Rhodobacter aestuarii sp. nov., a phototrophic alphaproteobacterium isolated from an estuarine environment

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An ovoid to rod-shaped, phototrophic, purple non-sulfur bacterium was isolated from a brown-coloured microbial mat from the brackish water of Bhitarkanika mangrove forest, Dangmal, Orissa, India. Cells of strain JA296^T were Gram-negative and motile, forming chains of four to eight cells. The colour of the cell suspension grown under anaerobic conditions in the light was yellowish green. Bacteriochlorophyll *a* and the carotenoids spheroidene and spheroidenone of the spirilloxanthin series were present as photosynthetic pigments. The bacterium was a facultative anaerobe and was able to grow photo-organoheterotrophically and chemo-organoheterotrophically. Thiamine was required as a growth factor. C_{18:1}ω7c was the dominant fatty acid. Internal cytoplasmic membranes were of the vesicular type. Strain JA296^T did not require NaCl for growth. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA296^T was most closely related to *Rhodobacter capsulatus* ATCC 11166^T (95.5% sequence similarity) and clustered with species of the genus *Rhodobacter* of the family *Rhodobacteraceae*, class *Alphaproteobacteria*. On the basis of 16S rRNA gene sequence analysis and morphological and physiological characteristics, strain JA296^T represents a novel species of the genus *Rhodobacter*, for which the name *Rhodobacter aestuarii* sp. nov. is proposed; the type strain is JA296^T (=JCM 14887^T =CCUG 55130^T).

At the time of writing, the genus *Rhodobacter* comprised ten recognized species, including five species that have been described by our group: *Rhodobacter vinaykumarii* (Srinivas *et al.*, 2007b), *Rba. changlensis* (Anil Kumar *et al.*, 2007), *Rba. ovatus* (Srinivas *et al.*, 2008), *Rba. maris* (Venkata Ramana *et al.*, 2008) and *Rba. megalophilus* (Arunasri *et al.*, 2008). Members of the genus *Rhodobacter* can be differentiated from those of other genera based primarily on internal cytoplasmic membranes, type of cytochromes, quinone components, lipopolysaccharides, NaCl requirement for optimal growth, 16S rRNA gene sequences and phenotypic characters (Imhoff, 2005). All species of the genus *Rhodobacter* except *Rba. vinaykumarii* and *Rba. maris* were described on the basis of freshwater isolates. *Rba. vinaykumarii* is a true marine species, requiring NaCl for growth, which necessitated emendation

of the genus description. *Rba. maris*, although isolated from marine waters, does not require NaCl for growth. During investigations of the purple bacterial diversity of brackish water, a purple non-sulfur bacterium (strain JA296^T) was isolated from water of a mangrove forest. Although isolated from salty waters, strain JA296^T is a non-marine isolate. On the basis of phenotypic and molecular analyses, it is proposed that strain JA296^T represents a novel species.

A brown-coloured microbial mat sample from the Bhitarkanika mangrove forest at Dangmal, Orissa, India (GPS position: 20° 49' N 86° 47' E), was collected in a sterile glass bottle. Strain JA296^T was isolated from enrichments under photo-organoheterotrophic conditions. Purification and polyphasic taxonomic analyses were carried out as described previously (Srinivas *et al.*, 2007a). Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (Microbial ID, MIDI; Agilent 6850) (Sasser, 1990) at the Royal Research Laboratories, Secunderabad, India. 16S rRNA gene sequences were aligned

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA296^T is AM748926.

An electron micrograph of an ultrathin section of strain JA296^T and absorption spectra of pigments of strain JA296^T are available as supplementary material with the online version of this paper.

Table 1. Differential characteristics of strain JA296^T and other species of the genus *Rhodobacter*

Strain/species: 1, *Rhodobacter aestuarii* sp. nov. JA296^T; 2, *Rba. capsulatus* (data from Imhoff, 2005); 3, *Rba. maris* (Venkata Ramana *et al.*, 2008); 4, *Rba. veldkampii* (Hansen & Imhoff, 1985; Imhoff, 2005); 5, *Rba. vinaykumarii* (Srinivas *et al.*, 2007b); 6, *Rba. ovatus* (Srinivas *et al.*, 2008); 7, *Rba. blasticus* (Imhoff, 2005); 8, *Rba. azotoformans* (Hiraishi *et al.*, 1996); 9, *Rba. megalophilus* (Arunasri *et al.*, 2008); 10, *Rba. sphaeroides* (Imhoff, 2005); 11, *Rba. changlensis* (Anil Kumar *et al.*, 2007). For all taxa, organic substrate utilization was tested during photo-organoheterotrophic growth. All taxa utilized pyruvate, but not benzoate or arginine. Motile species exhibit polar flagella. +, Substrate utilized or present; –, substrate not utilized or absent; ±, variable in different strains; (+), weak growth; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Cell width (µm)	0.7–1.0	0.5–1.2	0.6–1.0	0.6–0.8	0.8–1.2	0.9–1.2	0.6–0.8	0.6–1.0	1.2–1.5	2.0–2.5	0.8–1.0
Cell length (µm)	1.5–2.0	2.0–2.5	1.0–1.5	1.0–1.3	1.5–3.0	1.0–2.0	1.0–2.5	0.9–1.5	1.5–2.0	2.5–3.5	2.0–4.0
Cell shape*	O to R, C	O to R, C	O to R, C	O to R	R	O	O to R	O to R	O	S to O	O to R, C
Motility	+	+	+	–	–	–	–	+	–	+	–
Colour of cell suspension†	YB	YB	YB	YB	YB	YB	OB	YB	YB	GB	YB
Internal membrane system‡	V	V	V	V	V	V	L	V	V	V	V
Slime production	–	±	–	–	+	–	–	+	+	±	+
NaCl required	–	–§	–	–	+ (1–4%)	–	–	–	–	–¶	–
pH range	6.0–8.5	6.5–7.5	5.0–8.0	NR	6.0–8.0	6.0–8.0	NR	NR	NR	6.0–8.5	6.5–8.0
pH optimum	7.0	7.0	6.5–7.0	7.5	6.0–7.5	6.5–7.0	6.5–7.5	7.0–7.5	NR	7.0	6.5–7.5
Temperature optimum (°C)	25–30	30–35	25–30	30–35	20–30	25–30	30–35	30–35	5–40	30–34	20–30
Sulfate assimilation	+	+	+	–	+	+	+	+	+	+	+
Denitrification	–	–	–	–	–	–	–	+	NR	NR	–
Vitamins required#	t	t (b, n)	t	b, p-ABA, t	b	b, t	b, n, t, B ₁₂	b, n, t	t	b, t, n	b, n, t
DNA G + C content (mol%)	65.1	68.1–69.6	62.9	64.4–67.5	68.8	70.1	65.3	69.5–70.2	66.7	70.8–73.2	69.4
Carbon source/electron donor											
Hydrogen	–	+	–	–	–	–	+	NR	–	+	–
Sulfide	–	+	–	+	–	–	–	+	–	+	–
Thiosulfate	–	–	–	+	–	–	–	–	+	–	–
Sulfur	–	–	–	+	–	–	–	–	NR	–	–
Formate	–	+	–	–	–	–	–	+	+	–	–
Acetate	–	+	(+)	+	+	(+)	+	+	+	+	–
Propionate	–	+	(+)	+	–	(+)	+	+	+	+	–
Butyrate	–	+	(+)	+	(+)	+	+	+	+	+	–
Valerate	–	+	(+)	+	–	+	NR	NR	+	+	–
Caproate	–	+	(+)	+	–	+	NR	NR	+	+	–
Caprylate	–	+	–	+	–	–	NR	–	NR	+	–
Tartrate	–	–	–	–	–	–	–	–	+	+	–
Lactate	(+)	+	(+)	+	–	–	+	+	+	+	–
Malate	–	+	+	+	–	–	+	+	+	+	–
Fumarate	–	+	(+)	+	–	–	+	+	+	+	–
Citrate	–	±	–	–	–	–	+	NR	–	+	–
Aspartate	–	±	–	+	–	–	NR	NR	+	NR	–
Glutamate	–	+	–	+	+	–	+	+	+	+	+
Gluconate	–	–	–	NR	–	–	NR	NR	NR	NR	–
D-Glucose	–	+	–	+	+	–	+	+	NR	NR	+
Fructose	–	+	–	–	–	–	+	+	+	+	–
Mannitol	–	±	–	–	+	+	+	+	NR	+	+
Sorbitol	–	±	–	–	–	+	+	+	+	+	+
Glycerol	–	–	(+)	–	–	+	+	+	+	+	–
Methanol	–	–	–	–	–	–	–	–	NR	±	–
Ethanol	–	–	–	–	–	–	–	NR	+	+	–
Propanol	–	+	–	–	+	–	NR	NR	–	NR	–

Table 1. cont.

*C, Chains; O, ovoid; R, rod-shaped; S, spherical.

†GB, Greenish brown; OB, orange-brown; YB, yellowish brown.

‡L, Lamellar; V, vesicular.

§Data for salt tolerance of *Rba. capsulatus* from Hiraishi *et al.* (1996).

||Optimal growth occurs in the absence of NaCl, but able to grow in 5 % NaCl.

¶Optimal growth occurs in the absence of NaCl, but able to grow in 3 % NaCl.

#b, Biotin; B₁₂, vitamin B₁₂; n, niacin; p-ABA, p-aminobenzoic acid; t, thiamine; (b, n), a few strains require biotin and/or niacin.

using the program CLUSTAL_X (Thompson *et al.*, 1997) and the alignment was corrected manually. The dendrogram was constructed using the program PHYL (Guindon *et al.*, 2005) using 100 replicates of non-parametric bootstrap analysis, the GTR model of nucleotide substitution and four substitution rate categories.

The sample that yielded strain JA296^T had a pH of 7.2, salinity of 1–2 ‰ and temperature of 28–30 °C. Cells of strain JA296^T were ovoid to rod-shaped, 0.7–1.0 µm wide and 1.5–2.0 µm long, were motile, multiplied by binary fission and formed chains of four to eight cells. Electron micrographs of ultrathin sections of the cells revealed vesicular internal membrane structures (see Supplementary Fig. S1, available in IJSEM Online). Strain JA296^T was able to grow photo-organoheterotrophically [anaerobically, in the light (2400 lx) and in the presence of pyruvate (0.3 %, w/v)] and chemo-organoheterotrophically [aerobically, in the dark and in the presence of pyruvate (0.3 %, w/v)]. Photolithoautotrophic growth [anaerobically, in the light (2400 lx), with Na₂S·9H₂O (0.5 mM)/Na₂S₂O₃ (2 mM)/S⁰ (0.05 %, w/v)/H₂ (20 %, v/v) and NaHCO₃ (0.1 %, w/v)], chemolithoautotrophic growth [aerobically, in the dark, with Na₂S₂O₃ (0.5 mM) and NaHCO₃ (0.1 %, w/v)] and fermentative growth [anaerobically, in the dark, with

pyruvate (0.3 %, w/v)] could not be demonstrated. Substrates that were utilized as carbon sources and electron donors under photo-organoheterotrophic conditions included pyruvate and lactate (Table 1). Those that could not be utilized included formate, acetate, fumarate, malate, glycerol, valerate, caproate, propionate, butyrate, caprylate, gluconate, succinate, aspartate, ascorbate, benzoate, glutamate, propanol, glucose, fructose, mannitol, peptone, sucrose, Casamino acids, sorbitol, ethanol, tartrate, citrate, oxaloacetate, α-ketoglutarate, lactose, maltose, starch, pelargonate, arginine, yeast extract and oleic acid. Ammonium chloride, glutamine and molecular nitrogen were utilized as nitrogen sources, whereas urea, glutamate, aspartate, nitrate and nitrite did not support growth. Magnesium sulfate, thiosulfate, cysteine, thioglycolate and sulfide were utilized as sulfur sources, whereas elemental sulfur, methionine and sulfite did not support growth. NaCl was not obligatory for growth of strain JA296^T, but it tolerated up to 2 ‰ (w/v) NaCl. Strain JA296^T grew at pH 6.0–8.5 (optimum at pH 7.0) and 25–35 °C (optimum at 30 °C). Thiamine was required as a growth factor. The colour of the phototrophic culture was yellowish green, turning to reddish brown when exposed to air. The *in vivo* absorption spectrum of intact cells in sucrose exhibited maxima at 377, 476, 509, 590, 655 and 854 nm, thus

Fig. 1. Evolutionary-distance dendrogram depicting the phylogenetic relationships of strain JA296^T within the family Rhodobacteraceae, determined using 16S rRNA gene sequence analysis. The phylogenetic tree was constructed by using the PHYL method and was rooted with *Rhodospirillum rubrum* ATCC 11170^T as the outgroup. Numbers at nodes represent bootstrap values (based on 100 resamplings). GenBank accession numbers are shown in parentheses. Bar, 2 substitutions per 100 nucleotide positions.

confirming the presence of bacteriochlorophyll *a* (Supplementary Fig. S2a). The absorption spectrum for pigments extracted with acetone (Supplementary Fig. S2b) gave maxima at 431, 455 and 488 nm, indicating the presence of the carotenoids spheroidene and spheroidenone. Whole-cell fatty acid analysis of strain JA296^T revealed the predominance of C_{18:1}ω7c (62.2 %) with smaller amounts of C_{18:0} 2-OH (2.5 %), C_{18:0} (7.2 %), C_{16:0} (4.5 %) and C_{10:0} 3-OH (2.3 %).

The DNA G+C content of strain JA296^T was 65.1 mol% (by HPLC). The phylogenetic relationship of strain JA296^T to other purple non-sulfur bacteria was examined by 16S rRNA gene sequence analysis. The data obtained revealed that the novel isolate branched separately, but clustered with type strains of species of the genus *Rhodobacter* and was distinct from other genera of purple non-sulfur bacteria (Fig. 1). The highest sequence similarities of strain JA296^T were found with the type strains of *Rhodobacter capsulatus* (95.5 %), *Rba. maris* (94.6 %), *Rba. veldkampii* (91.6 %), *Rba. vinaykumarii* (91.0 %), *Rba. ovatus* (89.8 %), *Rba. blasticus* (89.2 %), *Rba. azotoformans* (88.5 %), *Rba. megalophilus* (87.8 %), *Rba. sphaeroides* (87.7 %) and *Rba. changlensis* (85.4 %). In addition to 16S rRNA gene sequence dissimilarity, strain JA296^T showed clear phenotypic differences (slime production, vitamin requirement, DNA G+C content, carbon and electron donor utilization) from its closest relative *Rba. capsulatus* (Table 1), thus justifying the description of this strain as a representative of a novel species of the genus *Rhodobacter*.

Description of *Rhodobacter aestuarii* sp. nov.

Rhodobacter aestuarii (aes.tu.a'ri.i. L. gen. n. *aestuarii* of the estuary, the environment from which the type strain was isolated).

Cells are ovoid to rod-shaped, 0.7–1.0 µm wide and 1.5–2.0 µm long, are motile, divide by binary fission and form chains of four to eight cells. Growth occurs under anaerobic conditions in the light (photo-organoheterotrophy) or under aerobic conditions in the dark (chemo-organoheterotrophy). Internal photosynthetic membranes are of the vesicular type. The colour of phototrophic cultures is yellowish green. Bacteriochlorophyll *a* and the carotenoids spheroidene and spheroidenone are present. Substrates that are utilized as carbon sources and electron donors under photo-organoheterotrophic conditions include pyruvate and lactate. NaCl is not required for growth, but is tolerated up to 2 % (w/v). Grows at pH 6.0–8.5 (optimum at pH 7.0) and 25–35 °C (optimum at 30 °C). Thiamine is required as growth factor. C_{18:1}ω7c is the dominant fatty acid; small amounts of C_{18:0} 2-OH, C_{18:0}, C_{16:0} and C_{10:0} 3-OH are also found. Photolithoautotrophy, chemolithoautotrophy and fermentative growth have not been demonstrated.

The type strain is JA296^T (=JCM 14887^T =CCUG 55130^T), isolated from a brown-coloured microbial mat from brackish water of Bhitarkanika mangrove forest, Dangmal, Orissa, India. The DNA G+C content of the type strain is 65.1 mol% (by HPLC).

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