7.0	7.5–8.0	8.5–9.5
Tolerance to sulfide	High	High	ND	High
G+C base content, mol %	58.8	65.5–69.6 for different strains	74.0–74.8	69.9

Notes: [1], Milford et al. 2000; [2], Bergey's manual of systematic bacteriology; [3], Suzuki et al. 1999; "+" stands for the presence of a feature; "–" stands for the absence of a feature; ND stands for no data.

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