Thiorhodococcus fuscus sp. nov., isolated from a lagoon

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A brown, moderately halophilic, photoautotrophic bacterium designated strain JA363 T was purified from a photoheterotrophic enrichment obtained from sediment from Chilika lagoon, Odisha, India. Cells of the isolate were coccoid, motile by means of single polar flagellum and Gram-stain-negative. Strain JA363 T had an obligate requirement for NaCl and could tolerate up to 7 % (w/v) NaCl. Strain JA363 T had complex growth factor requirements. Internal photosynthetic membranes were present as vesicles. Strain JA363 T contained bacteriochlorophyll a and spirilloxanthin series carotenoids with rhodopin as a major (>85 %) component. C16 : 1ω7c/C16 : 1ω6c, C18 : 1ω7c and C18 : 0 were the major fatty acids and phosphatidylglycerol and phosphatidylethanolamine were the major polar lipids. Q8 was the predominant quinone system of strain JA363 T. The DNA G+C content was 64 mol%. The highest 16S rRNA gene sequence similarity of strain JA363 T was found with the type strains of Thiorhodococcus kakinadensis (98.7 %), Thiohalobacter thiocyanaticus (98.2 %), Thiopeaeeococcus fuscus (97.4 %) and Thiorhodococcus bheemlicus (96.3 %). However, the phylogenetic trees generated firmly placed strain JA363 T in the genus Thiorhodococcus, which was further supported by phenotypic and chemotaxonomic evidence. Consequently, strain JA363 T is described as representing a novel species of the genus Thiorhodococcus as Thiorhodococcus fuscus sp. nov. The type strain is JA363 T (=KCTC 5701 T=NBRC 104959 T).

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Purple sulfur bacteria are often found in anoxic sediments and in shallow waters where there is the presence of both reduced sulfur compounds and solar light (Rabold et al., 2006). During our survey of one such water body at Chilika lagoon, a marine habitat with neutral pH, an unexpectedly rich diversity of cultivable purple anoxygenic phototrophs was revealed. Among the several bacteria isolated, a moderately halophilic bacterium affiliated to the genus Thiorhodococcus was isolated from the sediment of Chilika lake at Satpada, Odisha, India (GPS position: 19° 39’ E 85° 22’ N). The sample had a pH of 7.0 and salinity of 3.5 %. Members of the genus Thiorhodococcus are spherical to slightly ovoid, motile by binary fission and show obligate phototrophy. These strains are morphologically similar to species of the genera Thiocystis and Thiopeaeeococcus, but differ from them in their 16S rRNA gene sequences and several other aspects (Imhoff et al., 1998; Anil Kumar et al., 2008). This truly marine genus is currently represented by five species with validly published names; Thiorhodococcus minor (Guyoneaud et al., 1997), Thiorhodococcus mannitoliphagus (Rabold et al., 2006), Thiorhodococcus bheemlicus, Thiorhodococcus kakinadensis (Anil Kumar et al., 2007) and Thiorhodococcus modestalkaliphilus (Sucharita et al., 2010). The name of a sixth member ‘Thiorhodococcus drewsii’ (Zaar et al., 2003) has not been validly published.

Strain JA363 T was recovered from a photoheterotrophic enrichment in a medium described previously (Shivali et al., 2011; without supplementing sodium bicarbonate), incubated at 2400 lx, 30 °C for 7 days in 45 ml fully
DNA of strain JA363T, as determined by reverse-phase. Genomic DNA was extracted and purified according to the methods in the MEGA-5 software were used to reconstruct mum-likelihood (ML) and maximum-parsimony (MP) wise deletion procedure. The neighbour-joining (NJ), maxi-
Kimura two-parameter method (Kimura, 1980) in a pair
netic analyses. Distances were calculated by using the
slants or as lyophilized cultures preserved at 4
ditions described above. Cultures were maintained on agar
slants or as lyophilized cultures preserved at 4 °C.

Genomic DNA was extracted and purified according to the method of Marmur (1961) and the G+C content of the DNA of strain JA363T, as determined by reverse-phase HPLC, (Mesbah et al., 1989) was 64 mol%. Well isolated colonies were used for 16S rRNA gene amplification by using PCR master mix (GeNei) as described previously (Subhash et al., 2013a). 16S rRNA gene sequencing was performed on a 3130x ABI prism automated DNA sequencer (Applied Biosystems) as described previously (Subhash et al., 2013b). The 16S rRNA gene sequence of the strain was identified by BLAST search analysis on the EzTaxon-e server (Kim et al., 2012). The BLAST search analysis (1438 nt) indicated that JA363T shared highest 16S rRNA gene sequence similarity with the type strains of Thiorhodo-
coccus kakinadensis (98.79 %), Thiohalobacter thiocyana-
ticus (98.26 %), Thiophaeococcus fuscus (97.43 %) and Thiorhodo-
coccus bheemlicus (96.32 %). The CLUSTAL W algorithm of
MEGA-5 (Tamura et al., 2011) software was used for phyloge-
etic analyses. Distances were calculated by using the
Kimura two-parameter method (Kimura, 1980) in a pair
wise deletion procedure. The neighbour-joining (NJ), maxi-

mum-likelihood (ML) and maximum-parsimony (MP) methods in the MEGA-5 software were used to reconstruct phylogenetic trees and the combined phylogenetic tree (NJ, ML, MP; Fig. 1) revealed that strain JA363T clustered with the members of the genus Thiorhodococcus and was not specifically affiliated with lineages formed by members of the genera Thiophaeococcus and Thiohalobacter of the family Chromatiaceae, as otherwise indicated by BLAST search analysis. A more detailed analysis of the phylogenetic tree (Fig. S1 available in the online Supplementary Material) using some of the cloned sequences from NCBI further confirmed the affiliation of JA363T with the members of the genus Thiorhodococcus.

Further characteristics of strain JA363T were studied in detail as per recommended minimal standards (Imhoff & Caumette, 2004) together with those of the closely related strains Thiorhodococcus kakinadensis JCM 14150T (＝JA130T) and Thiorhodococcus bheemlicus JCM 14149T (＝JA132T). The taxonomic relationship between strain JA363T and the closely related type strains was examined using DNA–DNA hybridization which was performed using a membrane filter technique (Tourova & Antonov, 1987), using a nick translation kit supplied by BRIT, Jonaki, CCMB campus, Hyderabad. When strain JA363T was radioactively labelled, the levels of DNA–DNA reassociation with type strains of Thiorhodococcus kakinadensis and Thiorhodococcus bheemlicus were 45.5 % and 42.2 %, respectively. However, when the type strains of Thiorhodococcus kakinadensis and Thiorhodococcus bheemlicus were labelled and used for DNA–DNA hybridization with JA363T in the reciprocal reaction, the reassociation values were 43.3 % and 41.7 %, respectively. Based on the hybridization results, strain JA363T thus represents a novel species according to recommendations for delineating a bacterial species (Stackebrandt & Goebel, 1994).

Morphological properties (cell shape, cell division, cell size, motility) were observed under a phase-contrast microscope (BH-2, Olympus). Flagellar position was determined using a transmission electron microscope (H-7500, Hitachi). The internal membrane structures were also viewed with a transmission electron microscope, after the cells had been processed as described by Hanada et al. (2002). Cells of

![Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing phylogenetic relationships of strain JA363 (1438 nt) with a few members of the family Chromatiaceae. The tree was computed with MEGA 5.2 software and rooted by using Escherichia coli as the outgroup. GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bootstrap percentages refer to NJ/MP/ML analysis. Bar, 0.02 nucleotide substitutions per position.](http://ijs.microbiologyresearch.org)

http://ijs.microbiologyresearch.org

A novel species of Thiorhodococcus
strain JA363<sup>T</sup> were coccoid, 2–4 μm in diameter (Fig. S2), multiplied by binary fission and were motile by means of single polar flagella (Fig. S3A). Transmission electron microphotographs of ultrathin sections of the strain revealed the presence of a vesicular type of internal membrane structures (Fig. S3B).

Utilization of organic carbon compounds like formate, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol and ethanol as carbon sources/electron donors was tested at 0.1 % (v/v) along with NaHCO<sub>3</sub> (0.1 %, w/v), while for other substrates it was tested at 0.3 % (w/v) without NaHCO<sub>3</sub>. Growth was measured turbidometrically at 660 nm. Good growth was observed only on limited substrates such as acetate, benzoyl, caprylate, pyruvate, formate, fructose, lactate, malate, succinate, tartrate and valerate. Those which could not be utilized included ascorbate, aspartate, butanol, butyrate, caproate, citrate, crotonate, cysteine, ethanol, fumarate, glutamate, glucose, glycerol, gluconate, glycolate, 2-oxoglutarate, mannotol, methionine, methanol, peptone, propanol, propionate, sorbitol, sucrose, starch and yeast extract (Table 1).

For testing the utilization of sulfur sources, MgSO<sub>4</sub>·7H<sub>2</sub>O was replaced with MgCl<sub>2</sub>·6H<sub>2</sub>O (0.2 %) and various sulfur sources (sodium sulfide, sodium thiosulfate, thioglycolate, cysteine, magnesium sulfate, sodium sulfate, sodium sulfite all at 0.5 mM concentration) were added to the medium. While JA363<sup>T</sup> utilized sulfide, thiosulfate and thioglycolate as sulfur sources, methionine, cysteine, elemental sulfur and sulfate did not support growth. Nitrogen source requirements for growth were tested by replacing ammonium chloride with different nitrogen sources at a concentration of 7 mM. JA363<sup>T</sup> grew well with ammonium chloride, glutamine and glutamate as nitrogen sources while aspartate, nitrite, urea and nitrate did not support growth. Vitamin requirement was tested by replacing yeast extract with single vitamins and combinations of vitamins as growth factors, and JA363<sup>T</sup> showed a complex growth requirement.

| Table 1. Characteristics differentiating strain JA363<sup>T</sup> from the closely related species of the genus Thiorhodococcus |
|-----------------|-----------------|-----------------|
| Characteristic                                          | 1               | 2               |
| Cell diameter (μm)                                       | 2–3             | 3–5             | 4–6             |
| Source of isolation                                      | Sediment of lagoon | Water of marine aquaculture pond | Marine tidal waters |
| Colour of cell suspensions                                | Brown           | Purple–violet   | Purple–violet   |
| Growth factors                                           | y.e.            | n, b, p         | –               |
| pH optimum (range) (°C)                                  | 7.5 (6.0–8.5)   | 7.5 (6.5–8.0)   | 7.0–7.5 (6.5–8.0) |
| NaCl optimum (range) (w/v, %)                            | 2–4 (0.5–7)     | 1–2 (0.5–5)   | 1–3 (0.5–6)    |
| Photolithoautotrophy                                     | +               | –               | –               |
| Major carotenoid                                          | Rp              | Ly              | Ly              |
| Polar lipids                                              | UL1,2,3,4      | UL1,3,4        | UL1,2,3,4,5,6  |
| Substrates used as carbon/electron donors for growth     |                 |                 |
| Acetate                                                   | +               | –               | –               |
| Benzoate                                                  | +               | –               | –               |
| Casamino acids                                            | +               | –               | –               |
| Caprylate                                                 | +               | –               | –               |
| Formate                                                   | +               | –               | –               |
| Fructose                                                  | +               | –               | –               |
| Glucose                                                   | –               | –               | +               |
| Glucolyte                                                 | –               | –               | –               |
| Glycolate                                                 | –               | –               | +               |
| Lactate                                                   | +               | ( + )           | +               |
| Malate                                                    | +               | +               | –               |
| Propionate                                                | –               | +               | –               |
| Succinate                                                 | +               | +               | –               |
| Tartrate                                                  | +               | –               | –               |
| Valerate                                                  | +               | –               | ( + )           |
| DNA G + C content (HPLC) (mol%)                           | 64              | 60             | 65              |
Chemotrophic growth was determined by growing the cultures in Erlenmeyer flasks placed in an orbital shaker (in the dark) at 30 °C. It was observed that the JA363T was able to grow photoorganoheterotrophically [anaerobic, light (2400 lx) with pyruvate (0.03 %, w/v) as a carbon source/electron donor] and photolithoautotrophically [anaerobic, light (2400 lx), Na₂S₉H₂O/Na₂S₂O₃, 5H₂O (1 mM/5 mM) and NaHCO₃ (0.1 %, w/v)]. Chemolithoautotrophy [anaerobic, dark, Na₂S₂O₃, 5H₂O (5 mM) and NaHCO₃ (0.1 %, w/v)] and chemoorganoheterotrophy [anaerobic (respiration)/anaerobic (fermentation), dark and pyruvate (0.3 %, w/v)] could not be demonstrated in strain JA363T. JA363T had an obligate requirement of NaCl for growth, where optimum growth occurred at 2–4 %, but it could tolerate up to 7 % NaCl (range 0.5–7 %, w/v). The pH range for growth of strain JA363T was 6.0–8.5 with the optimum being pH 7.5. JA363T was a mesophile, growing optimally at 30 °C (range 25–35 °C).

Phototrophically grown cells were brown, while all the other five members of the genus *Thiorhodococcus* are red-shaded. The *in vivo* absorption spectrum of intact cells of strain JA363T as measured using a Genesys2 spectrophotometer (Spectronic) in sucrose solution (Trüper & Pfennig, 1981) exhibited maxima at 374, 491, 530, 590, 803 and 857 nm (Fig. S4) indicating the presence of bacteriochlorophyll *a*. Carotenoid composition of strain JA363T as determined by C₁₈-HPLC (eluted with acetonitrile/methanol/ethyl acetate; 5 : 4 : 1, v/v; flow rate 1 ml min⁻¹; absorption at 450 nm) using a photodiode array detector indicated the presence of rhodopin (>80 %) as the major carotenoid while the type strains of *Thiorhodococcus kakinadensis* and *Thiorhodococcus bheemlicus* contained lycopene (>85 %); both the carotenoids belong to the spirilloxanthin series.

For fatty acid analysis, cells were harvested by centrifugation (10 000 g for 15 min at 4 °C) on reaching a cell density of 70 % of the maximum optical density (100 %=0.9; OD₆₆₀) and the pellet was used for analysis. Cellular fatty acids were methylated, separated and identified according to the instructions for the Microbial Identification System (Microbial ID; MIDI 6.0 version; method, RTSB6A6) (Sasser, 1990) (www.midi-inc.com), which was outsourced to Royal Research Laboratories, Secunderabad, India. The fatty acid profile of JA363T was found to be in congruence with those of the type strains of species of the genus *Thiorhodococcus* with major proportions of C₁₆:1ω7c/C₁₆:1ω6c (48.2 %), C₁₈:1ω7c/C₁₈:1ω6c (19.1 %) and C₁₆:0 (18.0 %) and minor amounts of C₁₂:0 (5 %), C₁₄:0 (3.7 %), iso-C₁₇:1ω9c (1.4 %) and C₁₈:1ω5c (1.1 %; Table S1). However, strain JA363T differed from the other closely related type strains of the genus in the presence of iso-C₁₁:1ω9c and absence of C₁₈:1ω9c, anteiso-C₁₅:0, C₁₂:0 and anteiso-C₁₇:0. Members of the genera *Thiophaeococcus*, *Chromatium*, *Thiocapsa* and *Thiocystis*, which belong to the family *Chromatiaceae*, have also been reported to have similar fatty acid profiles with C₁₈:1ω7c and C₁₆:0 as major fatty acids (Divyasree et al., 2014; Imhoff & Bias-Imhoff, 1995); however, the position of bonds was not indicated for the latter three genera.

Polar lipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2 : 1 : 0.8, by vol.) as described by Kates (1986). Lipids were separated using silica gel TLC (Kieselgel 60 F₂₅₄, Merck) by two-dimensional chromatography using chloroform/methanol/water (65 : 25 : 4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.) in the second dimension (Tindall, 1990a, b; Oren et al., 1996). The total lipid profile was visualized by spraying with 5 % ethanolic molybdophosphoric acid and was further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff (quaternary nitrogen) or *z*-naphthol (specific for sugars) (Kates, 1972; Oren et al., 1996). The polar lipid profile of strain JA363T was observed to be in consonance with those of closely related type strains of species of the genus *Thiorhodococcus*, which contained phosphatidylglycerol and phosphatidylethanolamine as the major lipids with minor amounts of an aminophospholipid and four unidentified lipids that, based on their staining behaviour, did not contain either a sugar moiety or a phosphate or quaternary ammonium or an amino group (Fig. S5). The type strain of *Thiorhodococcus bheemlicus* additionally contained minor amounts of two other unidentified lipids. The lipid profile of species of the genus *Thiorhodococcus* (strain JA363T, *Thiorhodococcus kakinadensis* JCM 14150T and *Thiorhodococcus bheemlicus* JCM 14149T) differed from those of the species of the genus *Thiophaeococcus* (Divyasree et al., 2014) by the absence of diphosphatidylglycerol, phosphatidylcholine and phospholipids. Members of the genus *Thiorhodococcus* analysed in the present study additionally lacked glycolipids and cardiolipins which are otherwise reported to occur in the members of *Chromatiaceae* such as members of the genera *Chromatium*, *Thiocapsa* and *Thiocystis* (Imhoff et al., 1982). Quinones were extracted with a chloroform/methanol (2 : 1, v/v) mixture, purified by TLC and analysed by HPLC (Imhoff, 1984; Hiraishi & Hoshino, 1984; Hiraishi et al., 1984) and ubiquinone 8 (>90 %) was observed to be the predominant quinone system in strain JA363T and the type strains of *Thiorhodococcus kakinadensis* and *Thiorhodococcus bheemlicus*. This matched the quinone system found in the members of the genus *Thiophaeococcus* (Divyasree et al., 2014) and other phototrophic members of the family *Chromatiaceae* (Imhoff & Bias-Imhoff, 1995).

The phylogenetic distance of strain JA363T from type strains of closely related species, which is also reflected in a number of distinctive phenotypic (colour, growth factor requirement, organic substrate utilization, photolithoautotrophic growth; Table 1) and chemotaxonomic traits (polar lipids, carotenoids; Fig. S5, Table 1), justifies the description of strain JA363T as representing a novel species of the genus *Thiorhodococcus* for which the name *Thiorhodococcus fuscus* sp. nov. is proposed.
**Description of Thiorhodococcus fuscus** sp. nov.

*Thiorhodococcus fuscus* (fus’cus. L. masc. adj. fuscus tawny).

Individual cells are cocoid, 2–4 μm in diameter, Gram-stain-negative, motile by means of a single polar flagellum, multiply by binary fission and have internal photosynthetic membranes of the vesicular type. Photoorganoheterotrophy and photolithoautotrophy are the growth modes. Chemolithoautotrophy, chemoorganoheterotrophy and fermentative growth are absent. Phototrophically grown cultures are brown. The in vivo absorption spectrum of intact cells in sucrose exhibits maxima at 374, 491, 530, 590, 803 and 857 nm. Bacteriochlorophyll-α and rhodopin of the spirilloxanthin series are present as major pigments. The light absorption maxima are at 590, 803 and 857 nm. Bacteriochlorophyll-α in vivo is present as bacteriochlorophyll-α 590, 803 and 857 nm. Bacteriochlorophyll-α in vivo is present as bacteriochlorophyll-α 590, 803 and 857 nm. Bacteriochlorophyll-α in vivo is present as bacteriochlorophyll-α 590, 803 and 857 nm. Bacteriochlorophyll-α in vivo is present as bacteriochlorophyll-α 590, 803 and 857 nm. Bacteriochlorophyll-α in vivo is present as bacteriochlorophyll-α 590, 803 and 857 nm. Bacteriochlorophyll-α in vivo is present as bacteriochlorophyll-α 590, 803 and 857 nm.

Temperature for growth are pH 7.5 (range pH 6.0–8.5) and 2–4% NaCl (range 0.5–7%, w/v). Optimum pH and temperature for growth are pH 7.5 (range pH 6.0–8.5) and 30 °C (range 25–35 °C), respectively. Acetate, benzoate, caprylate, pyruvate, formate, fructose, lactate, malate, succinate, tartrate and valerate are good carbon sources/elec-

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**References**


Shivali, K., Ramana, V. V., Ramaprasad, E. V., Sasikala, Ch. & Ramana, Ch. V. (2011). *Marichromatium litoris* sp. nov.
Marichromatium chrysaorae sp. nov. isolated from beach sand and from a jelly fish (Chrysaora colorata). Syst Appl Microbiol 34, 600–605.


