Rhodovulum bhavnagarens sp. nov., a phototrophic alphaproteobacterium isolated from a pink pond

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An oval to rod-shaped, Gram-stain-negative, phototrophic bacterium, strain JA738^T, was isolated from a sediment sample collected from a pink pond. Strain JA738^T was non-motile and had vesicular-type intracellular photosynthetic membranes. Bacteriochlorophyll *a* and carotenoids of the spheroidene series were present as the major photosynthetic pigments. Strain JA738^T required thiamine and pantothenate for growth. The major cellular fatty acids were C_{18:1}ω7c, C_{18:1}ω5c, C_{18:0} and C_{18:1}ω7c11-methyl; minor amounts of C_{10:0} 3-OH and C_{16:0} were also present. The major quinone was Q-10 and major polar lipids were phosphatidylglycerol, phosphatidylethanolamine and two unidentified sulfolipids (SL1–2). Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA738^T clustered with species of the genus *Rhodovulum* in the class *Alphaproteobacteria*. Strain JA738^T was most closely related to *Rhodovulum adriaticum* DSM 2781^T (96.4 % 16S rRNA gene sequence similarity) and other members of the genus *Rhodovulum* (<96.1 %). On the basis of phenotypic and molecular genetic evidence, it is proposed that strain JA738^T should be classified as a novel species of the genus *Rhodovulum* for which the name *Rhodovulum bhavnagarens* sp. nov. is proposed. The type strain is JA738^T (=DSM 24766^T=KCTC 15110^T).

Purple, brown and pink ponds are commonly observed in the marine and freshwater habitats of India. Two new marine phototrophic species (*Rhodovulum lacipunice*, Kalyan Chakravarthy *et al.*, 2009; *Rhodovulum phaeolacus*, Lakshmi *et al.*, 2011) and a freshwater phototroph (*Rhodobacter ovatus*, Srinivas *et al.*, 2008) were previously reported from such ponds. In the present study, we report a novel species of the genus *Rhodovulum* which was isolated from a pink pond in Gujarat, India. Members of the genus *Rhodovulum* are representatives of isolates from marine habitats and, at the time of writing, there were 13 species with validly published names assigned to this genus (Lakshmi *et al.*, 2011).

Strain JA738^T was isolated from a sediment sample of a pink pond near Nari solar salterns at Bhavnagar in Gujarat, India (GPS position: 21° 78' N 72° 08' E). The sample that yielded strain JA738^T had a pH of 7 and salinity of 2 ‰

(w/v), and was collected on 12 May 2011. The culture was originally enriched in medium for photoheterotrophic bacteria in 50 ml fully filled screw-capped bottles incubated at 2400 lx fluorescent light, 28–30 °C for 7 days. The medium described by Lakshmi *et al.* (2009) was used for enrichment and growth of strain JA738^T. Sodium acetate (0.1 % w/v), sodium succinate (0.1 % w/v) and sodium pyruvate (0.1 % w/v) were used as carbon sources, yeast extract (0.03 % w/v) was used as a vitamin source and ammonium chloride (0.06 % w/v) was used as a nitrogen source. Na₂S·9H₂O (2 mM; neutralized under a stream of argon gas) was added to the medium after autoclaving, which was done in an argon atmosphere. Phototrophic incubation of the sample at 2400 lx and 28–30 °C for 4–5 days resulted in the formation of reddish brown enrichment. Purification was performed by repeated streaking on agar slants (25 × 150 mm test tubes sealed with butyl rubber corks). Purified cultures were grown in completely filled screw cap test tubes (10 × 100 mm) for photoheterotrophic growth. Comparative taxonomic analysis of strain JA738^T with the type strain of *Rhodovulum adriaticum* Imhoff 6H^T (=DSM 2781^T) was performed in the authors' laboratory.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA738^T is FR828479.

Four supplementary figures are available with the online version of this paper.

Genomic DNA was extracted and purified according to the method of Marmur (1961), and the G+C content of the DNA, determined by reverse-phase HPLC (Mesbah *et al.*, 1989), was 63.4 mol%. Cell material for 16S rRNA gene sequencing was taken from a colony. DNA was extracted and purified by using a genomic DNA extraction kit (Qiagen). Recombinant *Taq* polymerase (Genei) was used for PCR. The complete length of the 16S rRNA gene sequence was obtained by amplification with four primers, F'-27 (5'-GTTTGATCCTGGCTCAG-3'), F'-372 (5' TAC-GGGAGGCAGCAG-3'), F'-790 (5'-GGTTACCTGTTCGACT-3') and R'-1489 (5'-TACCTTGTTACGACTTCA-3') [Positions 11–27 and 1489–1506 for F'-27 and R'-1489 respectively, according to the *Escherichia coli* 16S rRNA numbering system of the International Union of Biochemistry (Brosius *et al.*, 1978; Lane *et al.*, 1985)]. PCR amplification was performed as described previously (Imhoff & Pfennig, 2001; Imhoff *et al.*, 1998) and 16S rRNA gene sequences were obtained (without gene cloning) by cycle sequencing with a SequiTherm sequencing kit (Biozym) and chain termination reaction using an automated laser fluorescence sequencer (Pharmacia). The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the NCBI-BLAST search (Altschul *et al.*, 1990) and EzTaxon server (Chun *et al.*, 2007). The CLUSTAL W algorithm of MEGA 4 software was used for sequence alignments and MEGA 4 software (Tamura *et al.*, 2007) was also used for phylogenetic analysis of the individual sequences. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Tamura *et al.*, 2007). Neighbour-joining (NJ) and minimum evolution (ME) methods in the MEGA 4 software were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. These phylogenetic data showed that the novel isolate branched separately but clustered with type strains of species of the genus *Rhodovulum* in the class *Alphaproteobacteria*. The highest sequence similarities for strain JA738^T were found with the type strains of *Rhodovulum adriaticum* DSM 2781^T (96.4 % 16S rRNA gene sequence similarity) and other members of the genus *Rhodovulum* (<96.1 %; Fig. 1; ME tree is shown as Fig. S1, available in IJSEM online).

Morphological properties (cell shape, cell division, cell size, flagella) were examined under a phase-contrast light microscope (BH-2; Olympus). Cells of strain JA738^T were oval to rod-shaped, 1–1.8 µm long and 0.5–0.9 µm wide, non-motile and multiplied by binary fission. The internal membrane structures were viewed with a transmission electron microscope (H-7500; Hitachi), after the cells had been processed as described by Hanada *et al.* (2002) and strain JA738^T was observed to have a vesicular type of internal membrane structures (Fig. S2).

The colour of the photosynthetically grown cell suspension was reddish brown. *In vivo* absorption spectra as measured in sucrose solution (Trüper & Pfennig, 1981) with a Spectronic Genesys 2 spectrophotometer, exhibited maxima at

380, 479, 590, 803 and 863 nm (Fig. S3) indicating the presence of bacteriochlorophyll *a*. The absorption spectrum for pigments extracted with acetone gave maxima at 303, 309, 348, 456 and 486 nm indicating the presence of carotenoids of the spheroidene series (Britton *et al.*, 2004). Carotenoid content was determined by C₁₈-HPLC analysis, (eluted with acetonitrile/methanol/ethylacetate, 5:4:1 by vol.; flow rate, 1 ml min⁻¹; absorption at 450 nm) with a Photo Diode Array (PDA) detector. Strain JA738^T had a carotenoid content (mol%) of spheroidene (16 %), demethylspheroidene (16 %), spheroidenone (48 %), OH-spheroidene (17 %) and neurosporene (3 %).

Growth was measured turbidometrically at 660 nm. NaCl improved growth yields of strain JA738^T [salinity range and optimum were 1–7 % and 1–3 % (w/v) NaCl, respectively]. Strain JA738^T grew over a pH range of 7.0–9.5 (optimum, 7.0–8.0) and at 25–35 °C (optimum, 28 °C). Strain JA738^T had an obligate requirement for sulfide as electron donor and photo- (anaerobic light; 2400 lx) or chemo- (aerobic dark) lithoheterotrophy [with Na₂S·9H₂O (1 mM) or Na₂S₂O₃ (2 mM) as electron donor and pyruvate (0.03 % w/v) as carbon source] is the preferred growth mode. Chemoorgano-heterotrophy [dark aerobic with pyruvate (0.3 %, w/v) as electron and carbon source], photolithoautotrophy [anaerobic light (2400 lx), with Na₂S (2 mM)/Na₂S₂O₃ (2 mM)/H₂ (20 %, v/v) as electron donors and NaHCO₃ (0.1 %, w/v) as carbon source], chemolithoautotrophy [dark aerobic with Na₂S₂O₃·5H₂O (1 mM) as electron donor and NaHCO₃ (0.1 %, w/v) as carbon source] and fermentative growth [dark anaerobic with pyruvate/glucose (0.3 %, w/v) as fermentable substrates] could not be demonstrated. Vitamin requirement was tested by replacing yeast extract with single or combinations of vitamins as growth factors; strain JA738^T required thiamine and pantothenate as growth factors.

Carbon source utilization was tested using propionate, butyrate, caproate, valerate, lactate, glycerol, and ethanol at a concentration of 0.1 % (v/v); other compounds tested were used at 0.3 % (w/v), with NaHCO₃ (0.1 %), and their utilization under photolithoheterotrophic conditions by strain JA738^T is shown in Table 1. For testing sulfur sources, MgSO₄·7H₂O was replaced by MgCl₂·5H₂O (0.2 %), and respective sulfur sources (sodium sulfide, sodium thiosulfate, sodium thioglycolate, cysteine, magnesium sulfate, sodium sulfite; all at 0.5 mM concentration) were added to the medium. Sulfide, thiosulfate or cysteine are obligatory and were used as sulfur sources, while sulfate, sulfite, thioglycolate and methionine did not support phototrophic growth of strain JA738^T. Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources at 0.06 % (w/v). Strain JA738^T could utilize ammonium chloride, glutamate and glutamine as nitrogen sources, but nitrate, nitrite, aspartate, asparagine and dinitrogen did not support growth.

For analysis of cellular fatty acids, photolithoheterotrophically grown cultures in the presence of Na₂S·9H₂O (1 mM) were harvested when growth of the cultures was

Fig. 1. Phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationship of strain JA738^T within the class Alphaproteobacteria. The trees were constructed by the neighbour-joining method using MEGA 4 software and rooted by using *Rhodospirillum rubrum* ATCC 11170^T as the outgroup. Numbers at nodes represent bootstrap values (based on 100 resamplings). The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bar, 1 nt substitution per 100 nt.

around 70 % of its maximal optical density. Cellular fatty acids were methylated, separated and identified according to the instructions for the Microbial Identification System (Sasser, 1990) (Microbial ID; MIDI; version 3V 6.0-2007; RTSBA6 database; 6850 series II gas chromatograph, Agilent) which was outsourced through Royal Research Laboratories, Secunderabad, India. The major fatty acids of strain JA738^T were C_{18:1}ω7c, C_{18:0}, C_{18:1}ω5c, and C_{18:1}ω7c 11-methyl, while C_{16:0} and C_{10:0} 3-OH are present in minor amounts (Table 1).

Polar lipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2:1:0.8, by vol.) as described by Kates (1986). Lipids were separated using silica gel TLC (Kieselgel 60 F₂₅₄; Merck) by two-dimensional chromatography using chloroform-methanol-water (75:32:4, by vol.) in the first dimension and chloroform-methanol-acetic acid-water (86:16:15:4, by vol.) in the second dimension (modified after Tindall, 1990a, b; Oren *et al.*, 1996). The total lipid profile was visualized by spraying with 5 % ethanolic molybdophosphoric acid and further characterized by spraying with ninhydrin (for amino groups), molybdenum blue (for phosphates), Dragendorff's reagent (for quaternary nitrogen) or α-naphthol (for sugars) (Kates, 1972; Oren *et al.*, 1996) and cresyl violet (for sulfolipids; Soto *et al.*,

2000). Strain JA738^T contained phosphatidylglycerol (PG), phosphatidylethanolamine (PE), two unidentified sulfolipids (SL1–2) and an unidentified polar lipid (L1; Table 1; Fig. S4). Strain JA738^T differed from its phylogenetically closest relative *R. adriaticum* Imhoff 6II^T by the absence of unidentified polar lipid L2 and an unidentified phospholipid (PL). The presence of various sulfolipids in strain JA738^T is in line with the genus description of *Rhodovulum* (Hiraishi & Ueda, 1994). Quinones were extracted with a chloroform-methanol (2:1, v/v) mixture, purified by TLC and analysed by HPLC (Hiraishi & Hoshino, 1984; Hiraishi *et al.*, 1984). Strain JA738^T contained Q-10 as the major quinone.

The 16S rRNA gene sequence similarity (<96.4 %) with members of the genus *Rhodovulum* allows strain JA738^T to be described as a novel species (Tindall *et al.*, 2010). Strain JA738^T is also distinct from all other members of the genus *Rhodovulum* (Lakshmi *et al.*, 2011) in its obligate requirement for pantothenate in addition to thiamine for growth. Further, the genotypic difference of strain JA738^T is also supported by phenotypic differences with its closest relative, *Rhodovulum adriaticum* Imhoff 6II^T. Major differences between the two are observed in NaCl tolerance, chemotrophic growth, colour of cell suspension, vitamin and organic substrate requirements, carotenoid and fatty acid

Table 1. Differentiating characteristics of strain JA738^T and *Rhodovulum adriaticum* Imhoff 6II^T (=DSM 2781^T)

All data from this study. For both taxa, organic substrate utilization was tested during photolithotrophic growth. Both taxa were non-motile and utilized pyruvate and succinate, but not benzoate and fructose. +, Substrate utilized or present; – substrate not utilized or absent. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; SL1–2, unidentified sulfolipids; PL, unidentified phospholipid; L1–2, unidentified lipids.

Characteristic	JA738 ^T	<i>Rhodovulum adriaticum</i> Imhoff 6II ^T
Cell size (width × length, μm)	0.5–0.9 × 1–1.8	0.5–0.8 × 1–2
NaCl range (%)	1–7	1–10
pH range (optimum)	7.0–9.5 (7.0–8.0)	6.0–8.5 (7.0–8.0)
Chemolithoheterotrophy	+	–
Temperature range °C (optimum)	25–35 (28)	25–30 (28)
Colour of cell suspension	Reddish brown	Brown
Vitamin requirement	Thiamine, pantothenate	Biotin, thiamine
DNA G + C content (mol%)	63.4	65.3
Organic substrates/electron donors supporting growth		
Propionate	–	+
Butyrate	–	–
Valerate	–	–
Caproate	–	+
Ethanol	–	+
Lactate	–	+
Fumarate	–	+
Malate	+	+
Citrate	–	–
Aspartate	+	–
Cysteine	+	–
Glutamate	+	–
Glucose	–	+
Glycerol	–	+
Mannitol	–	–
Carotenoid composition (mol%)		
Spheroidene	16	6
Spheroidenone	48	4
Demethylspheroidene	16	74
OH-spheroidene	17	9
Neurosporene	3	7
Fatty acid composition (mol%)		
C _{10:0} 3-OH	3.1	1.6
C _{14:0} 3-OH	0.1	1.4
C _{16:0}	5.8	2.4
C _{18:1} ω7c	38.6	56.8
C _{18:1} ω5c	19.2	25.7
C _{18:0}	15.4	3.6
C _{18:1} ω7c 11-methyl	13.3	3.2
Polar lipids	PG; PE; SL1–2; L1	PG; PE; SL1–2; L1–2; PL

composition (Table 1) and polar lipid composition (Fig. S4). Taken together these data justify the description of strain JA738^T as representative of a novel species in the genus *Rhodovulum*, for which the name *Rhodovulum bhavnagarens* sp. nov. is proposed.

Description of *Rhodovulum bhavnagarens* sp. nov.

Rhodovulum bhavnagarens (bhav.na.gar.en'se. N.L. neut. adj. *bhavnagarens* of or belonging to Bhavnagar, a coastal place in Gujarat, India famous for solar salterns).

Cells are oval to rod-shaped, 1.0–1.8 μm long and 0.5–0.9 μm wide, non-motile and divide by binary fission. Growth occurs under anaerobic conditions in the light. Internal photosynthetic membranes are of vesicular type. The colour of phototrophic cultures is reddish brown. Photosynthetic pigments are bacteriochlorophyll *a* and carotenoids of the spheroidene series. Optimum growth occurs at 28 °C (range, 25–35 °C), and pH 7.0–8.0 (range, pH 7.0–9.5). Optimal growth occurs at 1–3 % NaCl and can tolerate NaCl up to 7 %. Photo- or chemolithoheterotrophy with Na₂S·9H₂O as electron donor and a few organic compounds as carbon sources are the

growth modes. Carbon sources which support growth are malate, aspartate, cysteine, pyruvate, succinate and glutamate. Does not utilize fumarate, propionate, butyrate, valerate, caproate, ethanol, lactate, citrate, glucose, fructose, glycerol, mannitol or benzoate as carbon sources. Utilizes ammonium chloride, glutamate and glutamine as nitrogen sources, but not nitrate, nitrite, aspartate, asparagine or dinitrogen. Obligate requirement of sulfide or thiosulfate as e^- /sulfur source. Thiamine and pantothenate are required as growth factors. Predominant cellular fatty acids are $C_{18:1\omega7c}$, $C_{18:0}$, $C_{18:1\omega5c}$ and $C_{18:1\omega7c}$ 11-methyl with minor amounts of $C_{10:0}$ 3-OH and $C_{16:0}$ also present. Q-10 is the major quinone. Major polar lipids are phosphatidylglycerol, phosphatidylethanolamine, unidentified sulfolipids (SL1–2) and a polar lipid (L1).

The type strain, JA738^T (=DSM 24766^T=KCTC 15110^T) was isolated from a sediment sample of a pink pond, near Bhavnagar, Gujarat, India. The DNA G+C content of the type strain is 63.4 mol% (by HPLC).

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