Phaeovibrio sulfidiphilus gen. nov., sp. nov., phototrophic alphaproteobacteria isolated from brackish water

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Two strains (JA480^T and JA481) of phototrophic alphaproteobacteria were isolated from sediment samples collected from brackish water near Nagapattinam, India. They were Gram-negative, vibrioid cells containing bacteriochlorophyll *a* and rhodopin as major photosynthetic pigments. Most cells of both strains were non-flagellated; 1 % of cells showed two monopolar flagella. Cells showed positive phototaxis. Both strains showed chimeric intracellular photosynthetic membranes, where both lamellar stacks and vesicles were present together in a single cell. The major fatty acids were C_{18:1}ω7c and C_{16:0} in both strains. The genomic G + C content was 67.2–68.8 mol% and the two strains were closely related (mean DNA–DNA hybridization >85 %). 16S rRNA gene sequence comparisons indicated that the isolates represent members of the *Rhodospirillaceae* within the class *Alphaproteobacteria*. According to the sequence comparison data, strain JA480^T is positioned distinctly outside the group formed by the phototrophic genera *Rhodospirillum*, *Rhodocista*, *Phaeospirillum*, *Rhodovibrio*, *Rhodospira* and *Roseospira*, with only 80–92 % 16S rRNA gene sequence similarity. Distinct morphological, physiological and genotypic differences from previously described taxa support the classification of this isolate as a representative of a novel species in a new genus, for which the name *Phaeovibrio sulfidiphilus* gen. nov., sp. nov. is proposed. The type strain of *Phaeovibrio sulfidiphilus* is JA480^T (=KCTC 5825^T =NBRC 106163^T =DSM 23193^T).

Six (*Phaeospirillum*, *Rhodocista*, *Rhodospira*, *Rhodospirillum*, *Rhodovibrio* and *Roseospira*) of the 23 genera described so far (<http://www.bacterio.cict.fr>) in the family *Rhodospirillaceae* are represented by true phototrophs (Imhoff *et al.*, 1998a). The name of a seventh phototrophic genus, '*Roseospirillum*' (Glaeser & Overmann, 1999), is still not validly published (<http://www.bacterio.cict.fr/qr/roseospirillum.html>). Members of the genera *Rhodospirillum*, *Phaeospirillum* and *Rhodocista* are represented by freshwater forms, while *Rhodovibrio*, *Roseospira* and *Rhodospira* have representatives that are truly marine (Imhoff *et al.*, 1998a). In this

communication, we report two novel freshwater phototrophic strains associated with the family *Rhodospirillaceae* that have vibrioid cells and are obligate anaerobes that grow on a limited number of carbon substrates/sources.

Strain JA480^T was isolated from a sediment sample collected on 9 March 2003 from a brackish shrimp pond (water pH 8.2) at Vadkkupoygainallur, a village near Nagapattinam, Tamilnadu, India (GPS position of the sample collection site: 10° 44' 06.09" N 79° 50' 12.96" E). Strain JA481 was isolated from a brackish water sample collected on 12 September 2006 near another nearby village, Pappakovil (10° 44' 39.75" N 79° 49' 39.61" E). Original enrichment of the strains was obtained in a photoheterotrophic medium (pH 8.2) in 50 ml fully filled screw-capped bottles incubated at 2400 lx and 28–30 °C for 7 days. The medium contained NH₄Cl (18 mM), MgSO₄·7H₂O (1.2 mM), CaCl₂·2H₂O (1.3 mM), KH₂PO₄ (3.6 mM), NaCl (17 mM), sodium succinate (7.4 mM), yeast extract (2.0 g l⁻¹) and Na₂HPO₄ (2 mM).

Abbreviation: ICM, intracellular cytoplasmic membrane.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JA480^T and JA481 are FN391894 and FN543109, respectively.

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

Purification was done by repeated streaking on agar slants (25 × 150 mm test tubes sealed with butyl rubber corks). Anaerobic conditions were achieved by replacing the gas phase in the culture tubes with argon. The photoheterotrophic medium used for purification and further culturing contained KH_2PO_4 (3.6 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.8 mM), NaCl (6.8 mM), NH_4Cl (11 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.34 mM), sodium pyruvate (4.5 mM), sodium succinate (1.8 mM), sodium acetate (3.6 mM), yeast extract (0.3 g l^{-1}), Na_2S (1 mM), NaHCO_3 (100 mM), ferric citrate (0.2 mM) and trace-element solution SL 7 (1 ml l^{-1} ; Biebl & Pfennig, 1981). Purified cultures were grown in completely filled screw-capped test tubes (10 × 100 mm) for photoheterotrophic growth. For routine culturing and for physiological tests, strains JA480^T and JA481 were grown at pH 7, 30 °C and at a light intensity of 2400 lx. Morphological properties (cell shape, cell division, cell size, motility) were observed by light microscopy (Olympus BH-2). Flagella position and number were determined through transmission electron microscopy (Hitachi H-7500); the cell pellet was washed with phosphate buffer and a small drop of sample was placed on a carbon-coated EM grid. After 20 min, the grid was washed with distilled water, stained with 2 % uranyl acetate and air-dried. To observe intracytoplasmic structures, a centrifuged cell pellet was fixed with 5 % (v/v) glutaraldehyde and 1 % (v/v) osmium tetroxide. Ultrathin sections of the sample embedded in Spurr's resin were prepared with a glass knife on a Leica Ultracut UCT-GA-D/E-1/100 ultramicrotome. Samples were stained with uranyl acetate and counter-stained with lead citrate and examined using a Hitachi H-7500 transmission electron microscope. *In vivo* absorption spectra were measured with a Spectronic Genesys 2 spectrophotometer using sucrose solution for cell suspension (Trüper & Pfennig, 1981).

Fatty acid methyl esters were analysed according to the instructions for the Microbial Identification System (Microbial ID, MIDI; Agilent 6850) (Sasser, 1990); analysis was performed by Royal Research Laboratories, Secunderabad, India. All strains were cultured in the above-mentioned phototrophic medium with sodium pyruvate (4.5 mM), sodium succinate (1.8 mM), sodium acetate (3.6 mM) as carbon sources and Na_2S (1 mM) as electron donor at 28 °C and 2400 lx for 5 days. A loopful of each of the bacterial cultures was then picked and subjected to a series of four different reagents which enable saponification and methylation of fatty acids, enabling their cleavage from lipids. The fatty acid methyl esters thus obtained were analysed by gas chromatography equipped with Sherlock MIS software. The peaks obtained were then labelled and equivalent chain-lengths (ECL) were computed by the Sherlock software.

Genomic DNA was extracted and purified according to the method of Marmur (1961) and the G + C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989). Cell material for 16S rRNA gene sequencing was taken from a colony. DNA was extracted and purified by using a Qiagen genomic DNA extraction kit. Recombinant *Taq* polymerase (Genei) was used for PCR. The PCR used primers

5'-GTTTGATCCTGGCTCAG-3' (F'-27) (Chèneby *et al.*, 2000) and 5'-TACCTTGTTACGACTTCA-3' (R'-1489) (positions 11–27 and 1489–1506, 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 done as described previously (Imhoff & Pfennig, 2001; Imhoff *et al.*, 1998b) and 16S rRNA gene sequences were obtained (without gene cloning) by cycle sequencing with a SequiTherm sequencing kit (Biozym) and chain termination chemistry (Sanger *et al.*, 1977) using an automated laser fluorescence sequencer (Pharmacia). 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 was used for sequence alignments and MEGA 4 (Tamura *et al.*, 2007) software was used for phylogenetic analysis of individual sequences. Distances were calculated by using the Jukes and Cantor correction in a pairwise deletion manner (Tamura *et al.*, 2007). Neighbour-joining, minimum-evolution and maximum-parsimony methods in the MEGA 4 software (<http://www.megasoftware.net>) were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. The taxonomic relationship between strains JA480^T and JA481 was examined using DNA–DNA hybridization. Genomic relatedness was determined using a membrane filter technique (Seldin & Dubnau, 1985) using a DIG High Prime DNA labelling and detection starter kit II (Roche). Hybridization was performed with three replications for each sample (strains used for binding and labelling reversed) and mean values are quoted as DNA–DNA relatedness.

Cells of strains JA480^T and JA481 are vibrioid, 0.3–0.5 µm wide and 1.2–2.5 µm long (Supplementary Fig. S1a,

Fig. 1. Electron micrographs of ultrathin sections of cells of strain JA480^T, showing chimeric ICM structures. V, Vesicular; L, lamellar. Bars, 333 (a), 300 (b) and 330 (c) nm.

Table 1. Differentiating characteristics of *Phaeovibrio* gen. nov. (strains JA480^T and JA481)

Strains: 1, strain JA480^T and JA481 (data from this study); 2, *Rhodospirillum sulfurexigens* JA143^T and *Rhodospirillum rubrum* ATCC 11170^T (data from the authors' laboratory) and *Rhodospirillum photometricum* DSM 122^T; 3, *Phaeospirillum chandramohanii* JA145^T and *Phaeospirillum molischianum* DSM 120^T (data from the authors' laboratory) and *Phaeospirillum fulvum* DSM 113^T; 4, *Rhodocista centenaria* ATCC 43720^T and *Rhodocista pekingensis* 3-p^T; 5, *Rhodospira trueperi* 8316^T; 6, *Rhodovibrio salinarum* ATCC 35394^T and *Rhodovibrio sodomensis* DSI^T; 7, *Roseospira goensis* JA135^T and *Roseospira visakhapatnamensis* JA131^T (data from the authors' laboratory) and *Roseospira marina* CE2105^T, *Roseospira navarrensis* SE3104^T and *Roseospira mediosalina* BN280^T. Unless indicated, data were taken from Imhoff (2005a, b), Imhoff *et al.* (1998a), Kawasaki *et al.* (1992), Zhang *et al.* (2003), Pfennig *et al.* (1997), Kalyan Chakravarthy *et al.* (2007) and Guyoneaud *et al.* (2002). +, Substrate utilized or present; –, substrate not utilized or absent; (+), weak growth; ND, no data available; NR, not reported. All strains are motile. Organic substrate utilization was tested during photoheterotrophic growth. Pyruvate and acetate were utilized by all strains; arginine and citrate were not utilized by any of the strains.

Characteristic	1	2	3	4	5	6	7
Cell shape	Vibrio	Spiral	Spiral	Vibrio–spiral	Vibrio–spiral	Vibrio–spiral	Vibrio–spiral
Cell length (µm)*	1.2–2.5	4–10	3.5–8	1.0–2.0	0.6–0.8	0.6–0.9	1.5–8.0
Cell width (µm)*	0.3–0.5	1.5–4.0	0.5–1.0	0.5–1.0	0.3–0.5	0.3–0.5	0.5–1.0
Colour†	LB	PK–DAB	BR–O to DEB	PK	BG	PK–R	PK
Major carotenoid(s)‡	RP	RP/SP/LY	RP	SP	THSP	SP	SP
Carotenoid glycosides	–	–	+	ND	ND	ND	ND
NaCl tolerance (% w/v)	0–0.5	0–1.5	0–1.5	ND	0.5–5	3–24	0.5–15
Internal membrane system	Chimeric§	Stacks or vesicles	Stacks	Lamellae	Vesicles	Vesicles	Vesicles
DNA G + C content (mol%)	67.8–68.8	64–65	60–65	68.8–69.9	65.7	66.2–67.4	66.6–72.3
Growth factor(s)¶	B, PABA	T/B/NA	None or PABA	B, C/T	B, T, P	C	T, PABA, NA, YE
Photolithoautotrophic growth	–	–/ +	–	–	–	–	–/ +
Carbon source/electron donor used for growth							
Benzoate	–	–	–/ +	ND	ND	ND	–/ +
Butyrate	–	–/ +	(+)/ +	+	+	–	–/ +
Caproate	–	+ / –	(+)/ +	+	ND	ND	ND
Caprylate	–	–/ +	+	+	ND	ND	ND
Ethanol	–	–/ +	–/ +	ND	–	ND	ND
D-Fructose	–	(+)/ +	–/ +	–	ND	ND	–/ +
Fumarate	–	+	+	–	+	ND	–/ +/(+)
D-Glucose	–	–/ +	–	–	ND	ND	–/ +
L-Glutamate	–	+ / –	–	+	ND	ND	–/ +
Glycerol	–	–/ +	–	ND	ND	ND	–/ +
D-Lactate	–	(+)/ +	–/ +	+	+	ND	–/ +
Malate	–	+	+	–	+	+	+ / +/(+)
Mannitol	–	–/ +	(+)/ –	ND	ND	ND	–/ +
Methanol	–	–/ +	–/ +	ND	ND	ND	–/ ND
Propionate	–	–/ +	–/ +	ND	+	–	–/ +
Succinate	+	+	–/ +	–/ +	+	+	–/ +/(+)
Valerate	–	+	(+)/ +	+	+	ND	–/ +
Cellular fatty acid composition (%)							
C _{14:0}	2.6	0.7–3.5	0.4	ND	NR	NR	ND
C _{14:0} 3-OH	0.4	1.1	0.6	ND	NR	NR	ND
C _{16:0}	27.0	16.0–25.0	15.1–22.9	ND	27.9	7.4	ND
C _{16:0} 3-OH	3.9	1.3	–	ND	NR	NR	ND
C _{16:1ω5c}	1.0	–	–	ND	NR	NR	ND
C _{16:1ω7c} and/or C _{16:1ω6c}	3.6	22.0–30.0¶	22.2–25.8	ND	–	–	ND
C _{16:1ω7c} 3-OH	0.9	7.5	–	ND	–	–	ND
anteiso-C _{17:0}	0.9	0.1–1.0	0.3	ND	NR	NR	ND
C _{18:1ω5c}	1.3	0.9	4.1	ND	NR	NR	ND
C _{18:1ω7c}	50.0	41.0–51.0	36.0–54.5	ND	60.7	35.2	ND
C _{18:0}	1.5	0.9–2.3	18.3	ND	1.2	23.0	ND
11-Methyl C _{18:1ω7c}	–	0.9	15.8	ND	–	–	ND

*For spiral cells, dimensions are length and width of one complete turn of the spiral.

†BG, Beige; BR, brown; DAB, dark brown; DEB, deep brown; LB, light brown; O, orange; PK, pink; R, red.

‡LY, Lycopene; RH, rhodovibrin; RP, rhodopin; SP, spirilloxanthin; THSP, tetrahydrospirilloxanthin.

§Stacks and vesicles present in a single cell.

||PABA, *p*-Aminobenzoic acid; B, biotin; C, cobalamin; NA, nicotinamide; P, pantothenate; T, thiamine; YE, yeast extract.

¶C_{16:1}ω7c/C_{16:1}ω6c could not be detected in this study in *Rhodospirillum rubrum* ATCC 11170^T.

available in IJSEM Online). Both strains multiplied by binary fission. Flagellar motility could not be demonstrated at any stage of phototrophic growth under the phase-contrast microscope, and transmission electron microscope analysis also showed cells without flagella (Supplementary Fig. S1b). A more careful search confirmed the presence of monopolar biflagellate cells (Supplementary Fig. S1c) in both strains; however, the number was very small (1 out of 100). Soft agar (0.5 %, w/v) stabs were used to confirm motility; strains JA480^T and JA481 diffused throughout the medium, while the control *Phaeospirillum chandramohanii* JA145^T (where most of the cells are motile; Anil Kumar *et al.*, 2009) was confined to the stabbed area (Supplementary Fig. S2a). Phototaxis was demonstrated in strains JA480^T and JA481 (Supplementary Fig. S2b); tubes incubated in the dark (completely covered with aluminium foil) showed no growth, while, after 5 days of incubation, cells moved in the direction of the light in tubes that were illuminated only from the bottom. Transmission electron micrographs of ultrathin sections of both strains revealed a chimeric type of intracellular cytoplasmic membrane (ICM) structure, where both lamellar stacks and vesicles were present in a single cell (Fig. 1). To the best of our knowledge, this is the first report of chimeric ICM structures in phototrophic bacteria.

Strains JA480^T and JA481 were able to grow photoheterotrophically [anaerobic, light (2400 lx) with pyruvate (0.03 %, w/v) as carbon source/electron donor]. Photolithoautotrophy [anaerobic, light (2400 lx), Na₂S·9H₂O, Na₂S₂O₃·5H₂O (1 mM) and NaHCO₃ (0.1 %, w/v)], chemolithoautotrophy [aerobic, dark, Na₂S₂O₃·5H₂O (1 mM) and NaHCO₃ (0.1 %, w/v)] and chemoheterotrophy [aerobic, dark, pyruvate (0.3 %, w/v)] could not be demonstrated in either strain. Fermentative growth [anaerobic, dark with glucose/fructose/pyruvate (0.3 %, w/v)] could also not be demonstrated in either strain. Not many organic substrates supported growth of the strains (Table 1). The substrates that were utilized as carbon sources/electron donors under photoheterotrophic conditions included acetate, pyruvate and succinate. Those which could not be utilized included glucose, fructose, glutamate, lactate, malate, methanol, ethanol, caproate, caprylate, glycerol and butyrate. Strains JA480^T and JA481 could be grown with ammonium chloride and glutamine as nitrogen sources, while molecular nitrogen, urea, glutamate, aspartate, nitrite and nitrate did not support phototrophic growth. Biotin and *p*-aminobenzoic acid were required as growth factors by both strains. Sulfide was required and was used as a sulfur source, while thiosulfate and cysteine did not support phototrophic growth of either strain. There was no requirement for NaCl for growth, though both strains tolerated up to 0.5 % NaCl. Both strains grew at pH 7.0–8.0, with an

optimum at pH 6.8. Optimum growth of both strains occurred at 25–30 °C (range 20–40 °C). Phototrophically grown cell suspensions of both strains were light brown. The whole-cell absorption spectrum of strain JA480^T (JA481) showed absorption maxima at 488 (491), 524 (527), 593, 794 and 863 nm (Supplementary Fig. 3a). Acetone-extracted spectra of the two strains showed absorption maxima at 360, 474, 504 and 579 nm (Supplementary Fig. S3b), indicating the presence of the rhodopinal series of carotenoids (Britton *et al.*, 2004). The carotenoid composition of both strains, as determined by C₁₈ HPLC analysis [elution with acetonitrile/methanol/ethyl acetate (5 : 4 : 1 by vol.); flow rate 1 ml min⁻¹; absorption at 450 nm] with a PDA detector, showed the presence of rhodopin (80 mol%; *t*_r 9.37 min; absorption maxima 294, 443, 470 and 501 nm), 4,4'-diapolycopene (17 mol%; *t*_r 5.41 min; absorption maxima 225, 445, 470 and 502 nm) and tetrahydrolycopin (3 mol%; *t*_r 18.3 min; absorption maxima 294, 361, 447, 471 and 502 nm). Carotenoid glycosides could not be detected from acetone extracts of either strain, as tested by TLC [dichloromethane/hexane/methanol (3 : 1.5 : 0.5, by vol.); sprayed with anthrone reagent (0.2 % anthrone in 90 % H₂SO₄)] or HPLC, while they were detected from all members of the genus *Phaeospirillum* tested (Table 1). Major fatty acids were C_{18:1}ω7c and C_{16:0}, with minor quantities of C_{16:1}ω7c and/or C_{16:1}ω6c (Table 1).

The G + C contents of the genomic DNA of strains JA480^T and JA481 were 67.8 and 68.8 mol% (by HPLC), respectively. Phylogenetic analysis of strains JA480^T and JA481 with other purple non-sulfur bacteria, based on nearly complete (1450 nt) 16S rRNA gene sequences, revealed that the isolates belonged to the family *Rhodospirillaceae* of the class *Alphaproteobacteria*. The neighbour-joining tree is shown in Fig. 2; the minimum-evolution and maximum-parsimony trees had similar topologies (Supplementary Fig. S4). The highest sequence similarities were found between strain JA480^T and the type strains of *Rhodospirillum sulfurexigens* (92.2 %), *Rhodospirillum rubrum* (91.5 %) and *Rhodospirillum photometricum* (91.4 %). Similarity to other species of the genera *Rhodocista*, *Phaeospirillum*, *Rhodovibrio*, *Rhodospira* and *Roseospira* was only 80–91 %. Between strains JA480^T and JA481, the 16S rRNA gene sequence similarity was 100 % and DNA–DNA relatedness was 89 ± 1 % (triplicate analysis). Strain JA480^T was distinct from its phylogenetically close relatives (*Rhodospirillum*/*Phaeospirillum*) in the presence of a vibrioid cell shape, chimeric ICM structures and significant levels of C_{16:0} over C_{16:1}ω7c/C_{16:1}ω6c and by showing limited (pyruvate, succinate, acetate) utilization of carbon sources/electron donors for growth. Thus, based on morphological, physiological and genotypic differences, we propose a novel

Fig. 2. Phylogenetic tree based on almost-complete 16S rRNA gene sequences showing relationships of strains JA480^T and JA481 within the Alphaproteobacteria. The tree was reconstructed by the neighbour-joining method using MEGA4 software and rooted by using *Escherichia coli* ATCC 11775^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.

genus and species to accommodate strain JA480^T, with the name *Phaeovibrio sulfidiphilus* gen. nov., sp. nov.

Description of *Phaeovibrio* gen. nov.

Phaeovibrio (Pha.e.o.vib'ri.o. Gr. adj. *phaeos* brown; L. v. *vibro* to set in tremulous motion, move to and fro, vibrate; N.L. masc. n. *vibrio* that which vibrates, and also a genus name of bacteria possessing a curved rod shape; N.L. masc. n. *Phaeovibrio* brown vibrio).

Encompasses Gram-negative, vibrioid, mesophilic and freshwater phototrophic bacteria. Cells are motile by polar flagella and multiply by binary fission. Chimeric internal membranes of lamellar stacks and vesicles are present in a single cell. Bacteriochlorophyll *a* and carotenoids of rhodopinal series are the major photosynthetic pigments. Metabolism is strictly anaerobic and obligately phototrophic. A limited number of organic substrates can be photoassimilated. Growth factors are required. C_{18:1ω7c} and C_{16:0} are the major fatty acids. The G+C content of genomic DNA is 67.8–68.8 mol% (HPLC). Delineation of the genus is determined primarily by phylogenetic information from 16S rRNA gene sequences. The type species is *Phaeovibrio sulfidiphilus*.

Description of *Phaeovibrio sulfidiphilus* sp. nov.

Phaeovibrio sulfidiphilus [sul.fi.di.phi'lus. N.L. n. *sulfidum* sulfide; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*) friendly to, loving; N.L. masc. adj. *sulfidiphilus* sulfide-loving].

Displays the following characteristics in addition to those given for the genus. Cells are 0.3–0.5 µm wide and 1.2–2.5 µm long. Phototrophically grown cultures are light

brown. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 377–380, 488–491, 524–527, 593, 794 and 863 nm. The type strain is mesophilic. NaCl is not required for growth, but up to 0.5 % NaCl is tolerated. Optimum growth at pH 7 (range pH 7–8). Obligate photoheterotroph. Growth occurs with acetate, pyruvate and succinate. Ammonium salts and glutamine are used as good nitrogen sources. Sulfide is required as sulfur source. Biotin and *p*-aminobenzoic acid are required as growth factors.

The type strain, JA480^T (=KCTC 5825^T =NBRC 106163^T =DSM 23193^T), and an additional strain, JA481, were isolated from brackish waters of Nagapattinam district, Tamilnadu, India.

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