Rhodovulum lacipunicei sp. nov., an obligate sulfide-demanding phototrophic alphaproteobacterium isolated from a purple pond in India

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An ovoid-rod-shaped, phototrophic, purple non-sulfur bacterium, designated strain JA322^T, was isolated in pure culture from a water sample collected from a saline pond with purple-coloured water, located near Satpada in Orissa, India. Strain JA322^T was Gram-negative and non-motile and grew photoheterotrophically with a number of organic compounds serving as carbon sources or electron donors. Intracellular photosynthetic membranes were of the vesicular type. Bacteriochlorophyll *a* and carotenoids of the spheroidene series were present as photosynthetic pigments. Biotin and thiamine were required for growth of strain JA322^T. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA322^T was related most closely to the type strains of *Rhodovulum euryhalinum* (95.8% similarity) and *Rhodovulum strictum* (94.8%) and clustered with species of the genus *Rhodovulum* of the family *Rhodobacteraceae*. On the basis of phenotypic and molecular genetic evidence, it is suggested that strain JA322^T represents a novel species of the genus *Rhodovulum*, for which the name *Rhodovulum lacipunicei* sp. nov. is proposed. The type strain is JA322^T (=NBRC 104147^T=KCTC 5700^T).

At the time of writing, the genus *Rhodovulum* comprised ten species with validly published names, *Rhodovulum sulfidophilum*, *R. adriaticum*, *R. euryhalinum*, *R. strictum*, *R. iodosum* and *R. robiginosum*, and those described by our group, namely *R. marinum* (Srinivas *et al.*, 2006), *R. imhoffii* (Srinivas *et al.*, 2007a), *R. visakhapatnamense* (Srinivas *et al.*, 2007b) and *R. kholense* (Anil Kumar *et al.*, 2008). In the present communication, we describe a bacterial strain, designated JA322^T, isolated from a pond in India, and suggest that it represents a novel species of the genus *Rhodovulum* based on phenotypic and phylogenetic analyses.

Strain JA322^T was isolated from a water sample collected from a pond with purple-coloured water located near Satpada in Orissa, India (19° 47' N 85° 49' E), in May 2007.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA322^T is AM921780.

A phase-contrast micrograph of cells of strain JA322^T, and figures showing the sulfide tolerance of strain JA322^T and a whole-cell absorption spectrum of strain JA322^T and methanol and acetone spectra of extracted pigments of strain JA322^T are available as supplementary material with the online version of this paper.

The water sample that yielded strain JA322^T had a pH of 8.5–9, a temperature of 30 °C and a salinity of ~2% (w/v). The sample was directly streaked onto agar slants (25 × 150-mm test tubes sealed with suba seals and the gas phase was replaced with argon), which contained Pfennig medium (Pfennig & Trüper, 1992), supplemented with NaCl (2%, w/v), pyruvate (0.3%, w/v), NH₄Cl (0.06%, w/v), sodium ascorbate (0.05%, w/v) and Casamino acids (0.03%, w/v). Na₂S·9H₂O at 2 mM was added, after autoclaving, to the medium. Phototrophic incubation at 2400 lx and 28–30 °C for 4–5 days resulted in the formation of yellowish brown colonies. Purification was achieved by repeated streaking on agar slants and the purified culture was grown in medium containing [per litre distilled water, pH 7.0–7.5: 0.5 g KH₂PO₄, 2.0 g MgSO₄·7H₂O, 20.0 g NaCl, 0.6 g NH₄Cl, 0.15 g CaCl₂·2H₂O, 0.75 g sodium succinate, 0.75 g sodium acetate, 0.75 g sodium pyruvate, 0.275 µl lactic acid (0.33 g l⁻¹), 0.5 g sodium ascorbate, 0.3 g yeast extract, 0.3 g Casamino acids, 5 ml ferric citrate (from 0.1% (w/v) stock), 1 ml SL7 trace metal solution and 0.5 g peptone]. Na₂S·9H₂O at 0.75–1 mM was added, after autoclaving, to the medium. Purified cultures were grown in completely

filled screw-cap test tubes (10 × 100 mm) to test for photoheterotrophic growth.

Morphological properties (cell shape, cell division, cell size, motility) were observed by using phase-contrast light microscopy (BH-2; Olympus); for intracytoplasmic structures, such as the internal membrane system, ultrathin sections were viewed with a transmission electron microscope (H-7500; Hitachi). The internal membrane structures were viewed after the cells had been processed as described by Hanada *et al.* (2002). *In vivo* absorption spectra were measured in sucrose solution with a Spectronic Genesys 2 spectrophotometer (Trüper & Pfennig, 1981). Absorption spectra were also recorded from pigments extracted with acetone after eluting the cell suspension with acetone through a 10 × 200 mm column packed with aluminium oxide. The carotenoids present were detected by comparison with the absorption maxima of standards of Britton *et al.* (2004). Carotenoid composition was analysed by S. Takaichi (personal communication) by using HPLC. Utilization of other inorganic and organic compounds as electron donors for phototrophic growth was tested without any additional electron donor in the presence of yeast extract (0.03 %, w/v). Formate, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol, ethanol, propanol and butanol were used at a concentration of 0.1 % (v/v); crotonate, caprylate, glycolate, tartrate and gluconate were used at a concentration of 0.1 % (w/v) [for tests of growth with all the above substrates, NaHCO₃ (0.1 %, w/v) was also added, and a control with bicarbonate and without any carbon or electron donor was maintained]; other compounds tested were used at 0.3 % (w/v), with benzoate (1 mM) [NaHCO₃ (0.1 %, w/v) was also added while testing growth with benzoate and ascorbate]. For tests of sulfur sources, MgSO₄·7H₂O was replaced by MgCl₂·5H₂O (0.2 %); sulfur sources [Na₂S·9H₂O, Na₂S₂O₃, sulfite, sodium thioglycolate, cysteine, MgSO₄·7H₂O (all at 0.5 mM) or FeSO₄ (10 mM)] were added to the medium. Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources at 0.06 % (w/v). Vitamin requirements were tested by replacing yeast extract with combinations of vitamins as growth factors. Chemotrophy was determined by growing the cultures in Erlenmeyer flasks placed in an orbital shaker in the dark at 30 °C. Diazotrophy of the cultures was determined by growth under an N₂ atmosphere (the medium does not contain any other nitrogen source and yeast extract was also removed from the medium) and was confirmed by repeated subculturing (four times). Growth was measured turbidometrically at 660 nm.

Cell material for DNA isolation was taken from 1–2 ml of dense liquid cultures. DNA was extracted and purified by using the Qiagen genomic DNA buffer set. Recombinant *Taq* polymerase was used for PCR amplification by using the primers 5'-GTTTGATCCTGGCTCAG-3' and 5'-TACCTTGTTACGACTTCA-3' (*Escherichia coli* positions 11–27 and 1489–1506, respectively). PCR amplification

was performed as described by Imhoff & Pfennig (2001) and Imhoff *et al.* (1998) and 16S rRNA gene sequencing was outsourced through MWG, Biotech Pvt. Ltd, Bangalore, India. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved by using NCBI BLAST searches (Altschul *et al.*, 1990) and the EzTaxon server (Chun *et al.*, 2007), respectively. For phylogenetic analysis, 16S rRNA gene sequences were aligned by using the program CLUSTAL X (Thompson *et al.*, 1997) (multiple alignment option). Alignment was checked manually by using BioEdit software (Hall, 1999) and the file was saved in PHYLIP format. Phylogenetic analysis was carried out by using the PHYLIP package, version 3.5 (Felsenstein, 1989). A phylogenetic tree was constructed based on the neighbour-joining method (Saitou & Nei, 1987); distances were corrected according to the Kimura two-parameter nucleotide substitution model, by using sites corresponding to the pairwise deletion option, including transitions and transversions, substitutions and uniform substitution rates.

Cells of strain JA322^T were ovoid-rod-shaped, 0.7–1 µm wide and 1.0–3.0 µm long (see Supplementary Fig. S1 in IJSEM Online), non-motile and multiplied by binary fission. Electron micrographs of ultrathin sections of the cells revealed vesicular internal membrane structures. Strain JA322^T was able to grow photo-organoheterotrophically [anaerobically, in the light (2400 lx) and in the presence of pyruvate/succinate/acetate (0.075 %, w/v) or lactate (0.275 ml l⁻¹)], chemo-organoheterotrophically [aerobically, in the dark and in the presence of pyruvate/succinate/acetate (0.075 %, w/v) or lactate (0.275 ml l⁻¹)], photolithoheterotrophically [anaerobically, in the light (2400 lx) and in the presence of pyruvate/succinate/acetate (0.075 %, w/v) or lactate (0.275 ml l⁻¹) and Na₂S (1 mM)] and chemolithoheterotrophically [aerobically, in the dark and in the presence of pyruvate/succinate/acetate (0.075 %, w/v) or lactate (0.275 ml l⁻¹) and Na₂S₂O₃ (2 mM)]. Photolithoautotrophy [anaerobically, in the light (2400 lx), with Na₂S (1 mM), Na₂S₂O₃ (2 mM), S⁰ (0.05 %, w/v), ferrous iron (10 mM), thioglycolate (1 mM), sulfite (1 mM), H₂ (20 %, w/v) and NaHCO₃ (0.1 %, w/v)], chemolithoautotrophy [aerobically, in the dark, with Na₂S₂O₃ (2 mM) and NaHCO₃ (0.1 %, w/v)] and fermentative growth [anaerobically, in the dark, with glucose (0.3 %, w/v)] could not be demonstrated. Substrates that were utilized as carbon sources and electron donors under photo-organoheterotrophic conditions are detailed in Table 1. Ammonium chloride and glutamine were used as nitrogen sources by strain JA322^T, but not nitrate, nitrite, dinitrogen, urea or glutamate. Strain JA322^T was able to utilize sulfide, thiosulfate, thioglycolate and cysteine as sulfur sources, but not sulfite or sulfate. Elemental sulfur was also used as a sulfur source. There was no requirement of salt (NaCl) for growth of strain JA322^T but good growth was observed at up to 5 % (w/v) NaCl. Strain JA322^T grew at pH 6.5–9.0 (optimum at pH 7.0–8.5) and 25–35 °C (optimum at 30 °C). Biotin and thiamine were required as

Table 1. Differential characteristics between strain JA322^T and recognized species of the genus *Rhodovulum*

Strains: 1, JA322^T (*R. lacipunicea* sp. nov.); 2, *R. euryhalinum* KA-65^T (data from Kompantseva, 1985; Imhoff & Trüper, 1992); 3, *R. strictum* MB-G2^T (Hiraishi & Ueda, 1995); 4, *R. kholense* JA297^T (Anil Kumar *et al.*, 2008); 5, *R. marinum* JA128^T (Srinivas *et al.*, 2006); 6, *R. visakhapatnamense* JA181^T (Srinivas *et al.*, 2007b); 7, *R. sulfidophilum* W4^T (Hansen & Veldkamp, 1973; Imhoff & Trüper, 1992; Heising *et al.*, 1996); 8, *R. imhoffii* JA125^T (Srinivas *et al.*, 2007a); 9, *R. iodolum* N1^T (Straub *et al.*, 1999); 10, *R. robiginosum* N2^T (Straub *et al.*, 1999); 11, *R. adriaticum* BN 721(6 II)^T (Neutzing *et al.*, 1984). For all taxa, organic substrate utilization was tested during photoheterotrophic growth. All taxa utilized pyruvate and succinate, but not benzoate. Motile species exhibited polar flagella. +, Substrate utilized or present; –, substrate not utilized or absent; (+), weak growth; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Cell width (µm)	0.7–1	0.7–1.0	0.6–1.0	0.5–1	0.6–0.8	0.9–1.2	0.6–1.0	0.5–0.6	0.5–0.8	0.5–0.8	0.5–0.8
Cell shape*	O–R	O–R	O–R	O–R	O–R, C	O–R	O–R	R	O–R	O–R	R, C
Motility	–	+	+	+	–	–	+	–	–	–	–
NaCl range (%)	0–5	0.5–10	0.25–3	0.05–6	0.05–8	0–10	0–10	0.05–7	2.5–5	2.5–5	1–10
pH range	6.5–9.0	6.0–8.5	7.5–9.0	6.0–8.0	5.5–7.5	4.0–9.0	5.0–9.0	6.0–9.0	NR	NR	6.0–8.5
pH optimum	7.0–8.5	NR	NR	6.0–7.0	6.0–6.8	6.0–8.0	NR	7.0–8.0	6.5	6.5	NR
Chemo-organoheterotrophy	+	–	+	+	+	+	+	+	+	+	–
Temperature range (°C)	25–35	NR	30–35	20–35	25–35	20–35	NR	20–30	20–25	25–28	25–30
Temperature optimum (°C)	30	NR	NR	30	30	30	25	28	NR	NR	NR
Colour of cell suspension†	YB	YB	YB	YB	YB	YB	B	YB	YB	YB	B
Vitamin requirement‡	b, t	b, n, paba, t	b, paba, t	b, n, t	t	b, n, t, paba	b, n, paba, t	b	b, n	b, B ₁₂ , n	b, t
DNA G + C content (mol%)	68.6	62.1–68.6	67.3–67.7	63	62	61.2	66.3–66.6	58	66	69	64.9–66.7
Carbon source/electron donor											
Hydrogen	–	NR	NR	–	–	–	+	–	+	+	–
Sulfide	–	+	+	+	–	–	+	+	+	+	+
Thiosulfate	–	+	+	+	–	+	+	+	+	+	+
Sulfur	–	NR	NR	–	–	–	+	–	+	+	NR
Ferrous iron	–	–	NR	–	–	–	+	–	+	+	–
Formate	–	+	+	–	–	–	+	–	–	–	+
Propionate	+	+	+	–	–	+	+	–	+	+	+
Butyrate	+	+	+	+	–	+	+	–	+	–	–
Valerate	+	+	+	+	–	+	+	–	+	–	–
Caproate	+	NR	+	+	–	+	+	+	–	–	+
Ethanol	–	+	–	–	+	–	–	–	–	–	+
Lactate	+	+	+	+	+	+	+	–	+	+	+
Fumarate	+	+	+	+	+	+	+	+	+	–	+
Malate	+	+	+	+	–	+	+	+	+	+	+
Citrate	–	–	(+)	–	–	–	–	–	–	–	–
Aspartate	(+)	+	–	–	–	–	–	+	–	–	NR
Cysteine	(+)	NR	NR	–	–	–	NR	–	+	–	NR
Glutamate	(+)	+	–	+	–	+	+	+	+	+	NR
Glucose	(+)	+	+	+	+	+	+	–	–	–	+
Fructose	(+)	+	+	+	+	+	–	–	–	–	–
Glycerol	–	+	–	–	+	+	+	+	–	+	+
Mannitol	–	+	–	–	+	–	–	–	+	+	–

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

†B, Brown; YB, yellowish brown.

‡b, Biotin; B₁₂, vitamin B₁₂; n, nicotinic acid; paba, *p*-aminobenzoic acid; t, thiamine.

growth factors. Sulfide was obligately required for strain JA322^T as a source of sulfur, and was tolerated at up to 7 mM (Supplementary Fig. S2, in IJSEM Online).

The colour of phototrophic cultures was yellowish brown. The *in vivo* absorption spectrum of intact cells in sucrose exhibited maxima at 377, 449, 479, 512, 590, 806 and

860 nm, confirming the presence of bacteriochlorophyll *a* and carotenoids (Supplementary Fig. S3a). The absorption spectrum for pigments extracted with methanol showed a maximum at 770 nm, confirming the presence of bacteriochlorophyll *a* (Supplementary Fig. S3b). The absorption spectrum for pigments extracted with acetone showed

maxima at 430, 457 and 487 nm (Supplementary Fig. S3c), indicating the presence of carotenoids of the spheroidene series (Britton *et al.*, 2004). The carotenoid composition of strain JA322^T as detected by HPLC was spheroidenone (52 %), spheroidene (45 %) and hydroxyspheroidene (3 %). The DNA G+C content of strain JA322^T was 68.6 mol% (by HPLC).

The phylogenetic relationship between strain JA322^T and other purple non-sulfur bacteria was examined by means of 16S rRNA gene sequencing. The data showed that the novel isolate branched separately but clustered with the type strains of species of the genus *Rhodovulum*, being distinct from other genera of purple non-sulfur bacteria (Fig. 1). Highest levels of 16S rRNA gene sequence similarity for strain JA322^T were found with respect to the type strains of *R. euryhalinum* (95.8 %) and *R. strictum* (94.8 %). In addition to phylogenetic distinctiveness based on 16S rRNA gene sequence analysis, strain JA322^T showed clear phenotypic differences with respect to recognized species of the genus *Rhodovulum* (Table 1). Strain JA322^T is therefore considered to represent a novel species of the genus

Rhodovulum, for which the name *Rhodovulum lacipunicei* sp. nov. is proposed.

Description of *Rhodovulum lacipunicei* sp. nov.

Rhodovulum lacipunicei (la.ci.pu.ni'ce.i. L. n. *lacus* -us a lake, a pond; L. adj. *puniceus* -a -um purple-coloured; N.L. gen. n. *lacipunicei* of/from a purple pond).

Cells are ovoid-rod-shaped, 0.7–1 µm wide and 1.0–3.0 µm long, non-motile and divide by binary fission. Growth occurs under anaerobic conditions in the light (photo-organoheterotrophy/photolithoheterotrophy) or under aerobic conditions in the dark (chemo-organoheterotrophy/chemolithoheterotrophy). Assimilatory sulfate reduction is not observed. Internal photosynthetic membranes are of vesicular type. The colour of phototrophic cultures is yellowish brown. The *in vivo* absorption spectrum of intact cells in sucrose shows maxima at 377, 449, 479, 512, 590, 806 and 860 nm, indicating the presence of bacteriochlorophyll *a* and carotenoids. The absorption spectrum of pigments extracted with acetone shows maxima at 430, 457 and 487 nm, indicating the presence of carotenoids of the

Fig. 1. Phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationship between strain JA322^T and related species within the family *Rhodobacteraceae*. The tree was constructed by using the neighbour-joining method and was rooted by using *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, 1 nucleotide substitution per 100 nt.

spheroidene series (spheroidenone, spheroidene and hydroxy-spheroidene). Optimum growth occurs at 30 °C (range, 25–35 °C) and pH 7.0–8.5 (range, pH 6.5–9.0). Does not require NaCl for growth, but good growth occurs at up to 5% (w/v) NaCl. Photo-organoheterotrophy with a few organic compounds is the preferred mode of growth. Good growth is observed with propionate, butyrate, valerate, caproate, lactate, fumarate, malate, crotonate, pyruvate, 2-oxoglutarate, peptone, Casamino acids and yeast extract. Aspartate, glutamate, glucose, fructose, acetate, succinate, glycolate, ascorbate and cysteine are also utilized, but not formate, ethanol, citrate, glycerol, mannitol, caprylate, benzoate, tartrate, gluconate, sucrose, methanol, propanol, butanol or sorbitol. Photoautotrophic growth is not observed in the presence of sulfide, sulfite, thiosulfate, thioglycolate, elemental sulfur, ferrous iron or hydrogen as electron donor and NaHCO₃ as carbon source. Obligately requires sulfide (tolerates up to 7 mM) as sulfur source for growth. Chemoautotrophic growth is not observed in the presence of thiosulfate as electron donor and NaHCO₃ as carbon source. Fermentative growth is not observed in the presence of glucose as carbon source. Biotin and thiamine are required as growth factors. The DNA G+C content of the type strain is 68.6 mol% (by HPLC).

The type strain, JA322^T (=NBRC 104147^T=KCTC 5700^T), was isolated from a water sample collected from a saline pond with purple-coloured waters, located near Satpada in Orissa, India.

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