**Rhodovulum algae** sp. nov., isolated from an algal mat

E. V. V. Ramaprasad,¹ L. Tushar,¹ Bharti Dave,² Ch. Sasikala³ and Ch. V. Ramana¹

¹Department of Plant Sciences, School of Life Sciences, University of Hyderabad, P.O. Central University, Hyderabad 500 046, India
²Department of Life Sciences, Maharaja Krishnakumarsinhji Bhavnagar University, Bhavnagar 364002, Gujarat, India
³Bacterial Discovery Laboratory, Centre for Environment, IST, JNT University Hyderabad, Kukatpally, Hyderabad-500 085, India

A reddish-brown-pigmented, phototrophic bacterium, designated strain JA877\(^T\), was isolated from a brown algae mat sample collected from Jalandhar beach, Gujarat, India. On the basis of the 16S rRNA gene sequence, strain JA877\(^T\) belongs to the class Alphaproteobacteria and is closely related to the type strains *Rhodovulum viride* JA756\(^T\) (99.0 %), *Rhodovulum sulfidophilum* Hansen W4\(^T\) (98.9 %), *Rhodovulum visakhapatnamense* JA181\(^T\) (98.8 %), *Rhodovulum kholense* JA297\(^T\) (97.5 %) and *Rhodovulum salis* JA746\(^T\) (97.0 %). However, strain JA877\(^T\) showed only 20–45 % relatedness with its phylogenetic neighbours and had a \(\Delta T_m\) between 5.8 and 7.0 °C. The major respiratory quinone was ubiquinone-10 (Q10), and the polar lipid profile was composed of the major components phosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid, two unidentified sulfolipids and five unidentified lipids. The major fatty acids were C\(_{18:1}\), C\(_{18:2}\), C\(_{18:3}\)/C\(_{18:4}\)/C\(_{18:5}\), C\(_{16:0}\) and C\(_{18:0}\). The DNA G+C content was 64.5 mol%. On the basis of 16S rRNA gene sequence analysis, physiological data, and chemotaxonomic and molecular differences, strain JA877\(^T\) is significantly different from other species of the genus *Rhodovulum* and represents a novel species, for which the name *Rhodovulum algae* sp. nov. is proposed. The type strain is JA877\(^T\) (=LMG 29228\(^T\) = KCTC 42963\(^T\)).

Members of genus *Rhodovulum* (*Rdv.* ) are phototrophic, isolated from marine habitats and form a distinct monophyletic 16S rRNA gene cluster with no interspersing chemotrophs. At the time of writing, there were 19 species with validly published names assigned to this genus (Divyasree et al., 2016). In this study, we isolated a strain (JA877\(^T\)) from a brown algae mat sample and found that the 16S rRNA gene sequence of the isolate was related to those of species of the genus *Rhodovulum*. Here we describe the phenotypic, physiological and chemotaxonomic features of this isolate.

Strain JA877\(^T\) was isolated from a brown algae mat sample collected from Jalandhar beach, Gujarat, India (GPS position of the sample collection site: 20° 709’ N 70° 985’ E), on 21 December 2014. One gram brown algae mat was serially diluted [10-fold dilution in saline (0.9 % NaCl)] and plated on a Zobell’s marine agar (MA) medium (Himedia, India, cat no. M384). Three distinct colony morphologies were observed from plates incubated at 30 °C for 6 days. Reddish-brown-pigmented colonies were purified by subsequent streaking on the same medium. The purified isolate was designated strain JA877\(^T\) and further the strain was preserved as glycerol stocks and stored at 4 °C. For routine culturing and for physiological tests, strain JA877\(^T\) was grown in a medium described previously (Srinivas et al., 2014) in 50 ml fully filled, screw-capped bottles incubated at 30 °C under 200 W m\(^{-2}\) of illumination by a tungsten lamp for 7 days under anaerobic conditions.

Genomic DNA was isolated and purified by the method described by Marmur (1961), and the G+C content (mol%)
of the DNA as determined by HPLC (Mesbah et al., 1989) was 64.5 mol%. Cell material for 16S rRNA gene sequencing was taken from a well-isolated colony, and the gene was PCR-amplified and sequenced as described previously (Ramaprasad et al., 2015a). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using EzTaxon-e BLAST analysis (Kim et al., 2012). Ez-Taxon-e BLAST analysis of a 1386 bp length of the 16S rRNA gene indicated that strain JA877T is related phylogenetically to the members of the genus *Rhodovulum*, with its closest relatives being *Rhodovulum viride* JA756T (99.0 %), *Rdv. sulfidophilum* Hansen W4T (98.9 %), *Rdv. visakhapatnamense* JA181T (98.8 %), *Rdv. kholense* JA297T (97.5 %), *Rdv. salis* JA746T (97.0 %) and other members of the genus *Rhodovulum* (<97 %). 16S rRNA gene sequences were aligned using the SILVA incremental aligner (SINA; http://www.arb-silva.de), and the MEGA 6 (Tamura et al., 2013) software was used for phylogenetic analyses. For reconstructing neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) phylogenetic trees, the following statistical methods were used. For NJ, the Kimura two-parameter model (Kimura, 1980), with uniform rates and pairwise deletion was used. For ML, the Kimura two-parameter model with uniform rates and the heuristic search algorithm nearest-neighbour-interchange (NNI) with complete deletion was used. For MP, subtree-pruning-regrafting (SPR) as heuristic algorithm with complete deletion was used. The combined phylogenetic tree (NJ, ML, MP) confirmed the clustering of strain JA877T with members of the genus *Rhodovulum* (Fig. 1).

DNA–DNA reassociation between strains was determined using a membrane filter technique as described previously (Ramaprasad et al., 2015b). When strain JA877T was labelled radioactively, the level of DNA–DNA reassociation with *Rdv. viride* JA756T was 45.6±2 % (reciprocal 44.8 %), with *Rdv. sulfidophilum* DSM 1374T was 43±1.6 % (reciprocal 42.6 %), with *Rdv. visakhapatnamense* JA181T was 38.7±2.4 % (reciprocal 39±1 %), with *Rdv. kholense* JA297T was 36±3 % (reciprocal 36.2 %) and with *Rdv. salis* JA746T was 20±1 % (reciprocal 20.5 %). On the basis of the hybridization results, strain JA877T represents a novel species according to recommendations for delineating a bacterial species (Stackebrandt & Goebel, 1994; Wayne et al., 1987). The degree of DNA–DNA relatedness was also estimated using a fluorimetric method with SYBR Green-I, by measuring the divergence between the thermal denaturation midpoint of homoduplex and heteroduplex DNA (ΔTm) as described by Diysasree et al. (2016). DNA–DNA relatedness estimates using a ΔTm method confirmed that strain JA877T should be classified as a representative of a different species. The ΔTm value of strain JA877T with its closest phylogenetic neighbours was 5.8–7.0 °C, which is significantly above the 5 °C cut-off level recommended for species delineation (Rosselló-Mora & Amann, 2001; Wayne et al., 1987). *Rdv. viride* JA756T, *Rdv. salis* JA746T, *Rdv. visakhapatnamense* JA181T and *Rdv. sulfidophilum* DSM 1374T (=Hansen W4T) were used for comparative taxonomic analysis under the authors’ laboratory conditions.

Morphological properties (cell shape, cell division, cell size, flagella) were examined under a phase-contrast light microscope (BH-2; Olympus). Cells of strain JA877T were oval to rod-shaped, 1–1.4 μm long and 0.6–0.9 μm wide, motile and multiplied by binary fission (Fig. S1, available in the online Supplementary Material). 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 JA877T 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 (Truper & Pfennig, 1981) with a Spectronic Genesys 2 spectrophotometer exhibited maxima at 854, 804, 479 and 375 nm (Fig. S3) indicating the absence of bacteriochlorophyll a. Carotenoid composition of strain JA877T, *Rdv. viride* JA756T, *Rdv. sulfidophilum* DSM 1374T, *Rdv. visakhapatnamense* JA181T, *Rdv. kholense* JA297T and *Rdv. salis* JA746T as determined by C8-HPLC (Ramaprasad et al., 2013) indicated the presence of hydroxyspheroidenone (6 %), demethylspheroidenone (9 %), spheroidene (84 %) and neuropsorene (1 %) (Fig. S4). Neuropsorene was the sole carotene identified in *Rdv. viride* JA756T. Strain JA877T differed from *Rdv. viride* JA756T by the presence of the carotenoids spheroidene, hydroxyspheroidenone and demethylspheroidene. From the other strains it differed in the relative abundance of different carotenoids, and the carotenoids profiles of the other four strains were similar.

Growth was measured turbidometrically at 660 nm. NaCl improved growth yields of strain JA877T (salinity range and optimum were 1–7 % and 2 %, w/v, NaCl, respectively). Strain JA877T grew over a pH range of 7.0–9.0 (optimum, pH 7.5) and at 20–35 °C (optimum, 30 °C). Strain JA877T had no requirement for sulfide for growth and could tolerate atmospheric levels of oxygen. Photo- (anaerobic, light; 2400 lx) and chemo- (aerobic, dark) organoheterotrophy [with pyruvate (0.03 %, w/v) as carbon source/electron donor] was the preferred growth mode. 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, SH2O (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 JA877T required biotin as a growth factor.

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 JA877ᵀ 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. Sulfate, sulfide and thiosulfate were used as sulfur sources by strain JA877ᵀ. Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources at 0.06 % (w/v). Strain JA877ᵀ could utilize ammonium chloride, nitrate, nitrite and glutamine as nitrogen sources, but glutamate, aspartate, asparagine and N₂ did not support growth.

Cells grown photoheterotrophically were harvested when growth of the cultures had reached around 70 % (early stationary phase) of the maximal optical density and were used for analysis of cellular fatty acids, polar lipids, quinones and cyclic triterpenoids (hopenoids and steroids), which was done as described previously (Ramaprasad et al., 2015c, d; Soto et al., 2000). Major (>5 %) fatty acids were C₁₈:₁ω₅c, C₁₈:₁ω₇c, C₁₈:₁ω₆c, C₁₈:₀, C₁₆:₀ with minor (>1 to <5) amounts of C₁₀:₀ 3OH, C₁₆:₁ω₇c/C₁₆:₁ω₆c, C₁₆:₁ω₇c 11-methyl and C₁₅:₀ 2OH. The differences in fatty acid profile of strain JA877ᵀ from the phylogenetically most closely related type strains are shown in Table S1.

All species of the genus Rhodovulum have phosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid, unidentified sulfolipids and two unidentified lipids as polar lipids. The major polar lipids of strain JA877ᵀ were in coherence with our earlier report (Srinivas et al., 2014); however, the absence of an unidentified phospholipid (PL2) and the presence of additional unidentified lipids (L3, L4 and L5; Fig. S5) differentiated the novel strain from the rest of the species of the genus Rhodovulum (Srinivas et al., 2014). Strain JA877ᵀ contained Q-10 as the sole quinone.

Cyclic triterpenoids (hopenoids and steroids) were extracted as previously described (Ramaprasad et al., 2015d) from 0.2 g lyophilized cells. Cells were sonicated and extracted twice with 10 ml methanol/dichloromethane.
All data are from the present study. Organic substrate utilization was tested during photoheterotrophic growth. +, Substrate utilized/present; –, substrate not utilized/absent; B, brown; YB, yellowish brown; G, green; RB, reddish brown; b, biotin; n, niacin; paba, p-aminobenzoic acid; t, thiamine. All the strains are oval-rod shaped, divide by binary fission, have vesicles as intracytoplasmic membrane structures and grow optimally at 30°C (range 25–35°C); Q10 is the only quinone; they utilize malate, glucose and pyruvate, but not aspartate; they do not contain hopanoids, but contain steroid (cholest-5-en-3-ol; except for *Rdv. kholense* JA297T); they contain spheroidene as the major carotenoid (exception for *Rdv. viride* JA756T which accumulates neurosporene).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter (µm)</td>
<td>0.6×1.5</td>
<td>0.8×2-3</td>
<td>0.6×1</td>
<td>1×2-3</td>
<td>0.5–1×1.5–1.8</td>
<td>0.9×1.2</td>
</tr>
<tr>
<td>NaCl range (optimum)</td>
<td>1–7 (2)</td>
<td>0–9 (1)</td>
<td>0–10 (2)</td>
<td>1–8 (2)</td>
<td>0–6 (1–4)</td>
<td>0–10 (2)</td>
</tr>
<tr>
<td>pH range</td>
<td>7.0–9.0</td>
<td>6–8.5</td>
<td>5.0–9.0</td>
<td>6.0–7.5</td>
<td>6.0–8.0</td>
<td>4.0–9.0</td>
</tr>
<tr>
<td>Colour of cell suspension</td>
<td>RB</td>
<td>G</td>
<td>B</td>
<td>YB</td>
<td>YB</td>
<td></td>
</tr>
<tr>
<td>Vitamin requirement</td>
<td>b</td>
<td>–</td>
<td>b, n, paba, t</td>
<td>t, b, paba</td>
<td>b, n, t</td>
<td>b, n, t, paba</td>
</tr>
<tr>
<td>Carbon/electron donors</td>
<td>Propionate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Valerate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Fumarate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>64.5</td>
<td>63.3</td>
<td>66.3</td>
<td>62.4</td>
<td>63</td>
<td>61.2</td>
</tr>
</tbody>
</table>

(0.5:4, by vol.) and the pooled supernatants were finally extracted with DCM. The organic phase was collected and evaporated to dryness, and the extracted lipids were dissolved in 1ml DCM. The extract was acetylated using acetic anhydride and analysed using GC-MS as previously described (Tushar *et al.*, 2014). Strain JA877T did not contain any detectable hopanoids; however, it contained the steroid, cholest-5-en-3-ol. Strain JA877T, *Rdv. viridiae* JA756T, *Rdv. sulfidophilum* DSM 1374T, *Rdv. visakhapatnamense* JA181T, *Rdv. salis* JA756T, *Rdv. imhoffii* JA125T and *Rdv. bhanugoreense* JA738T contained cholest-5-en-3-ol (Table S2). However, *Rhodovulum adriaticum* DSM 2781T, *Rdv. phaeocolus* JA580T, *Rdv. euryhalinum* DSM 4868T, *Rdv. robiginosum* DSM 12329T, *Rdv. strictum* DSM 11289T, *Rdv. steppense* VKB B-2489T have hopanoids (Table S2). Neither hopanoids nor sterols were detected from *Rdv. kholense* JA297T or *Rhodovulum marinum* JA128T. Unlike the genus *Rhodoplanes* where all the members have hopanoids (Tushar *et al.*, 2015), heterogeneity in cyclic triterpenoids was observed among species of the genus *Rhodovulum*; while a few species have hopanoids, some have steroids and others do not have either of these.

Strain JA877T is also distinct from all other members of the genus *Rhodovulum* (Srinivas *et al.*, 2014) in its obligate requirement for biotin for growth. Further, the genotypic difference of strain JA877T is also supported by phenotypic differences with its closest phylogenetic relatives, *Rdv. viridiae* JA756T and *Rdv. sulfidophilum* DSM 1374T. Major differences between the three strains are observed in vitamin requirements, NaCl tolerance, colour of cell suspension, organic substrate requirements, carotenoids, fatty acid composition (Tables 1 and S1) and polar lipid composition (Fig. S5). Taken together, these data justify the description of strain JA877T as a representative of a novel species in the genus *Rhodovulum*, for which the name *Rhodovulum algae* sp. nov is proposed.

**Description of *Rhodovulum algae* sp. nov.**

*Rhodovulum algae* (al’gae. L. fem. gen. n. alge of an alga, referring to the isolation source of the type strain).

Cells are oval to rod-shaped, 1.0–1.4 µm long and 0.6–0.9 µm wide, motile and divide by binary fission. Growth occurs under anaerobic conditions. 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 30°C (range, 20–35°C) and pH 7.5 (range, pH 7.5–9.0). Optimal growth occurs...
with 2% NaCl and it can tolerate NaCl up to 7%. Carbon sources which support growth are lactate, propionate, ethanol, citrate, glycerol, mannan, malate, glucose and pyruvate. Does not utilize valerate, fumarate, glutamate or aspartate. Utilizes ammonium chloride, nitrate, nitrite and glutamine as nitrogen sources, but not glutamate, aspartate, asparagine or dinitrogen. Sulfide or thiosulfate are not required for growth as electron or sulfur sources. Biotin is required as a growth factor. Predominant cellular fatty acids are C18:1ω5c, C18:1ω7c, C16:0 and C18:0. Q-10 is the sole quinone. Polar lipids are phosphatidylglycerol, phosphatidylethanolamine, two unidentified sulfoleipids, an unidentified phospholipid and five unidentified polar lipids. Contains cholest-5-en-3-ol as cyclic triterpenoid.

The type strain is JA877T (=LMG 29228T=KCTC 42963T) and was isolated from a brown alga sample collected from Jalandhar beach, Gujarat, India. The DNA G+C content of the type strain is 64.5 mol%.

Acknowledgements

Funds received from Ministry of Earth Sciences, Government of India are duly acknowledged (grant no. MoES/16/06/2013-RDEAS). CSIR, Government of India is acknowledged for funding the hopanoids part of the work of this manuscript. DST and UGC are acknowledged for providing infrastructural facilities supported through FIST and SAP-DRS programs. Ch. V. R. thanks the Department of Biotechnology, Government of India for the award of Tata Innovative Fellowship.

References


