**Elioraea tepidiphila** gen. nov., sp. nov., a slightly thermophilic member of the *Alphaproteobacteria*

Luciana Albuquerque,¹ Fred A. Rainey,² M. Fernanda Nobre¹ and Milton S. da Costa³

¹Department of Zoology and Center for Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal  
²Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA  
³Department of Biochemistry, University of Coimbra, 3001-401 Coimbra, Portugal

A bacterial isolate, strain TU-7T, with an optimum growth temperature of about 45–50 °C and an optimum pH for growth between 8.0 and 8.5, was recovered from a hot spring in the Furnas area of the island of São Miguel in the Azores. The new isolate is non-pigmented, facultatively chemolithoorganotrophic, strictly aerobic and catalase- and oxidase-positive. The organism oxidized thiosulfate to sulfate with enhancement of growth. This organism assimilated organic acids but did not assimilate carbohydrates or polyols. 16S rRNA gene sequence analysis placed strain TU-7T within the radiation of the *Alphaproteobacteria* as a deep branch of the family *Acetobacteriaceae*. The major fatty acids of strain TU-7T are 18:1ω7c, 18:0, 19:0 cyclo ω8c and an aliphatic chain with an equivalent chain-length of 20.195, identified as 19:0 2-OH cyclo ω8 (Δ11:12). Ubiquinone 10 is the major respiratory quinone and the major polar lipids are phosphatidylcholine, phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol in addition to two unidentified aminolipids. Bacteriochlorophyll *a* and *puf* genes were not detected. Based on 16S rRNA gene sequence analysis and physiological and biochemical characteristics, we describe a novel species of a new genus represented by strain TU-7T, for which we propose the name *Elioraea tepidiphila* gen. nov., sp. nov. The type strain of *Elioraea tepidiphila* is strain TU-7T (=DSM 17972T =CIP 109115T).

The *Alphaproteobacteria* comprises many species with diverse characteristics; some organisms are strictly organotrophic or facultative chemolithoorganotrophic, while others are chemolithoautotrophic. A large number of organisms possess bacteriochlorophyll (*BChl*) *a* and some have been shown to be photoautotrophic. However, only a few species isolated from hot springs, such as *Rubritepida flocculans* (Alarico et al., 2002), *Porphyrobacter tepidarius* (Hanada et al., 1997), *Porphyrobacter cryptus* (Rainey et al., 2003) and *Albidovulum inexpectatum* (Albuquerque et al., 2002), have optimum growth temperatures of around 50 °C, but do not grow at 55 °C. With the exception of *Albidovulum inexpectatum*, which is closely related to the species of the genus *Rhodovulum* and appears to have lost phototrophic metabolism, all other slightly thermophilic alphaproteobacteria reported to date are red-pigmented and contain *BChl* *a*.

We recently isolated a slightly thermophilic, non-pigmented organism, designated TU-7T, from a hot spring on the island of São Miguel in the Azores, which, on the basis of phenotypic characteristics and phylogenetic position, we believe to represent a novel species of a new genus. Strain TU-7T was isolated from a hot spring in the Furnas area of S. Miguel. Water samples were maintained without temperature control for 6 days and then filtered through membrane filters (Gelman type GN-6; pore size 0.45 μm, diameter 47 mm). The filters were placed on the surface of solidified *Thermus* medium (Williams & da Costa, 1992), wrapped in plastic bags and incubated at 50 °C for up to 4 days. Cultures were purified by subculturing and the isolates were stored at −70 °C in *Thermus* medium with 15% (w/v) glycerol. Culture in R3A medium (Reaoner & Geldreich, 1985) was later adopted because it resulted in higher growth yields. *Rubritepida flocculans* H-8T, *Rhodovulum sulfidophilum* DSM 1374T and *Albidovulum inexpectatum* FRR-10T were used for comparative purposes.

Cell morphology and motility were examined by phase-contrast microscopy during the exponential growth phase. Flagella were visualized by light microscopy after staining....

Abbreviation: BChl, bacteriochlorophyll.
of the cells with the Ryu stain (Heimbrook et al., 1989). Unless otherwise stated, all biochemical and tolerance tests were performed, as described previously (Pires et al., 2005), in R3A liquid medium without starch and glucose or on R3A agar without starch and glucose at 50 °C for up to 6 days. The growth temperature range of the strains was examined at 25, 30, 35, 45, 50 and 55 °C by measuring the turbidity (610 nm) of cultures incubated in 300 ml metal-capped Erlenmeyer flasks, containing 100 ml medium in a rotary water-bath shaker at 150 r.p.m. (Albuquerque et al., 2005). The pH range for growth was examined at 50 °C in the same medium by using 50 mM MES, HEPES, TAPS or CAPSO over a pH range from 5.5 to 10.5 in a rotary water-bath shaker (Albuquerque et al., 2005). Catalase, oxidase and DNase activities were examined as described previously (Pires et al., 2005). Additional enzyme activities were assessed using the API ZYM system (bioMérieux) at 50 °C. Anaerobic growth was assessed in cultures in R3A medium without starch and glucose, containing KNO3 (1.0 g l−1) incubated in anaerobic chambers (GENbox anaer; bioMérieux). Single-carbon source assimilation tests were performed in a minimal medium composed of Degryse basal salts (Degryse et al., 1978) to which filter-sterilized ammonium sulfate (0.5 g l−1) was added. Cultures containing 0.1 g yeast extract l−1 without H2 or any of the reduced sulfur compounds were used as controls.

Autotrophic growth of the organism on H2, sodium thiosulfate (1.0 g l−1), sodium tetrathionate (1.0 g l−1), sodium sulfite (1.0 g l−1) and sodium sulfide (0.5 g l−1) was investigated in DSMZ medium 27 lacking ethanol, vitamin B12, sodium sulfide or organic substrates, with 0.1 g yeast extract l−1 (http://www.dsmz.de/media/med027.htm) in the dark under aerobic conditions; cultures to assess autotrophic growth on H2 were placed in sealed chambers to which this gas was added. Cultures containing 0.1 g yeast extract l−1 without H2 or any of the reduced sulfur compounds were used as controls.

Phototrophic growth was assessed in DSMZ medium 27 (lacking ethanol and vitamin B12) supplemented with neutralized sodium sulfide solution (0.1 g l−1) or with sodium thiosulfate (0.1 g l−1) in completely filled screw-capped bottles under incandescent illumination (1000 lx) along with Rhodobacter capsulatus DSM 1374T as a control.

Chemolithoorganotrophic growth on sodium thiosulfate was assessed under aerobic conditions at the optimum growth temperature in DSMZ medium 27 lacking sulfate and containing yeast extract (0.3 g l−1), sodium succinate (1.0 g l−1) and sodium acetate (0.5 g l−1). Filter-sterilized sodium thiosulfate was added to the medium at concentrations that varied between 0.5 and 5.0 g l−1. At appropriate intervals, the turbidity of the cultures was determined and the cells were harvested and centrifuged. Levels of thiosulfate and sulfate in the supernatants were determined using the methods described by Westley (1987) and Sörbo (1987), respectively.

Cultures for polar lipid analysis were grown in 1 l Erlenmeyer flasks containing 250 ml medium at 50 °C in a rotary water-bath shaker until the late exponential phase. Harvesting of cultures, extraction of lipids and one-dimensional TLC were performed as described previously (Moreira et al., 2000). Lipoquinones were extracted, purified by TLC and separated by HPLC (Tindall, 1989). Cultures for fatty acid analysis were grown on solidified R3A medium without starch and glucose, in sealed plastic bags submerged in a water bath at 50 °C for 48 h. Fatty acid methyl esters (FAMEs) were obtained from fresh wet biomass and separated, identified and quantified with the standard MIS Library Generation Software (Microbial ID, Inc.) as described previously (Albuquerque et al., 2005).

Cultures for carotenoid analysis were harvested during the exponential phase of growth and washed twice by centrifugation with MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl2, 0.001 M; pH 7.5). Carotenoids were extracted with chloroform/methanol (1:2, v/v) in the dark to avoid photo-oxidation of the pigments (Liaaen-Jensen & Jensen, 1971). One-dimensional TLC was performed on silica gel G plates (Merck no. 5626) using a solvent system composed of chloroform/methanol (95:12.5, v/v). Bacteriochlorophylls were extracted with acetone/methanol (7:2, v/v) and the absorption spectrum of the extract was examined on a UVikon spectrophotometer 942.

The isolation of DNA from strain TU-7T and Rut. floculans H-8T was performed as described by Marmur (1961). PCR amplification of puf genes was performed with a pair of degenerate primers (Alarico et al., 2002), based on conserved regions from homologous genes in related organisms and used to amplify a continuous nucleotide stretch corresponding to a 1.5 kb fragment that included pufL and pufM. A PCR product was visualized by agarose gel electrophoresis, purified from agarose with a Wizard SV Gel DNA kit (Promega) and ligated in the pGEM T-Easy vector (Promega). Transformation of Escherichia coli XL1-Blue was carried out with standard procedures described in Sambrook et al. (1989). After confirmation of positive clones, cells were grown overnight in ampicillin-containing (50 μg ml−1) LB medium and plasmids were isolated (High Pure plasmid isolation kit; Roche). The inserts were sequenced by Agowa GmbH (Berlin, Germany).

DNA for determination of the G+C content was isolated as described previously (Nielsen et al., 1995). The G+C content of DNA was determined by HPLC as described by Mesbah et al. (1989).

Extraction of genomic DNA for 16S rRNA gene sequence determination, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried...
Strain TU-7\textsuperscript{T} was isolated from a hot spring with a temperature of 70 °C and pH of 7.5. Only strain TU-7\textsuperscript{T} was recovered from one plate containing 21 colonies. The other organisms isolated from the same sample included strains of Truepera radiovictrix, Meiothermus timidus and Tepidicella xavieri (Albuquerque et al., 2005; Pires et al., 2005; França et al., 2006). Phylogenetic analysis of a nearly complete 16S rRNA gene sequence showed strain TU-7\textsuperscript{T} to represent a distinct lineage within the Alphaproteobacteria (Fig. 1). Strain TU-7\textsuperscript{T} clustered with an environmental clone sequence designated CCSD_DF730_B11, with which it shared 99.7 % pairwise sequence similarity (Fig. 1). 16S rRNA gene sequence similarity values between strain TU-7\textsuperscript{T} and cultured representatives of the closest relatives were in the range 88.3–91.8 %. Phylogenetic analysis using the neighbour-joining, maximum-likelihood and parsimony methods showed the position of strain TU-7\textsuperscript{T} at the root of the family Acetobacteriaceae supported by 100 % bootstrap values. Based on 16S rRNA gene sequence similarity, the closest named relative of strain TU-7\textsuperscript{T} is Acidisphaera rubrifaciens. This relationship is reflected in the short branch of this species, but indicates no close relationship between strain TU-7\textsuperscript{T} and Acidisphaera rubrifaciens. Based on its deep branching position, the novel organism could be considered to represent a new family, but it would be better to obtain additional isolates before proposing a new family for this lineage. The G+C content of the DNA of strain TU-7\textsuperscript{T} was 70.9 ± 0.6 mol%.

Strain TU-7\textsuperscript{T} formed very short, Gram-negative, rod-shaped cells (0.5–1.0 μm wide and 1.0–1.5 μm long) that were motile by means of single polar flagella. Colonies were translucent and non-pigmented on R3A agar without starch and glucose. The organisms had an optimum growth temperature between 45 and 50 °C and did not grow at 25 or 55 °C. The optimum pH for growth of strain TU-7\textsuperscript{T} was pH 8.0–8.5, and no growth was detected at pH 5.5 or 10.0. The organism was oxidase- and catalase-positive and hydrolysed hippurate, starch and xylan weakly. Aesculin, arbutin, gelatin, casein and elastin were not hydrolysed. Strain TU-7\textsuperscript{T} used organic acids as single carbon and energy sources, but was unable to utilize any of the carbohydrates or polyols examined. Weak growth was observed with proline and glutamine. Yeast extract was not necessary for growth in minimal medium. Nitrate was reduced to nitrite but anaerobic growth with nitrate as an electron acceptor was not observed. The organism produced acid from D-ribose, D-fructose, L-sorbose, D-tagatose and potassium 5-ketogluconate.

![Fig. 1. 16S rRNA gene sequence-based phylogenetic dendrogram showing the relationships of strain TU-7\textsuperscript{T} and related taxa. The dendrogram was constructed from distance matrices using the neighbour-joining method. Numbers at branching points represent bootstrap percentages from 1000 data samplings. Bar, 2 inferred substitutions per 100 nucleotides.](http://ijs.sgmjournals.org)
The addition of thiosulfate to DSMZ medium 27 containing yeast extract, succinate and acetate led to an increase in the biomass of strain TU-7T, indicating that thiosulfate was used as an energy source in the presence of organic substrates (results not shown). Determination of the levels of sulfate and thiosulfate in the medium during growth indicated that thiosulfate was oxidized completely to sulfate (results not shown). Photoautotrophic growth and chemolithotrophic growth with H₂, thiosulfate, tetrathionate, sulfite or sulfide were not observed.

In contrast to the type strain of *Rhodothermus sulfidophilum*, we did not detect the presence of BCHl *a* in strain TU-7T under aerobic or anaerobic growth conditions. Carotenoid pigments were not detected in extracts under any experimental conditions. The presence of *pufL* and *pufM* genes was not detected by PCR amplification in strain TU-7T.

The polar lipid pattern on TLC revealed the presence of phosphatidylcholine, phosphatidylethanolamine, diposphatidylglycerol and phosphatidylglycerol in addition to two unidentified aminolipids (Supplementary Fig. S1, available in IJSEM Online). The major respiratory lipoquinone was ubiquinone 10. The fatty acid composition of strain TU-7T was dominated by 18∶1ω7c, 18∶0, 19∶0 cyclo ω8c and a rare fatty acid with an equivalent chain-length (ECL) of 20.195, tentatively identified as 19∶0 2-OH cyclo ω8 (A11∶12) (Supplementary Table S1).

Unexpectedly, strain TU-7T was not able to assimilate any of the carbohydrates examined, even though acid was produced from a few sugars in the API 50 CH system. The polar lipids and respiratory quinone were as one would expect for members of the *Alphaproteobacteria*. High levels of a rare fatty acid with an ECL of 20.195, identified as 19∶0 2-OH cyclo ω8 (A11∶12) (Ralf Paisley, Microbial ID, Inc., personal communication), were found in this organism. This fatty acid, along with the less rare 19∶0 cyclo ω8c, have been found previously in the slightly thermophilic alphaproteobacterium *Rut. flocculans*, but not in the phylogenetically closely related organisms *Roseococcus thiosulfatophilus*, *Craurococcus roseus* and *Paracraurococcus ruber* (Alarico et al., 2002) (Table 1).

The fact that the 16S rRNA gene sequence of strain TU-7T shows high similarity (99.7 %) to an environmental clone sequence designated CCSD_DF730_B11, which was recovered from drilling fluid (Zhang et al., 2005), is interesting from an ecological and diversity standpoint. The environmental clone sequence was recovered from drilling fluid at 730 m, where the *in situ* temperature was 43 °C, which is close to the optimum growth temperature of strain TU-7T. With such high 16S rRNA gene sequence similarity, it could be considered that clone CCSD_DF730_B11 represents a strain or species of the novel genus to which strain TU-7T is assigned. Based on the phylogenetic analysis and the phenotypic characteristics, which are distinct from those of members of the family *Actinobacteria* (Table 1), we are of the opinion that the organism designated TU-7T represents a new genus and species of the *Alphaproteobacteria*.

**Description of Elioraea gen. nov.**


Form motile, rod-shaped cells that stain Gram-negative. Endospores are not formed. Facultatively chemolithoorganotrophic, strictly aerobic, oxidase- and catalase-positive. BCHl *a* is not present. Major fatty acids are straight-chain saturated and unsaturated fatty acids including hydroxy derivatives; major phospholipids are phosphatidylcholine, phosphatidylethanolamine, diposphatidylglycerol and phosphatidylglycerol; ubiquinone 10 is the major respiratory quinone. Thiosulfate is oxidized to sulfate. Organic compounds serve as carbon and energy sources. The genus *Elioraea* belongs to the *Alphaproteobacteria*. The type species is *Elioraea tepidiphila*.

**Description of Elioraea tepidiphila sp. nov.**

*Elioraea tepidiphila* (te.pi.di.phi/la. L. adj. tepidus warm; Gr. adj. philos loving; N.L. fem. adj. tepidiphila loving warmth).

Displays the following properties in addition to those described for the genus. Cells are 1.0–1.5 μm long and 0.5–1.0 μm wide. Colonies on R3A medium without starch and glucose are not pigmented. Slightly thermophilic; the optimum growth temperature is about 45–50 °C and growth does not occur at 25 or 55 °C. The optimum pH is between 8.0 and 8.5; growth does not occur at pH 5.5 or 10.0. Grows in media with up to 1.5 % NaCl. Yeast extract is required for growth. Nitrate is reduced to nitrite. Major fatty acids are 18∶0 (24.8 %), 18∶1ω7c (19.0 %), 19∶0 2-OH cyclo ω8 (A11∶12) (18.6 %), 19∶0 cyclo ω8c (12.4 %), 18∶1ω7c 11-methyl (8.0 %), 16∶0 (5.4 %) and 18∶0 3-OH (3.8 %). Starch, xylan and hippurate are degraded; aesculin, arbutin, gelatin, casein and elastin are not degraded. DNase-positive. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase test positive in API ZYM; other activities test negative. Succinate, α-ketoglutarate, lactate, malate, pyruvate, glutamate, fumarate, glutonate, citrate, proline and glucose are assimilated; glucose, mannose, galactose, fructose, sorbose, D- and L-arabinose, ribose, xylose, sucrose, maltose, lactose, trehalose, cellobiose, melibiose, melezitose, L-rhamnose, raffinose, L-fucose, ribitol, xylitol, sorbitol, erythritol, mannitol, myo-inositol, glycerol, acetate, aspartate, formate, glucuronate, alanine, asparagine, phenylalanine, glycine, histidine, isoleucine, leucine, lysine, methionine, arginine, serine, threonine, valine, ornithine and cysteine are not assimilated. Acid is produced from the following carbohydrates using API 50CH: ribose, fructose, L-sorbose, tagatose and potassium 5-ketogluconate. The G+C content of the DNA is 70.9 ± 0.6 mol%.

The type strain, TU-7T (=DSM 17972T =CIP 109115T), was isolated from a hot spring at Furnas on the island of São Miguel in the Azores.
Table 1. Characteristics that distinguish strain TU-7T and the genera Acetobacter, Acidocella, Craurococcus, Roseococcus and Rubritepida


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain TU-7T</th>
<th>Acetobacter</th>
<th>Acidocella</th>
<th>Craurococcus</th>
<th>Roseococcus</th>
<th>Rubritepida</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Cocci</td>
<td>Cocci</td>
<td>Rods</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.5–1.0 × 1.5</td>
<td>0.6–0.9 × 1.0–4.0</td>
<td>0.5–0.8 × 1.0–2.0</td>
<td>0.8–2.0</td>
<td>0.9–1.3 × 1.3–1.6</td>
<td>0.7–1.2 × 1.4–1.7</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Not pigmented</td>
<td>Not pigmented, brown or pink</td>
<td>White, cream or light brown</td>
<td>+ or −</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+ or −</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth temperature (°C)</td>
<td>30–50</td>
<td>≤ 42</td>
<td>ND</td>
<td>18–37</td>
<td>ND</td>
<td>≤ 50</td>
</tr>
<tr>
<td>Optimum</td>
<td>45–50</td>
<td>28–30</td>
<td>Mesophilic</td>
<td>28–32</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>pH for growth</td>
<td>6.0–9.8</td>
<td>3.0–9.0</td>
<td>2.5–6.0</td>
<td>7.2–8.0</td>
<td>ND</td>
<td>6.5–9.5</td>
</tr>
<tr>
<td>Presence of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BChl a</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>−</td>
<td>− or (+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+ or +</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Major quinone</td>
<td>Q-10</td>
<td>Q-9</td>
<td>Q-10</td>
<td>Q-10</td>
<td>Q-9</td>
<td>Q-9</td>
</tr>
<tr>
<td>Presence of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19:0 cyclo ω8c</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>19:0 2-OH cyclo ω8</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>(A11 : 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>70.9</td>
<td>50.5–60.3</td>
<td>58–65</td>
<td>70.5</td>
<td>70.4</td>
<td>70.2</td>
</tr>
</tbody>
</table>

*Except Acetobacter peroxidan.

Acknowledgements

This work was supported by the Fundação para a Ciência e a Tecnologia (FCT), Portugal, and FEDER, project A005/2005, action V.5.1, and by the Governor’s Biotechnology Initiative of the Louisiana Board of Regents (BOR#021 – Moving an Established Marine Biotechnology Program to the Next Level: Natural Product Screening and Development). We also wish to thank Jean Euzéby (Toulouse, France) for the etymology of the name.

References


