Rhodomicrobium udaipurense sp. nov., a psychrotolerant, phototrophic alphaproteobacterium isolated from a freshwater stream

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Two strains (JA643T and JA755) of Gram-stain-negative, facultatively anaerobic phototrophic, bacteria capable of growth at low temperatures (10–15 °C) were isolated from freshwater streams from different geographical regions of India. Both strains contain bacteriochlorophyll a and carotenoids of the spirilloxanthin series. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, an unidentified phospholipid (PL), unidentified amino lipids (AL1–AL6, AL9) and an unidentified lipid (L1) were the polar lipids present in both strains. The major cellular fatty acid was C18 : 1ω7c (76–79 % of the total). Bacteriohopane derivatives (BHD1,2), unidentified hopanoids (UH1–5), diplopterol (DPL) and diploptene (DPE) were the major hopanoids of both strains. The DNA G+C content was 64.2–64.5 mol%. 16S rRNA gene sequence-based phylogenetic analysis showed that both strains are closely related to the genus Rhodomicrobium and clustered with Rhodomicrobium vannielii DSM 162T (99 % sequence similarity). However, both strains exhibited only 46.1 % DNA–DNA hybridization with R. vannielii DSM 162T. Strains JA643T and JA755 shared >99 % 16S rRNA gene sequence similarity and were >85 % related on the basis of DNA–DNA hybridization; they are therefore considered to represent a novel species in the genus Rhodomicrobium, for which the name Rhodomicrobium udaipurense sp. nov. is proposed. The type strain is JA643T (=KCTC 15219T=NBRC 109057T).

The family Hyphomicrobiaceae in the class Alphaproteobacteria contains three phototrophic genera: Blastochloris, Rhodomicrobium and Rhodoplanes. The genus Rhodomicrobium can be distinguished from Blastochloris and Rhodoplanes mainly on the basis of cell morphology, including long prosthecae and a characteristic vegetative growth cycle. Rhodomicrobium vannielii (Duchow & Douglas, 1949) is the single recognized species of the genus Rhodomicrobium; in the present communication, we propose a novel species based on two strains (JA643T and JA755) isolated from different geographical locations in India.

Strain JA643T was isolated from a freshwater stream sample collected from Udaipur in Himachal Pradesh [GPS of the sampling site is 32° 43’ 30.55” N 76° 39’ 55.45” E; altitude of 8970 feet (2734 m) above sea level], in the western Himalayas, India. Strain JA755 was isolated from a freshwater stream sample collected from Rottikadai town near the Annamalai hills [10° 35’ N 76° 97’ E; altitude of 3500 feet (1067 m) above sea level] in the Western Ghats, Tamil Nadu, India. Both strains were isolated after enrichment in PE medium (Hanada et al., 1995) at pH 7.0, in fully filled 50 ml screw-capped bottles incubated under phototrophic (2500 lx) conditions at 30 ± 2 °C for 15 days. Purification of the isolates and the media used for growth of the organisms are as previously described (Lakshmi et al., 2011a, b).

Genomic DNA was extracted and purified according to the method of Marmur (1961) and the DNA G+C content of strains JA643T and JA755 was 62.4 and 62.8 mol% as determined by HPLC (Mesbah et al., 1989). Cell material

Abbreviation: FT-IR, Fourier transform infrared.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains JA643T and JA755 are FN995218 and HE863941, respectively.

Five supplementary figures are available with the online version of this paper.
for 16S rRNA gene sequencing was taken from a colony and DNA was extracted and purified using a Qiagen genomic DNA extraction kit. Recombinant Taq polymerase (Genei) was used for PCR and the complete length of the 16S rRNA gene sequence was obtained by sequencing with primers F'-27 (5'-GTTTGATCCTGGCTCAG-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 (Brosius et al., 1978; Lane et al., 1985)]. PCR amplification and sequencing was done as previously described (Ramana et al., 2012). On the basis of Eztaxon-e BLAST search analysis (Kim et al., 2012) of the nearly complete 16S rRNA gene sequences of strains JA643T and JA755 (1343 and 1355 bp, respectively), they shared 99% similarity with R. vannielii DSM 162T. The CLUSTAL W algorithm of MEGA 4 was used for sequence alignments and MEGA 4 (Tamura et al., 2007) software was used for phylogenetic analysis of the individual sequences. Distances were calculated by using the Kimura two-parameter model (Kimura, 1980). Neighbour-joining and minimum-evolution methods in the MEGA 4 software (http://www.megasoftware.net/mega4/mega4.pdf) were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. The phylogenetic relationship between strains JA643T, JA755 and other closely related bacteria revealed that the new isolates clustered with, but were distinct from, R. vannielii DSM 162T [the neighbour-joining tree is shown as Fig. 1; the tree topology was similar using the minimum-evolution, unweighted pair group method with arithmetic averages (UPGMA) and maximum-likelihood methods (data not shown)]. Sequence similarity between strains JA643T and JA755 was 99.8%.

The taxonomic relationship between members of the genus Rhodobium was examined using whole genome DNA–DNA hybridization. Genomic relatedness was determined using a membrane filter technique (Seldin & Dubnau, 1985; Tourova & Antonov, 1988) and the experimental details are as described previously (Ramana et al., 2012). Strains JA643T and JA755 were related closely (>85% DNA–DNA relatedness) to each other, while both strains were more distantly (46.1%; average of both forward and reverse) related to R. vannielii DSM 162T. The hybridization values were below the recommended threshold to delineate a bacterial species (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). To support the

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**Fig. 1.** Dendrogram depicting the phylogenetic relationships between strains JA643T and JA755, the type strain of *Rhodomicrobium vannielii* along with interspersing chemotrophs, as determined using 16S rRNA gene sequences. The tree was constructed (1343/1355 gap-free sites were compared) using the neighbour-joining method utilizing MEGA 4 software. Bootstrap percentages from 500 replicates are shown (only values >70%) next to the corresponding branch. Bar, 2 nucleotide substitutions per 100 nt.
genomic data, comparative chemotaxonomic and physiological analyses was performed using strains JA643\textsuperscript{T}, JA755 and \textit{R. vannielii} DSM 162\textsuperscript{T}.

For analysis of the cellular fatty acids, quinones, polar lipids and hopanoids and Fourier transform infrared (FT-IR) fingerprinting, cells grown in the above medium were harvested when growth of the cultures was around 70\% of its maximal optical density. Cellular fatty acids were analysed as their methyl esters after separation and identified according to the instructions for the Microbial Identification System [Microbial ID; MIDI version 6.0;

### Table 1. Differential characteristics between strains JA643\textsuperscript{T} and JA755 and \textit{Rhodomicrobium vannielii} DSM 162\textsuperscript{T}

Strains: 1, JA643\textsuperscript{T}; 2, JA755; 3, \textit{R. vannielii} DSM 162\textsuperscript{T}. All data from the present study. For all taxa organic substrate utilization was tested during photoheterotrophic growth. Acetate, butyrate, fumarate, lactate, malonate, benzoate and pyruvate are utilized by all strains. Citrate, D-glucose, D-fructose, sorbitol and mannitol are not utilized. All strains have bacteriochlorophyll-\textalpha, ubiquinone and rhodoquinone as major quinones, no salt or vitamin requirements, assimilate sulfate and do not ferment glucose/fructose. All the strains are motile (individual cells), and have tube-type budding, rosette formation and a lamellar intracellular membrane. +, Substrate utilized/present; –, substrate not utilized/absent; (+), weak growth.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Cell size (width \times length, μm)</td>
<td>1.2–1.4 × 2.8–3.5</td>
<td>1.2–1.4 × 2.8–3.5</td>
<td>1.2–1.5 × 2.6–2.8</td>
</tr>
<tr>
<td>Broth culture colour</td>
<td>Reddish-brown</td>
<td>Reddish-brown</td>
<td>Red</td>
</tr>
<tr>
<td>Optimal pH (range)</td>
<td>6.5–7.5 (5.5–8.0)</td>
<td>6.5–7.5 (5.5–8.0)</td>
<td>5.5–6.5 (5.0–7.5)</td>
</tr>
<tr>
<td>Temperature range (optimum; °C)</td>
<td>10–40 (30)</td>
<td>15–40 (30)</td>
<td>25–35 (30)</td>
</tr>
<tr>
<td>Photoautotrophic growth with</td>
<td>H₂, sulfide, thiosulfate</td>
<td>H₂, sulfide, thiosulfate</td>
<td>H₂, sulfide</td>
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<tr>
<td>Major polar lipids*</td>
<td>PE, DPG, PC, PL, AL1–6,9</td>
<td>PE, DPG, PC, PL, AL1–6,9</td>
<td>PE, DPG, PC, PL, L1–2, AL1–9</td>
</tr>
<tr>
<td>Major hopanoids†</td>
<td>BHD1, BHD2, DPL, DPE, UH1–2,4,5</td>
<td>BHD1, BHD2, DPL, DPE, UH1–5</td>
<td>BHD1, BHD2, DPL, UH1–4</td>
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<tr>
<td>C\textsubscript{14}:0</td>
<td>2.4</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>C\textsubscript{15}:0 2-OH</td>
<td>2.5</td>
<td>2.4</td>
<td>–</td>
</tr>
<tr>
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<td>3.2</td>
<td>3.5</td>
<td>7.0</td>
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<td>–</td>
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<tr>
<td>C\textsubscript{16}:1\textsubscript{ω7c}</td>
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<td>C\textsubscript{17}:0 10-methyl</td>
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<td>5.3</td>
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<tr>
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<td>79</td>
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<td>Growth on:</td>
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<tr>
<td>Aspartate</td>
<td>(+)</td>
<td>(+)</td>
<td>–</td>
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<tr>
<td>Caproate</td>
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<tr>
<td>Glutamate</td>
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<td>+</td>
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<tr>
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<tr>
<td>Malate</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
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<tr>
<td>Propanol</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Butanol</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Propionate</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>Succinate</td>
<td>–</td>
<td>–</td>
<td>(+)</td>
</tr>
<tr>
<td>Tartrate</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Valerate</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
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<tr>
<td>Utilization of sulfur sources</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioglycolate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cysteine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)‡</td>
<td>62.4</td>
<td>62.8</td>
<td>64.2</td>
</tr>
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</table>

*AL1–9, Unidentified aminolipids; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PG, phosphatidylycerol; PL, unidentified phospholipid; L1,2, unidentified lipids.
†BHD1,2, Bacteriohopane derivatives; DPL, diplopterol; DPE, diploptene; UH1–5, unidentified hopanes.
‡HPLC method.
were the common polar lipids detected in all three strains (AL1–AL6, AL9) and an unidentified phospholipid (PL) (DPG), phosphatidylcholine (PC), unidentified aminolipids Phosphatidylethanolamine (PE), diphosphatidylglycerol
1984; Hiraishi purification by TLC (Imhoff, 1984; Hiraishi & Hoshino, all the three strains as analysed by HPLC after extraction none-10 (RQ10; 35–40 mol%) were the major quinones of

dried cells as described previously (Ramana
[144] differences from
[144] JA643T and JA755. An unidentified hopane (UH3) was
[144] present in strain JA755 and
[144] JA643T and JA755 also differed from
[144] R. vannielii DSM 162T. FT-IR fingerprinting of all three strains was done as previously described (Mashego et al., 2007, Ramana et al., 2006; Srinivas et al., 2007; Kumar et al., 2007, Arunasri et al., 2008) and the labelled transmission peaks were considered as FT-IR barcodes (Fig. S3). The fingerprints of strains
[144] JA643T and JA755 were almost identical and showed clear differences from
[144] R. vannielii DSM 162T, particularly in the region 1650–2000 cm
[144] In vivo absorption spectra of intact cells of all three strains in sucrose (Trüper & Pfennig, 1981) exhibited maxima at 377, 512, 595–605, 797, 805 and
[144] 869 nm (Fig. S4), indicating the presence of bacteriochlorophyll a and carotenoids. Acetone extracts of all three strains showed absorption maxima at 445, 475 and 502 nm, indicating the presence of carotenoids of the spirilloxanthin series (Britton et al., 2004). The colour of phototrophically grown cultures of strains
[144] JA643T and JA755 was reddish-brown, while
[144] R. vannielii DSM 162T was red. Cell morphology (cell shape, cell division, cell size, flagella and visible internal or external structures) was observed by phase-contrast microscopy (BH-2; Olympus) and transmission electron microscopy (Hitachi H-7500). Cells of strains
[144] JA643T and JA755 were ovoid to swollen rods 1.2–1.4 μm wide and 2.8–3.5 μm long that multiplied by tube-type budding and formed complex aggregates due to their long and slender branched filaments (Fig. S5a). Resting cells of strains
[144] JA643T and JA755 formed budded exospore-like structures (Fig. S5b), similar to the cell morphology of
[144] R. vannielii DSM 162T (Whittenbury & Dow, 1977). Individual cells were motile and motility was arrested in older cells after their aggregation. For transmission electron microscopy, cells were subjected to negative staining with phosphotungstic acid and grids were examined under an Hitachi H-7500 electron microscope. Transmission electron micrographs of ultrathin sections of all three strains revealed lamellar-type internal membrane structures parallel to the cytoplasmic membrane. Gram-staining was performed as described by Smibert & Krieg (1994) and all three strains stained Gram-negative. The three strains were motile as observed by the hanging-drop method (Suzuki et al., 2001).

Growth was measured turbidometrically at 660 nm in a colorimeter (Systronics) and all physiological tests were performed in triplicate. Growth at various concentrations of NaCl [0–10 % (w/v), at intervals of 0.5 %], pH (pH 5.0–
10.0, at intervals of 0.5 pH units) and temperature (5–
45 °C, at intervals of 5 °C) was investigated in the medium described above. NaCl was not obligatory for growth of all three strains, but strains
[144] JA643T and JA755 were able to tolerate up to 2 %, which is in contrast to
[144] R. vannielii DSM 162T, which tolerated only up to 0.5 % NaCl. Growth pH optima for strains
[144] JA643T and JA755 were pH 6.5–7.5 (range 5.5–8.0), while
[144] R. vannielii DSM 162T required an acidic medium of pH 5.5–6.5 (range 5.0–7.5). Growth at low temperatures (10–15 °C) also differentiated strains
[144] JA643T and JA755 from
[144] R. vannielii DSM 162T (Table 1).

Organic acids (0.35 %, w/v, benzoic acid 0.5 mM), alcohols/fatty acids (0.1 %, v/v) and inorganic compounds (0.5–1.0 mM) such as sulfide, sulfate, elemental sulfur, H₂ (20 % of the gas phase) and thiosulfate were tested for their utilization as electron donor and/or carbon source under photoheterotrophic conditions. In addition, 0.1 % (w/v) bicarbonate was supplemented in the medium for fatty acids, alcohols, thiolsulfate, sulfide, sulfate, benzoate, H₂, elemental sulfur and thioglycollate. Strains JC643T and JC755 were capable of growing photo-organo-heterotrophically [anaerobically in the light (2400 lx) with sodium pyruvate (0.3 %, w/v) as carbon source] and chemo-organo-heterotrophically [anaerobically in the dark with sodium pyruvate (0.3 %, w/v) as carbon source]. Photolithoautotrophy [anaerobically in the light (2400 lx)] with Na₂S·9H₂O (1 mM)/Na₂S⁰₂·5H₂O (3 mM)/H₂ as electron donor and NaHCO₃ (0.1 %, w/v) as carbon source] was demonstrated
in strains JA643T and JA755, while thiosulfate was not used as an electron donor by *R. vannielii* DSM 162T. Chemolithoautotrophic growth [aerobically in the dark with Na2S2O3, 5H2O (0.5 mM) as electron donor and NaHCO3 (0.1%, w/v) as carbon source] and fermentative growth [anaerobically in the dark with glucose/fructose/pyruvate (0.3%, w/v)] could not be demonstrated in any of the strains. The preferred mode of growth was photo-organoheterotrophy with a number of organic compounds, which are listed in the species description and the differences between strains JA643T and JA755 and *R. vannielii* DSM 162T are shown in Table 1. Nitrogen source utilization was tested by replacing ammonium chloride with urea, nitrate, nitrite, glutamate or glutamine at 0.06% (w/v). Strains JA643T and JA755 were able to grow with ammonium chloride, glutamine and urea as nitrogen sources while nitrite, nitrate and glutamate could not support growth. Vitamin requirements were tested by replacing yeast extract with different vitamins [vitamin B12, biotin, niacin, p-aminobenzoic acid, pantothenate, pyridoxal phosphate, riboflavin, thiamine and a mixture of all the above (0.02%, w/v)] as growth factors; neither of the novel strains had vitamin requirements.

The DNA–DNA hybridization data between strain JA643T and *R. vannielii* DSM 162T were supported strongly by differences in the growth temperature range, whole-cell fatty acid profile, polar lipid composition, metabolite fingerprints, hopanoid composition, pH optima and range, colony colour and organic substrates utilized for growth (Table 1). Therefore, we suggest that strains JA643T and JA755 represent a single novel species of the genus *Rhodomicrobium*, for which the name *Rhodomicrobium udaipurense* sp. nov. is proposed.

**Description of Rhodomicrobium udaipurense sp. nov.**

*Rhodomicrobium udaipurense* (u.dai.pur.en’se. N.L. neut. adj. udaipurense of or belonging to Udaipur, a place in the Indian Himalayas, from where the type strain was isolated). Cells are ovoid to swollen rods 1.2–1.4 μm wide and 3.0–3.5 μm long, and individual cells are motile and multiply by budding. Exosporalike structures are present. Phototrophic cultures are reddish-brown. Older cells form complex aggregates due to entanglement of long prosthecae. Internal cytoplasmic membranes are of the lamellar type. In vivo absorption spectra of intact cells exhibit maxima at 377, 512, 595 and 860 nm. Bacteriochlorophyll α and carotenoids of the spirilloxanthin series are present. NaCl or vitamins are not required for growth. Growth occurs over a wide range of temperature (10–40 °C) with an optimum at 30 ± 2 °C. The optimum pH for the growth is 6.5–7.5 (range 5.5–8.0). The preferred mode of growth is photo-organoheterotrophy with a few organic compounds. Good growth occurs on pyruvate, glutamate, benzoate, formate, glutamate, malate, succinate, acetate, butyrate, fumarate, lactate, malonate, tartrate, caprylate and propionate. Weak growth occurs with aspartate, caproate, propanol, butanol and valerate. No growth occurs with citrate, d-glucose, d-fructose, sorbitol, mannitol, glycerol or ethanol. Ammonium chloride, glutamine and urea are used as sole nitrogen source while nitrite, nitrate and glutamate are not. Sulfate, thioglycolate and cysteine are used as sulfur sources, while sulfite is not. Growth occurs by photo-organoheterotrophy and chemo-organoheterotrophy. Photolithoautotrophy is possible with Na2S, 9H2O (1 mM)/Na2S2O3, 5H2O (3 mM) or H2/NaHCO3 (0.1%, w/v). Fermentative growth is not possible with glucose or pyruvate. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, diphasphatidylglycerol, an unidentified phospholipid (PL), unidentified amino lipids (AL1–AL6, AL9) and an unidentified lipid (L1) are present. The major cellular fatty acid is C18:1ω7c. Bacteriohopane derivatives (BHD1, 2), unidentified hopanoids (UH1–5), deplopterol (DPL) and deploptene (DPE) are the major hopanoids.

The type strain is JA643T (=KCTC 15219T=NBRC 109057T), isolated from a freshwater sample collected from a stream at Udaipur, Himachal Pradesh in the western Himalayas. JA755 is a second strain of the species. The DNA G+C content of the type strain JA643T is 64.2 mol% (by HPLC).

**Acknowledgements**

We thank Professor J. Euzéby for help with the species epithet and Latin etymology. V. V. R. acknowledges the UGC, Government of India, for the award of a Dr D. S. Kothari postdoctoral fellowship. P. S. R. acknowledges JNTU Hyderabad for award of a research fellowship. CSIR, Government of India is acknowledged for financial assistance. L. T. acknowledges the UGC for the award of JRF fellowship.

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