**Thiophaeococcus fuscus** sp. nov., isolated from a lagoon

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A brown-coloured bacterium, designated strain JA633ᵀ, was purified from a photoheterotrophic enrichment culture obtained from black sand of a lagoon. Cells of strain JA633ᵀ were coccoid–spherical, Gram-stain-negative and motile by means of polar flagella. Strain JA633ᵀ had an obligate requirement for NaCl and could tolerate up to 4 % (w/v) NaCl. Internal photosynthetic membranes were present as vesicles. Photo-organoheterotrophy was the only growth mode observed. Strain JA633ᵀ contained bacteriochlorophyll a and a major (>85 %) unidentified carotenoid of the spirilloxanthin series. Thiamine and p-amino benzoic acid were required for growth. Major fatty acids were C₁₈ : 1₀⁻⁷C/C₁₈ : 1₀⁻⁶C, C₁₆ : 0 and C₁₆ : 1₀⁻⁷C/C₁₆ : 1₁⁻⁶C. Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylylcholine and an unknown aminophospholipid were the major polar lipids in strain JA633ᵀ. The DNA G+C content of strain JA633ᵀ was 64.5 mol%. Strain JA633ᵀ shared highest 16S rRNA gene sequence similarity with the type strains of *Thiorhodococcus kakinadensis* (96.9 %), *Thiophaeococcus mangrovi* (96.3 %) and *Thiorhodococcus bheemlicus* (96.2 %), which belonged to the class *Gammaproteobacteria*. However, phylogenetic analysis based on 16S rRNA gene sequences showed that strain JA633ᵀ formed a separate clade along with *Thiophaeococcus mangrovi* JA304ᵀ whereas the members of the genus *Thiorhodococcus* remained as two distinct phylogenetic lineages. Based on morphological, physiological, chemotaxonomic and molecular evidence, strain JA633ᵀ was significantly different from the type strain of *Thiophaeococcus mangrovi* of the family *Chromatiaceae*. It is thus proposed that the strain be classified as a representative of a novel species, for which the name *Thiophaeococcus fuscus* sp. nov. is proposed. The type strain is JA633ᵀ (=KCTC 15337ᵀ=NBRC 109958ᵀ).

At the time of writing, there are 24 phototrophic genera in the family *Chromatiaceae* (http://www.bacterio.net/classifgenerafamilies.html#Chromatiaceae). 16S rRNA gene sequence comparisons have revealed genetic divergence between members of the *Chromatiaceae* that originate from freshwater sources and those of truly marine and halophilic nature (Imhoff et al., 1998). The genus *Thiophaeococcus* is a truly marine taxon represented by a single species (*Thiophaeococcus mangrovi*; Anil Kumar et al., 2008) showing obligate phototrophy and spherical, motile cells that multiply by binary fission. In this study we describe a novel species of the genus *Thiophaeococcus*, isolated from a lagoon.

Strain JA633ᵀ was isolated from a phototrophic enrichment of black sand sample which was collected from Muthupet lagoon, Tamil Nadu, India, on 25 May 2009 (GPS position: 10° 2’ N 79° 32’ E). Strain JA633ᵀ was recovered from an enrichment in medium described by Shivali et al. (2011; but without supplementary sodium bicarbonate), incubated at 2400 lx and 30 °C for 7 days in 45 ml fully filled screw-capped bottles. Purification was done by repeated streaking on agar slants (Lakshmi et al., 2011). Purified cultures were grown in completely filled screw-capped test tubes (10 × 100 mm) under phototropic conditions 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 JA633ᵀ as determined by reversed-phase HPLC (Mesbah et al., 1989) was 64.5 mol%. Well-isolated colonies were used for 16S rRNA gene amplification by using PCR master mix (GeNei) as described previously.
16S rRNA gene sequencing was performed on a 3130xl Applied Biosystems ABI prism automated DNA sequencer as described by Subhash et al. (2013b). The 16S rRNA gene sequence of strain JA633T was compared with others identified by BLAST search analysis on the EzTaxon-e server (Kim et al., 2012). The BLAST search analysis indicated that strain JA633T shared highest 16S rRNA gene sequence similarity with *Thiorhodococcus kakinadensis* JA130T (96.9 %), *Thiophaeococcus mangrovi* JA304T (96.3 %) and *Thiorhodococcus bheemicus* JA132T (96.2 %), which belonged to the class Gammaproteobacteria. The CLUSTAL W algorithm within the MEGA 5 (Tamura et al., 2011) software was used for phylogenetic analyses. Distances were calculated according to Kimura's two-parameter model (Kimura, 1980) in a pairwise deletion procedure. The neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) methods in the MEGA 5 software were used to construct phylogenetic trees and a combined phylogenetic tree (NJ, ML, MP; Fig. 1) revealed that strain JA633T along with *Thiophaeococcus mangrovi* JA304T formed a cluster that is distinct from the phylogenetic lineages formed by other members of the family Chromatiaceae.

Characteristics of strain JA633T were studied in detail as per recommended minimal standards (Imhoff & Caumette, 2004; Logan et al., 2009) together with *Thiophaeococcus mangrovi* JC M 14889T (=JA304T). Morphological properties (cell shape, cell division, cell size, motility) were observed under a phase-contrast microscope (Olympus BH-2). 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 strain JA633T were coccoid–spherical, 2.5–3.0 μm in diameter (Fig. S1, available in the online Supplementary Material), multiplied by binary fission and were motile by means of polar flagella. Transmission electron micrographs of ultrathin sections of cells revealed the presence of vesicular-type internal membrane structures.

Utilization of organic carbon compounds such as formate, propionate, butyrate, caproate, valerate, lactate, glycerol, methanol and ethanol as carbon sources/electron donors was tested with each at 0.1 % (v/v) along with NaHCO₃ (0.1 %, w/v), while for other substrates it was tested at

![Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain JA633T and members of the family Chromatiaceae. The tree was computed with MEGA 5.2 software and rooted by using *Escherichia coli* ATCC 11775T as the outgroup. GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bootstrap percentages refer to NJ/MP/ML analyses. Bar, 0.02 nt substitutions per position.](http://ijs.sgmjournals.org)
0.3 % (w/v) without NaHCO₃. Growth was measured turbidometrically at 660 nm. Good growth was possible with acetate, butyrate, pyruvate, fructose, fumarate, malate, valerate, propionate and crotonate. Mannitol, citrate, sorbitol, succinate and sucrose were utilized weakly. Methionine, thioglycolate, methanol, Casamino acids, benzoate, propanol, glucose, gluconate, peptone, aspartate, glycerol, glycolate, tartrate, lactate and ethanol could not be utilized for growth (Table 1).

For tests of the utilization of sulfur sources, MgSO₄·7H₂O was replaced with MgCl₂·6H₂O (0.2 %) and respective sulfur sources (sodium sulfide, sodium thiosulfate, sodium thioglycolate, cysteine, magnesium sulfate, sodium sulfate and sodium sulfit e, all at 0.5 mM) were added to the medium. Strain JA633T utilized sulfate, sulfite, sulfide, elemental sulfur and thiosulfate as sulfur sources but not methionine or cysteine. Nitrogen requirements for growth were tested by replacing ammonium chloride with different nitrogen sources at 7 mM. Strain JA633T grew well with ammonium chloride, glutamate and aspartate as nitrogen sources while glutamine, nitrite, urea and nitrate did not support growth. Vitamin requirements were tested by replacing yeast extract with single and also combinations of

<table>
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<tr>
<th>Table 1. Differential characteristics between strain JA633T and Thiophaeococcus mangrovi JCM 14889T</th>
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<tr>
<td><strong>Characteristic</strong></td>
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<td>Cell size (µm)</td>
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<td>Colour of broth culture</td>
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<td>pH optimum (range)</td>
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<td>Temperature optimum (range) (°C)</td>
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<td>NaCl optimum (range) (w/v, %)</td>
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<td>Photolithoautotrophy</td>
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<td>Minor polar lipids</td>
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<td>Substrates used as carbon/electron donors</td>
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<td>Casamino acids</td>
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<td>Valerate</td>
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<td>Fatty acid composition (%)</td>
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<td>C₁₂ : 0</td>
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<td>C₁₄ : 0</td>
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<td>C₁₆ : 0</td>
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<tr>
<td>C₁₆ : 1ω7c/C₁₆ : 1ω6c</td>
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<td>C₁₈ : 0</td>
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<td>anteiso-C₁₈ : 9ω/C₁₈ : 6ω6,9c</td>
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<tr>
<td>C₁₈ : 1ω6c</td>
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<td>C₁₈ : 1ω7c/C₁₈ : 1ω6c</td>
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<td>C₁₈ : 1ω9c</td>
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<td>DNA G + C content (mol%)</td>
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</table>

All data are from the present study. Both strains are coccoid–spherical, motile by polar flagella and divide by binary fission, and have lycopene-related molecules as carotenoids, Q8 quinone system and polar lipids diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unknown aminophospholipid (APL). Organic substrate utilization was tested during photoheterotrophic growth. Malate, fructose and pyruvate were utilized by both strains. Benzoate, lactate, arginine, aspartate, caproate, caprylate, glycolate, ethanol, methanol, tartrate, formate, peptone, yeast extract, glutarate and propanol were not utilized. +, Good growth; −, no growth; (+), weak growth; PL1, PL2, PL3, unidentified polar lipids.
vitamins as growth factors, and thiamine and p-amino-
benzoic acid were observed to be obligate for growth of
strain JA633<sup>T</sup>.

Chemotrophy was determined by growing the cultures in
Erlenmeyer flasks placed in an orbital shaker (in the
dark) at 30 °C. Strain JA633<sup>T</sup> was able to grow photo-
organoheterotrophically [anaerobically, in the light (2400 lx)
with pyruvate (0.03 %, w/v) as a carbon source/electron
donor] only. Photolithoautotrophy [anaerobically, light
(2400 lx), Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5 H<sub>2</sub>O (1 mM) and
NaHCO<sub>3</sub> (0.1 %, w/v)] chemolithoautotrophy [anaerobically,
dark, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 5H<sub>2</sub>O (5 mM) and NaHCO<sub>3</sub>
(0.1 %, w/v)] and chemo-organoheterotrophy [anaerobically
(fermentation), dark, pyruvate (0.3 %, w/v)] could not be
demonstrated for strain JA633<sup>T</sup>. The strain showed
an obligate requirement for NaCl for growth, with
optimum growth at 2 %, and it was able to tolerate up to 4 %
(range 1–4 %, w/v). The pH range for growth of strain
JA633<sup>T</sup> was 6.5–7.5, with optimum growth at pH 7.0. Strain
JA633<sup>T</sup> was mesophilic, growing optimally at 30 °C (range
25–35 °C).

Phototrophically grown cells were brown. The in vivo
absorption spectrum of intact cells of strain JA633<sup>T</sup> as
measured using a Spectronic Genesys2 spectrophotometer
in sucrose solution (Trüper & Pfennig, 1981) exhibited
maxima at 374, 458, 488, 593, 800 and 860 nm (Fig.
S2), indicating the presence of bacteriochlorophyll a.
The carotenoid composition of the strain as determined by C<sub>18</sub>-
HPLC (eluted with acetone/methanol/ethyl acetate at
5:4:1, v/v; flow rate of 1 ml min<sup>−1</sup> absorption at 450 nm)
using a photodiode array detector indicated the presence of
a major (>85 %) unidentified carotenoid whose peak
retention time of 6.4 min was very close to that of lycopene
(6.0 min) and distinct from rhodopin (hydroxylycopene;
4.2 min), both of which belong to the spirilloxanthin series
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overlapping at 6.4 min were 208, 295, 442, 469 and 500 nm
showing the presence of bacteriochlorophyll a.

For fatty acid analysis, cells were harvested by centrifu-
gation (10 000 g for 15 min at 4 °C) on reaching a cell
density of 70 % of the maximum optical density (OD<sub>660</sub>
of 0.9) 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, RTSBA6)
(Sasser, 1990; www.midi-inc.com), which was outsourced
to Royal Research Laboratories, Secunderabad, India. The
fatty acid profile of strain JA633<sup>T</sup> was similar to that of
<em>Thiophaeococcus mangrovi</em> JCM 14889<sup>T</sup>, with major propor-
tions of C<sub>18</sub>:1<sup>ω7</sup> C<sub>18</sub>:1<sup>ω6c</sup> (36.2 %), C<sub>16</sub>:0 (25.1 %) and
C<sub>16</sub>:1<sup>ω9c</sup> C<sub>16</sub>:1<sup>ω6c</sup>, C<sub>16</sub>:1<sup>ω9c</sup> (21.1 %) and minor amounts of C<sub>12</sub>:0
(6.4 %), C<sub>14</sub>:0 (2.3 %) and C<sub>18</sub>:1(1.7 %) (Table 1). Members
of the genera <em>Chromatiun</em>, <em>Thiocapsa</em> and <em>Thiocystis</em>
belonging to the family <em>Chromatiaceae</em> were also reported
to contain similar fatty acid profiles with C<sub>18</sub>:1<sup>ω7</sup> C<sub>16</sub>:1<sup>ω6c</sup>
and C<sub>16</sub>:1<sup>ω9c</sup> as major components, although the position of bonds
was not indicated (Imhoff & Bias-Imhoff, 1995).

Polar lipids were extracted from 1 g of 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 F<sub>254</sub>; Merck) by two-dimensional chromatography with 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 reagent (quaternary nitrogen)
or a-naphthol (specific for sugars) (Kates, 1972; Oren
et al., 1996). The polar lipid profile of strain JA633<sup>T</sup>
matched that of <em>Thiophaeococcus mangrovi</em> JCM 14889<sup>T</sup>,
which contained diphasphatidylglycerol (DPG), phospha-
tidylglycerol (PG), phosphatidylethanolamine (PE), phos-
phatidylcholine (PC) and an unknown aminophospholipid
(APL) as major lipids with minor amounts of unknown
phospholipid PL1. However, strain JA633<sup>T</sup> differed based on
the presence of unknown phospholipids PL2 and PL3
(Fig. S3). The absence of cardiolipin and glycolipids and
the presence of diphasphatidylglycerol and phosphatidyl-
choline differentiated strain JA633<sup>T</sup> (and <em>Thiophaeococcus
mangrovi</em> JCM 14889<sup>T</sup>) from its nearest phylogenetic
relatives in the genera <em>Chromatiun</em>, <em>Thiocapsa</em> and
<em>Thiocystis</em> (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 as the predominant quinone system in strain
JA633<sup>T</sup> and <em>Thiophaeococcus mangrovi</em> JCM 14889<sup>T</sup>.

In addition to showing 16S rRNA gene sequence divergence,
strain JA633<sup>T</sup> also showed distinct phenotypic (growth
factor requirements, organic substrate utilization, no
photolithoautotrophic growth; Table 1) and chemotaxo-
nomic properties (polar lipids, Fig. S3) as compared with
<em>Thiophaeococcus mangrovi</em> JCM 14889<sup>T</sup>. Therefore, strain
JA633<sup>T</sup> is considered to represent a novel species of the
genus <em>Thiophaeococcus</em>, for which the name <em>Thiophaeococcus
fusus</em> sp. nov. is proposed.

**Description of Thiophaeococcus fusus sp. nov.**

<i>Thiophaeococcus fusus</i> (fusus L. masc. adj. fusus tawny).

Cells are coccoid–spherical, 2.5–3 μm in diameter, Gram-

dian-negative, motile by means of peritrichous flagella and

multiply by binary fission. Photo-organoheterotrophic

growth occurs and internal photosynthetic membranes are

of the vesicular type. Photolithoautotrophy, chemolithoau-
totrophy, chemo-organoheterotrophy and fermentative

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growth are absent. Phototrophically grown cultures are brown. The in vivo absorption spectrum of intact cells in sucrose exhibits maxima at 374, 458, 488, 524, 593, 800 and 860 nm. Bacteriochlorophyll \( a \) and an unidentified major (>85%) carotenoid of the spirilloxanthin series are present. Optimum pH and temperature for growth are pH 7.0 (range pH 6.5–7.5) and 30 °C (range 25–35 °C), NaCl is required for growth (1–4%, w/v), with optimum growth at 2%. Acetate, butyrate, pyruvate, fructose, fumarate, malate, valerate, propionate and crotonate are good carbon sources/electron donors for growth. Sulfate, sulfite, sulfide, elemental sulfur and thiosulfate are used as sulfur sources and ammonium chloride, glutamate and aspartate are used as nitrogen sources. Thiamine and \( p \)-aminobenzoic acid are required for growth. Major fatty acids are \( \text{C}_{18:1} \omega 7c/\text{C}_{18:3} \omega 6c, \text{C}_{16:0} \) and \( \text{C}_{16:1} \omega 7c/\text{C}_{16:1} \omega 6c \) with minor amounts of \( \text{C}_{12:0} \omega 0, \text{C}_{14:0} \) and \( \text{C}_{18:0} \). Diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unknown aminophospholipid are the major polar lipids with minor amounts of unknown phospholipids (PL1–3). Q8 is the predominant quinone system.

The type strain is JA633\(^T\) (=KCTC 15337\(^T\)=NBRC 109958\(^T\)), isolated from phototrophic enrichment of a black sand sample collected from Muthupet lagoon, Tamil Nadu, India. The DNA G+C content of the type strain is 64.5 mol% (by HPLC).

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