Phaeospirillum chandramohanii sp. nov., a phototrophic alphaproteobacterium with carotenoid glycosides

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A Gram-negative, spiral-shaped, phototrophic, purple non-sulfur bacterial strain, designated JA145^T, was isolated from a freshwater habitat. Cells of strain JA145^T were motile by means of a monopolar flagellum. Intracellular photosynthetic membranes were of the stacked type. Bacteriochlorophyll *a* and the carotenoid lycopene and its glucosides were present as photosynthetic pigments. There was no vitamin requirement for strain JA145^T. The predominant cellular fatty acids were C_{16:1}ω7c/C_{16:1}ω6c (22.24 %), C_{16:0} (22.97 %) and C_{18:1}ω7c (43.24 %). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain JA145^T clustered with species of the genus *Phaeospirillum*, in the class *Alphaproteobacteria*. The highest sequence similarities of strain JA145^T were found with the type strains of *Phaeospirillum fulvum* (96.12 %) and *Phaeospirillum molischianum* (96.19 %). Based on the 16S rRNA gene sequence analysis and the morphological and physiological characteristics, strain JA145^T is considered to represent a novel species, for which the name *Phaeospirillum chandramohanii* sp. nov. is proposed. The type strain is JA145^T (=JCM 14933^T=KCTC 5703^T=NBRC 104961^T).

The genus *Phaeospirillum* was created by the transfer of two species of the freshwater representatives of the genus *Rhodospirillum* that show marked physiological and phylogenetic differences (Imhoff *et al.*, 1998a). The genus *Phaeospirillum* comprises two recognized species, *Phaeospirillum fulvum* and *Phaeospirillum molischianum* (Imhoff *et al.*, 1998a). In this paper, based on phenotypic and phylogenetic analysis we propose a novel species of the genus *Phaeospirillum*, isolated from a freshwater reservoir in India, which has properties that were distinct from those of the recognized species of the genus *Phaeospirillum*.

Strain JA145^T was isolated from a water sample (pH 7.2, 30 °C), collected on 18 March 2007 from the freshwater reservoir at Mudasarlova, Visakhapatnam, India (17° 45' N

83° 17' E). Two purple non-sulfur bacteria (strains JA143^T and JA145^T) were isolated from photolithoheterotrophic enrichments obtained from the water sample during a study of the diversity of cultured purple anoxygenic phototrophic bacteria. Isolation of strain JA145^T was performed as described previously for strain JA143^T (*Rhodospirillum sulfurexigens*; Anil Kumar *et al.*, 2008b). Subsequent culturing, purification and characterization were done using a medium (Pfennig & Trüper, 1974, 1989) supplemented with Na₂S₂O₃ (4 mM). Purification and polyphasic taxonomic studies (Srinivas *et al.*, 2007) and spectral analysis (Anil Kumar *et al.*, 2008a) were carried out as described previously. Utilization of organic compounds as carbon source/electron donors for phototrophic growth was determined without any additional carbon source/electron donor in the presence of yeast extract (0.02 %, w/v). The concentrations of the compounds used were 0.1 %, v/v (formic acid, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol and ethanol), 0.3 %, w/v (other organic compounds tested) and 1 mM sodium benzoate. For the determination of sulfur sources,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JA145^T is AM779061.

A phase-contrast micrograph of cells and whole-cell absorption and acetone spectra of strain JA145^T and a table giving the ¹H NMR spectral data for hydroxylycopene β-D-glucoside in CDCl₃ are available as supplementary material with the online version of this paper.

MgSO₄·7H₂O was replaced by MgCl₂·5H₂O; sulfur sources [Na₂S·9H₂O, Na₂S₂O₃, sodium thioglycolate, cysteine and MgSO₄·7H₂O (all at 1 mM)] were added to the medium. Nitrogen source utilization was determined by replacing ammonium chloride with various nitrogen sources (0.068 %, w/v). The requirement for vitamins was determined using different vitamins [µg l⁻¹: biotin (100), thiamine (300), niacin (350), *p*-aminobenzoate (200), pyridoxal phosphate (100), pantothenate (100), inositol (100) and B₁₂ (20)], both singly and in combination. Negative controls did not have any of the vitamins or yeast extract. One control was supplemented with yeast extract alone. NaCl tolerance was determined by adding various concentrations of NaCl (0.05, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 %) and also without NaCl in the medium. Chemotrophic growth was determined by growing the cultures in Erlenmeyer flasks placed in an orbital shaker in the dark and at 30 °C. Microaerobic growth was tested by growing the cultures in agar dilution tubes without airtight stoppers. Diazotrophy of the culture was determined as growth under a nitrogen atmosphere by repeated sub-culturing for four times. Carotenoid composition was analysed by using C₁₈-HPLC (Takaichi *et al.*, 2001). After purification of carotenoids using silica gel and DEAE-Toyopearl column chromatography, and C₁₈-HPLC, they were identified based on spectral data (Takaichi *et al.*, 2001). Fatty acid methyl esters were prepared, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI; Agilent: 6850) (Sasser, 1990) at the Royal Research Laboratories, Secunderabad, India.

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). DNA was extracted and purified by using a Qiagen genomic DNA extraction kit. PCR amplification and 16S rRNA gene sequencing were performed as described previously (Imhoff *et al.*, 1998b). Recombinant *Taq* polymerase was used for PCR, which was initiated with the primers 5'-GTTTGATCCTGGCTCAG-3' and 5'-TACCTTGTTACGACTTCA-3' (*Escherichia coli* positions 11–27 and 1489–1506, respectively). Sequences were obtained by using cycle sequencing with a SequiTherm sequencing kit (Biozym) and the chain termination reaction (Sanger *et al.*, 1977) with an automated laser fluorescence sequencer (Pharmacia). Next relatives and sequence similarities were determined by using NCBI-BLAST search (Altschul *et al.*, 1990) and EzTaxon (Chun *et al.*, 2007). For phylogenetic analysis, 16S rRNA gene sequences of representative type strains of *Rhodospirillales* (purple non-sulfur bacteria) species and of strain JA145^T were aligned using the program CLUSTAL_X (Thompson *et al.*, 1997) (multiple alignment option). The alignment was checked manually using BioEdit software (Hall, 1999) and the file was saved in PHYLIP format. The PhyML online version (Guindon *et al.*, 2005) was used for phylogenetic calculations. The maximum-likelihood tree was calculated using the GTR model (Lanave *et al.*, 1984),

based on the hill-climbing principle and estimated proportion of invariable sites, as well as the Gamma distribution parameter. The parameters in the program PhyML were as follows: input sequences are interleaved, with 100 non-parametric bootstrap analysis, GTR model of nucleotide substitution, 4 substitution rate categories, fixed Gamma distribution parameter (alpha=2.00).

Cells of strain JA145^T were Gram-negative, spiral shaped, 0.8–1.0 µm wide and 4–8 µm long and one single turn is 6–8 µm long; cells also formed long chains and the length of the chains could reach up to 30 µm or more (see Supplementary Fig. S1, available in IJSEM Online). The cells multiply by binary fission, are motile by means of a monopolar flagellum and intracellular photosynthetic membranes were of the stacked type. Strain JA145^T was able to grow photo-organoheterotrophically [anaerobically in the light (2400 lx) with pyruvic acid (0.3 %, w/v)]. Photolithoautotrophy [anaerobically in the light (2400 lx) with Na₂S·9H₂O, Na₂S₂O₃·5H₂O (1.0 mM) and NaHCO₃ (0.1 %, w/v)], chemolithoautotrophy [aerobically in the dark with Na₂S₂O₃·5H₂O (1.0 mM) and NaHCO₃ (0.1 %, w/v)], chemoorganoheterotrophy [aerobically in the dark with pyruvate (0.3 %, w/v)] and fermentative growth [anaerobically in the dark with pyruvate (0.3 %, w/v)] could not be demonstrated. Organic substrates utilized for photoorganoheterotrophy (Table 1) include acetate, butyrate, valerate, caproate, caprylate, crotonate, pyruvate, malate, fumarate and mannitol. Those substrates that could not be utilized by strain JA145^T include formate, propionate, glycolate, benzoate, tartrate, lactate, succinate, citrate, D-glucose, D-fructose, glutamate, arginine, aspartate, sorbitol, glycerol, methanol, ethanol, propanol, peptone, yeast extract and Casamino acids. Strain JA145^T could utilize ammonium chloride and dinitrogen as nitrogen sources, whereas glutamate, glutamine, urea, nitrate and nitrite did not support growth. Growth factors were not required for growth of strain JA145^T. Sulfate, sulfite, elemental sulfur and sulfide are used as sulfur sources by the strain. NaCl was not required for growth of strain JA145^T. The pH range for growth of strain JA145^T was 6.5–8.0, with an optimum at pH 7.0. The temperature range for growth was 25–35 °C, with optimum growth at 30 °C. The colour of photosynthetically grown cell suspensions of the strain was brown-orange. The whole-cell absorption spectrum (Supplementary Fig. S2a, in IJSEM Online) of strain JA145^T showed absorption maxima at 383, 407, 461, 491, 527, 593, 803, 860 nm, confirming the presence of bacteriochlorophyll *a* and the absorption spectrum (Supplementary Fig. S2b, in IJSEM Online) for pigments extracted with acetone gave absorption maxima at 448, 473 and 506 nm, indicating the presence of carotenoids. The carotenoid composition of strain JA145^T, as determined by using HPLC analysis, was lycopene (10 %), hydroxylycopene (77 %), hydroxylycopene glucoside (11 %), and dihydroxylycopene diglucoside (2 %). They were identified based on an absorption spectrum (293, 361, 444, 468, 499 nm) of the C₁₈-HPLC

Table 1. Differential characteristics of species of the genus *Phaeospirillum*

Taxa: 1, strain JA145^T (*Phaeospirillum chandramohanii* sp. nov.); 2, *Phaeospirillum fulvum*; 3, *Phaeospirillum molischianum*. Most of the data for reference species 2 and 3 were taken from Imhoff (2005a, b); carotenoid composition and fatty acid methyl ester analysis for species 1 and 3 was done in the authors' laboratory under identical conditions. All strains were motile and contain carotenoids: lycopene, hydroxylycopene, hydroxylycopene glucoside and dihydroxylycopene diglucoside. Organic substrate utilization was tested during photoheterotrophic growth. Pyruvate, fumarate, malate and caprylate were utilized by all the taxa. Arginine, citrate, glycerol and fructose were not utilized by any of the taxa. +, Substrate utilized or present; –, substrate not utilized or absent; +/–, variable in different strains; (+), weak growth; μ, growth under micro-oxic conditions; NA, information not available; ND, not determined/not detected.

Characteristic	1	2	3
Cell size (width of one complete turn of a spiral) (μm)	0.8–1.0 × 4–8 (6–8), form chains, length may reach up to 30 μm or more	0.5–0.7 × 3.5 (1–1.6)	0.7–1.0 × 4–6 (1.5–2.5)
Colour of cell suspension	Brown-orange	Deep brown	Brown-red to dark brown
DNA G + C content (mol%)	60.54	64.3–65.3	60.5–64.8
Growth factors	–	PABA*	–
Aerobic growth in the dark	–	μ	–†
Whole-cell absorption maxima (nm)	383, 407, 461, 491, 527, 593, 803, 860	NA	375, 465, 488–491, 520–528, 590–595, 803–807, 850–855
Carbon source/electron donor			
Acetate	(+)	+	+
Aspartate	–	+/–	+/–
Benzoate	–	+	–
Butyrate	(+)	+	+
Caproate	(+)	+	+
Ethanol	–	+	+
D-Glucose	–	+/–	–
Lactate	–	–	+/–
Mannitol	(+)	–	–
Methanol	–	+/–	–
Propionate	–	+	+
Succinate	–	+	+
Valerate	(+)	+	+
Fatty acids (%)‡			
C _{12:0}	2.05	ND	2.48
C _{12:0} aldehyde (unknown)	1.34	ND	1.38
iso-C _{14:0} 3-OH	1.57	ND	–
C _{14:0} 3-OH/iso-C _{16:1} I	1.34	ND	1.38
C _{14:0} 2-OH	–	ND	1.0
C _{16:0}	22.97	ND	17.82
C _{16:0} 3-OH	2.0	ND	3.04
iso-C _{16:1} G	–	ND	1.57
C _{16:1} ω7c/C _{16:1} ω6c	22.24	ND	22.43
C _{17:0} 10-methyl	1.4	ND	–
C _{18:0}	–	ND	2.33
C _{18:1} ω7c	43.24	ND	36.3
C _{18:1} ω5c	–	ND	2.05

*PABA, *p*-Aminobenzoic acid.

†Tested in our laboratory.

‡Fatty acids less than 1 % were not considered.

eluent of methanol, the retention times on the C₁₈-HPLC, and the relative molecular masses (536, 554, 716, and 896, respectively), and the proton nuclear magnetic resonance spectrum (Supplementary Table S1, available in IJSEM Online; Takaichi *et al.*, 2001). Carotenoids of the recognized species of *Phaeospirillum*, *P. fulvum* and *P.*

molischianum, were reported to be lycopene, hydroxylycopene (rhodopin), dihydroxylycopene and polar carotenoid (Takaichi, 1999), but their identification was insufficient. The presence of lycopene glucosides is limited to a few bacterial species (Takaichi, 1999) and all the species of the genus *Phaeospirillum* have them. Whole-cell fatty acid

analysis showed that the fatty acids $C_{16:1\omega7c}/C_{16:1\omega6c}$ (22.24%), $C_{16:0}$ (22.97%) and $C_{18:1\omega7c}$ (43.24%) predominated in strain JA145^T (Table 1).

The DNA base composition of strain JA145^T was 60.54 mol% (by HPLC). The phylogenetic relationship of strain JA145^T to other purple non-sulfur bacteria was examined based on 16S rRNA gene sequences (1400 bases). The data obtained revealed that the new isolate clustered with the type strains of *Phaeospirillum* species, but were distinct from other genera of purple non-sulfur bacteria. The highest sequence similarities of strain JA145^T were with the type strains of *P. fulvum* (96.12%) and *P. molischianum* (96.19%) (Fig. 1). Strain JA145^T also showed clear phenotypic differences with other *Phaeospirillum* species (Table 1) that justify the description of strain JA145^T as representing a novel species, for which the name *Phaeospirillum chandramohanii* sp. nov. is proposed.

Description of *Phaeospirillum chandramohanii* sp. nov.

Phaeospirillum chandramohanii (chan.dra.mo.ha'ni.i. N.L. masc. gen. n. *chandramohanii* of Chandramohan, named

after Dr D. Chandramohan, an Indian marine microbiologist, who has played a crucial role in transforming microbiological research at the National Institute of Oceanography, India into technologically rewarding activities).

Cells are Gram-negative, spiral-shaped, 0.8–1.0 µm wide and 4–8 µm long; one single turn is 6–8 µm long. Cells also form long chains of more than 30 µm in length, are motile with monopolar flagella and divide by binary fission. Growth occurs under anaerobic conditions in the light (photoorganoheterotrophy). Internal photosynthetic membranes are of the stacked type. The colour of the phototrophic culture is brown-orange. The *in vivo* absorption spectrum of intact cells in sucrose exhibits maxima at 383, 407, 461, 491, 527, 593, 803 and 860 nm. Bacteriochlorophyll *a* and the carotenoid lycopene and its glucosides are the photosynthetic pigments. Mesophilic (30 °C) and has a pH optimum of 7.0 for optimal growth. Photoorganoheterotrophy with a few organic compounds is the only mode of growth. Pyruvate, crotonate, malate and fumarate are good carbon sources. Growth also occurs on acetate, butyrate, valerate, caproate, caprylate and mannitol. Photolithoautotrophic, chemolithoautotrophic,

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain JA145^T within the order Rhodospirillales. The tree was constructed using the PhyML program. Numbers at nodes are bootstrap percentages. Values <50% are not shown. Bar, 2 nucleotide substitutions per 100 nucleotides.

chemoorganoheterotrophic and fermentative growth could not be demonstrated. Sulfate, sulfite, elemental sulfur and sulfide are used as sulfur sources. Growth factors are not required. Predominant fatty acids are C_{16:1}ω7c/C_{16:1}ω6c, C_{16:0} and C_{18:1}ω7c. The DNA G+C content of the type strain is 60.54 mol% (by HPLC). Natural habitats are freshwater.

The type strain, JA145^T (=JCM 14933^T=KCTC 5703^T=NBRC 104961^T), was isolated from a water sample from freshwater reservoir at Mudasarlova, Visakhapatnam, India.

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