Marichromatium fluminis sp. nov., a slightly alkaliphilic, phototrophic gammaproteobacterium isolated from river sediment

K. Sucharita,¹ E. Shiva Kumar,¹ Ch. Sasikala,¹ B. B. Panda,² S. Takaichi³ and Ch. V. Ramana⁴

Correspondence

Ch. Sasikala

sasi449@yahoo.ie

¹Bacterial Discovery Laboratory, Centre for Environment, Institute of Science and Technology, J. N. T. University, Kukatpally, Hyderabad 500 085, India

²Department of Botany, Bharampur University, Bharampur, Orissa, India

³Department of Biology, Nippon Medical School, Kosugi-cho, Nakahara, Kawasaki 211-0063, Japan

⁴Department of Plant Sciences, School of Life Sciences, University of Hyderabad, PO Central University, Hyderabad 500 046, India

An anoxygenic, phototrophic gammaproteobacterium designated strain JA418^T was isolated from a sediment sample collected from the Baitarani River, Orissa, India. The bacterium was a Gram-negative, motile rod with a single polar flagellum. Bacteriochlorophyll *a* and rhodopin were the major photosynthetic pigments. The organism grew best at slightly alkaline pH (8–8.5) and lacked chemotrophic growth. The major fatty acids were C_{16:0}, C_{16:1ω7c}/C_{16:1ω6c} and C_{18:1ω7c}. A phylogenetic tree based on 16S rRNA gene sequence analysis showed that strain JA418^T clusters with species of the genus *Marichromatium* belonging to the class *Gammaproteobacteria*. The highest 16S rRNA gene sequence similarities of strain JA418^T were found with the type strains of *Marichromatium gracile* (95.9%), *Marichromatium indicum* (95.6%), *Marichromatium purpuratum* (95.5%) and *Marichromatium bheemlicum* (95.6%). The DNA base composition of strain JA418^T was 71.4 mol% G + C (by HPLC). Based on the 16S rRNA gene sequence analysis and physiological and chemotaxonomic characteristics, strain JA418^T is sufficiently different from other *Marichromatium* species to merit the description of a novel species, *Marichromatium fluminis* sp. nov., to accommodate it. The type strain is JA418^T (=KCTC 5717^T =NBRC 105221^T).

The genus *Marichromatium* comprises four species with validly published names, *Marichromatium gracile*, *Mch. purpuratum*, *Mch. indicum* (Arunasri *et al.*, 2005) and *Mch. bheemlicum* (Anil Kumar *et al.*, 2007). *Mch. gracile* biotype *thermosulfidophilum* is a newly described biotype of the species *Mch. gracile* (Serrano *et al.*, 2009). In this paper, we propose a novel species of the genus *Marichromatium* based on a strain that grows optimally at slightly alkaline pH; alkaliphilic growth is not widespread among the phototrophic members of the family *Chromatiaceae* (Imhoff, 2005).

Strain JA418^T was isolated from phototrophic [anaerobic, light (30 μE m⁻² s⁻¹)] enrichments of a sediment sample

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA418^T is FM210274.

Details of the effects of NaCl concentration and pH on growth and absorption spectra from cells of JA418^T are available as supplementary material with the online version of this paper.

collected from the Baitarani River in Kalibanj forest, Orissa, India, by using the medium (pH 6.8) of Pfennig (Pfennig & Trüper, 1992) supplemented with sodium chloride (1% w/v) and with pyruvate (0.3% w/v) as carbon source/electron donor and ammonium chloride (0.12%) as nitrogen source. Samples were collected on 31 May 2008 and the GPS position of the sample collection site is 20° 47.284' N 86° 52.646' E. The sample that yielded strain JA418^T had a pH of 6.8 (the pH of the lagoon is known to vary temporally between 6.5 and 9), a salinity of 2.5% NaCl and a temperature of 30 °C. Purification was achieved by repeated streaking on agar slants (25 × 150 mm test tubes sealed with butyl rubber stoppers and the gas phase replaced with argon). Purified cultures were grown in completely filled screw-capped test tubes (10 × 100 mm) for photoheterotrophic growth. For routine culturing and for physiological tests, strain JA418^T was grown at pH 8, 30 °C and at a light intensity of 30 μE m⁻² s⁻¹. Growth experiments at pH 6–12 were performed

(protocol of Aino *et al.*, 2008) using the basal medium of Pfennig (Pfennig & Trüper, 1992) buffered with 100 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 6–7.5), 100 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (pH 8–9.5) or 100 mM $\text{Na}_2\text{CO}_3/\text{NaOH}$ (pH 10–12). Morphological properties (cell shape, cell division, cell size, flagella) were observed by light microscopy (Olympus BH-2). To study the ultrastructure of the flagella, cells were stained with 1 % phosphotungstic acid; ultrathin sections were viewed through a transmission electron microscope (H-7500; Hitachi) to examine intracytoplasmic structures such as the internal membrane system. *In vivo* absorption spectra were measured with a Spectronic Genesys 2 spectrophotometer using a sucrose solution for cell suspension (Pfennig & Trüper, 1992). Carotenoid composition was analysed by using HPLC (Takaichi & Shimada, 1992). The utilization of different carbon substrates and electron donors (0.3 % w/v or v/v, unless otherwise mentioned) was tested in the medium of Pfennig (Pfennig & Trüper, 1992) containing 1 mM $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$.

Ammonium chloride was replaced with different nitrogen sources (at 0.12 % w/v) in order to test for their utilization. Diazotrophy of the culture was determined by growing strain JA418^T under a N_2 atmosphere. Growth was measured turbidometrically at 660 nm. Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; Agilent 6850) (Sasser, 1990); this procedure was carried out by Royal Life Sciences Pvt Ltd (Secunderabad, India).

Genomic DNA was obtained from 1–2 ml well-grown liquid culture using a Genomic DNA extraction kit (Qiagen). Recombinant *Taq* polymerase (Bangalore GeNei) was used for PCR. The PCR was primed with primers F-27 (5'-GTTTGATCCTGGCTCAG-3') and R-1489 (5'-TACCTTGTTACGACTTCA-3') (positions 11–27 and 1489–1506, respectively, according to the *Escherichia coli* 16S rRNA numbering system of the International Union of Biochemistry). PCR amplification was done as described previously (Imhoff *et al.*, 1998) and 16S rRNA gene sequences were obtained by cycle sequencing with a SequiTherm sequencing kit (Biozym) and the chain

termination reaction (Sanger *et al.*, 1977) using an automated laser fluorescence sequencer (Pharmacia). Sequences were aligned using the CLUSTAL_X program and a neighbour-joining tree was constructed using MEGA 4.0 software using 500 replicates for non-parametric bootstrap analysis (Tamura *et al.*, 2007). Genomic DNA was extracted and purified according to the method of Marmur (1961) and the G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989).

Individual cells of strain JA418^T were rod-shaped (Fig. 1a), 0.6–1.0 μm wide and 3–6 μm long. The cells were highly motile with single polar flagella (Fig. 1b) and multiplied by binary fission. Electron photomicrographs of ultrathin sections of the cells revealed a vesicular type of internal membranes (Fig. 1c). Strain JA418^T grows well photo-organoheterotrophically [anaerobically in light ($30 \mu\text{E m}^{-2} \text{s}^{-1}$) with pyruvate as carbon source/electron donor]. Strain JA418^T was able to grow photolithoautotrophically [anaerobically in the light ($30 \mu\text{E m}^{-2} \text{s}^{-1}$) with $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (1.0 mM) or $\text{Na}_2\text{S}_2\text{O}_3$ (5.0 mM) as electron donors with NaHCO_3 (0.1 % w/v) as carbon source] and photolithoheterotrophically [anaerobically in the light ($30 \mu\text{E m}^{-2} \text{s}^{-1}$) with $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (1.0 mM) or $\text{Na}_2\text{S}_2\text{O}_3$ (1 mM) as electron donors and organic substrates (0.3 % w/v) as carbon source]. Chemolithoautotrophy [aerobic growth in the dark with thiosulfate (5.0 mM) as electron donor and NaHCO_3 (0.1 % w/v) as carbon source], chemo-organoheterotrophy and fermentative growth [anaerobically in the dark with pyruvate as fermentable substrate (0.3 % w/v)] could not be demonstrated. Utilization of substrates as carbon/electron donors under photolithoheterotrophic conditions is detailed in Table 1. Thiosulfate, thioglycolate, elemental sulfur and sodium sulfide (each at 1 mM) were utilized as sulfur sources under phototrophic conditions.

Ammonium chloride, glutamate, glutamine and urea were utilized as nitrogen sources, while molecular nitrogen, nitrate and nitrite did not support growth. Acetylene reduction activity as a marker for the enzyme nitrogenase was also not observed in strain JA418^T. Salt was required for growth of strain JA418^T, and growth occurred in the

Fig. 1. (a) Phase-contrast photomicrograph of cells of strain JA418^T. Bar, 16.6 μm . (b) Electron photomicrograph showing monopolar flagellum of strain JA418^T. Bar, 1 μm . (c) Transmission electron photomicrograph showing vesicular intracytoplasmic membrane structures of strain JA418^T. Bar, 869 nm.

Table 1. Differentiating characteristics of type strains of species of the genus *Marichromatium*

Strains: 1, strain JA418^T; 2, *Mch. bheemicum* JA124^T; 3, *Mch. indicum* JA100^T; 4, *Mch. gracile* DSM 203^T; 5, *Mch. purpuratum* DSM 1591^T. Data were obtained in this study. Organic substrate utilization was tested during photolithoheterotrophic growth in the presence of Na₂S·9H₂O (1 mM). Organic substrate utilization, vitamin requirements and nitrogen source utilization were examined and FAME analysis was carried out for all five strains under identical conditions from cells grown at pH 7.5. +, Substrate utilized; –, substrate not utilized/fatty acid not detected. DNA G + C contents were determined by HPLC. All five strains were positive for utilization of acetate, pyruvate and malate and negative for utilization of glutamate, glucose, tartrate, mannitol and benzoate. All five strains were similar in the presence of rod-shaped cells with single polar flagella and internal membranes of the vesicular type and cell division by binary fission.

Characteristic	1	2	3	4	5
Cell width (µm)	0.4–0.5	0.8–1	0.8–1	1–1.3	1.2–1.7
Cell length (µm)	4–6	2–4	2–7	2–6	3–4
Rosette formation	Absent	Absent	Present	Absent	Absent
Sulfur granules per cell (n)	1–3	≥ 2	1	≥ 2	≥ 2
NaCl concentration for growth (% w/v)					
Range	0.5–6.5	1.5–11	0.05–13	0.5–8	2–7
Optimum	1.5–3	1.5–8.5	1–4	2–3	5
Optimum growth temperature (°C)	30–35	30–35	30–35	25–30	30–35
Optimum pH	8–8.5	6.5–8.5	6.0–7.5	6.5–7.6	7.2–7.6
Growth modes*	PLH, PLA, POH	PLH, PLA, POH	PLH, PLA, CLH, POH (with cysteine)	POH, PLA, COH, CLA, CLH	POH, PLA, COH, CLA, CLH
Colour of cell suspension	Reddish brown	Reddish brown	Reddish brown	Reddish brown	Purple–red
Carotenoids (major component)	Spirilloxanthin series (rhodopin)	Spirilloxanthin series (rhodopin)	Spirilloxanthin series	Spirilloxanthin series	Okenone series
DNA G + C content (mol%)	71.4	67	66.7	71.2	68.2
Vitamin requirement	None	Pyridoxal phosphate	None	None	None
Nitrogen sources utilized	NH ₄ Cl, Glu, Gln, urea	NH ₄ Cl, Gln, Glu	NH ₄ Cl, urea, N ₂	NH ₄ Cl, Glu, N ₂	NH ₄ Cl
Sulfide tolerance (mM)	4	8	4	4	4
Organic substrates/electron donors utilized†					
Propionate	+	–	+	+	+
Butyrate	+	–	+	+	+
Valerate	+	+	–	–	+
Caproate	+	+	–	–	–
Citrate	+	–	–	–	–
Succinate	+	+	–	+	+
Fumarate	+	+	–	+	+
Fructose	+	–	+	–	–
Lactate	–	–	+	+	+
Glycerol	–	+	–	–	–
Methanol	+	+	+	–	–
Ethanol	–	+	–	–	–
Whole-cell fatty acids (%)					
C _{12:0}	2.4	12.5	3.0	9.2	1.8
C _{12:0} anteiso	–	–	2.0	–	–
C _{14:0} anteiso	–	–	3.1	–	–
C _{14:0}	–	–	1.8	–	–
C _{14:0} 2-OH	–	–	2.1	–	–
C _{16:0}	25.7	–	2.7	15.6	20.5
C _{16:0} n-OH‡	–	–	1.7	–	–
C _{16:0} iso	–	–	1.8	–	–
C _{16:1} ω9c	–	–	3.6	–	–
C _{18:0}	–	–	–	4.2	–
C _{18:1} iso OH‡	–	–	3.7	–	–
C _{18:3} ω6,9,12c	–	33.4	2.7	–	–

Table 1. cont.

Characteristic	1	2	3	4	5
C _{16:1} ω7c/C _{16:1} ω6c	28.4	—	—	10.5	29.4
C _{16:1} ω7c alcohol	—	—	—	2.1	—
C _{18:1} ω7c	37.0	—	1.8	49.2	40.4
C _{18:1} ω5c	0.3	54.0	68.5	—	—

*CLA₃, Chemolithoautotrophy; CLH₃, chemolithoheterotrophy; COH₃, chemo-organoheterotrophy; PLA₃, photolithoautotrophy; PLH₃, photolithoheterotrophy; POH₃, photo-organoheterotrophy.

†Results were obtained after three subcultures with the respective substrates and compared against a control without the test substrate.

‡Position of hydroxyl group and/or double bond not identified.

presence of 0.5–6.5 % (w/v) NaCl, with optimum growth at 1.5–3.0 % (w/v) NaCl (Supplementary Fig. S1a, available in IJSEM Online). Strain JA418^T grew at pH 6.5–10.0 with optimum growth at pH 8.0–8.5 (Supplementary Fig. S1b) and at 25–30 °C with optimum growth at 30 °C. Yeast extract was required as a growth factor (0.03 % w/v). The predominant cellular fatty acids were C_{16:0}, C_{16:1}ω7c/C_{16:1}ω6c and C_{18:1}ω7c.

Photosynthetically grown cultures of strain JA418^T were reddish brown. The whole-cell absorption spectrum (Supplementary Fig. S2a) of strain JA418^T showed absorption maxima at 374, 488, 593, 803 and 857 nm. The peak in the methanolic spectrum at 770 nm confirmed the presence of bacteriochlorophyll *a* and the absorption spectrum of pigments extracted with acetone (Supplementary Fig. S2b) gave absorption maxima at 473 and 503 nm, indicating the presence of the spirilloxanthin series of carotenoids. HPLC analysis of the carotenoid indicated that strain JA418^T contained rhodopin as the major pigment.

The DNA base composition of strain JA418^T was 71.4 mol% G + C (by HPLC). The phylogenetic relationship of strain JA418^T to other purple sulfur bacteria was examined by 16S rRNA gene sequencing. The data obtained revealed that the

sequence (1434 bp) of the new isolate was included in the cluster of the genus *Marichromatium* (Fig. 2), but was distinct from those of other species of the genus. The highest 16S rRNA gene sequence similarities of strain JA418^T were found with the type strains of *Mch. gracile* (95.9 %), *Mch. indicum* (95.6 %), *Mch. purpuratum* (95.5 %) and *Mch. bheemlicum* (95.6 %).

In addition to 16S rRNA gene sequence dissimilarity, strain JA418^T showed clear phenotypic differences from other *Marichromatium* species (Table 1). Important phenotypic traits that distinguish strain JA418^T from other members of the genus *Marichromatium* include the requirement for slightly alkaline pH (8–8.5) for optimal growth and the lack of chemotrophy in strain JA418^T, characters that distinguish it from other *Marichromatium* species except *Mch. bheemlicum*. Strain JA418^T differs from *Mch. bheemlicum* in vitamin requirements, fatty acid composition, sulfide tolerance, urea utilization, organic substrate utilization and genomic DNA G + C content (Table 1). These major phenotypic differences, supported by large differences in 16S rRNA gene sequences, justify the description of a novel species, *Marichromatium fluminis* sp. nov., to accommodate strain JA418^T.

Fig. 2. Neighbour-joining dendrogram depicting the phylogenetic relationships of strain JA418^T within the family Chromatiaceae determined using the sequence of the 16S rRNA gene; 1350 gap-free sites were compared. Bootstrap percentages from 500 replicates appear next to the corresponding branch. Bar, 1 substitution per 100 nucleotide positions.

Description of *Marichromatium fluminis* sp. nov.

Marichromatium fluminis (flu'mi.nis. L. gen. n. *fluminis* of a river, referring to the isolation of the type strain from sediment of the Baitarani River, located in Kalibanj forest, Orissa, India).

Cells are rod-shaped, 0.6–1.0 µm wide and 3–6 µm long, motile and multiply by binary fission. Gram-negative. Growth occurs under anaerobic conditions in the light (photolithoautotrophy, photolithoheterotrophy and photo-organoheterotrophy). Internal photosynthetic membranes are of the vesicular type. Phototrophic cultures are reddish brown. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 374, 488, 593, 803 and 857 nm. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spirilloxanthin series (with rhodopin as the major component). Mesophilic (temperature range 20–35 °C), exhibits optimum growth at pH 8.0–8.5 and requires 0.5–6.5 % (w/v) NaCl for growth. The major fatty acids are C_{16:0}, C_{16:1}ω7c/C_{16:1}ω6c and C_{18:1}ω7c. Photolithoheterotrophy with Na₂S·9H₂O and various organic compounds is the preferred mode of growth. Good growth with acetate and pyruvate. Growth also occurs on butyrate and caproate. Thiosulfate, sulfate, sulfite, thioglycolate, cysteine and sodium sulfide are utilized as sulfur sources under phototrophic conditions. Photolithoautotrophic growth occurs. Diazotrophic growth and acetylene reduction activity are absent. The DNA base composition of the type strain is 71.4 mol% G + C (by HPLC).

The type strain, JA418^T (=KCTC 5717^T =NBRC 105221^T), was isolated from sediment samples collected from the Baitarani River, located in Kalibanj forest, Orissa, India.

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