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

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A bacterial isolate, strain TU-7^T, 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-7^T within the radiation of the *Alphaproteobacteria* as a deep branch of the family *Acetobacteriaceae*. The major fatty acids of strain TU-7^T are 18:1 ω 7c, 18:0, 19:0 cyclo ω 8c and an aliphatic chain with an equivalent chain-length of 20.195, identified as 19:0 2-OH cyclo ω 8 (Δ 11:12). Ubiquinone 10 is the major respiratory quinone and the major polar lipids are phosphatidylcholine, phosphatidylethanolamine, diphosphosphatidylglycerol 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-7^T, for which we propose the name *Elioraea tepidiphila* gen. nov., sp. nov. The type strain of *Elioraea tepidiphila* is strain TU-7^T (=DSM 17972^T =CIP 109115^T).

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*.

Abbreviation: BChl, bacteriochlorophyll.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TU-7^T is EF519867.

Results of one-dimensional TLC of polar lipids and analysis of the fatty acid composition of strain TU-7^T are available as supplementary material with the online version of this paper.

We recently isolated a slightly thermophilic, non-pigmented organism, designated TU-7^T, 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-7^T 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 μ m, 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 (Reasoner & Geldreich, 1985) was later adopted because it resulted in higher growth yields. *Rubritepida flocculans* H-8^T, *Rhodovulum sulfidophilum* DSM 1374^T and *Albidovulum inexpectatum* FRR-10^T 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

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), yeast extract (0.1 g l⁻¹) and the carbon source (2.0 g l⁻¹) were added. Growth of strains on single carbon sources was examined by measuring the turbidity of cultures incubated at 50 °C in 20 ml screw-capped tubes containing 10 ml medium for up to 6 days. Acid production from carbohydrates was determined by using the API 50 CH system (bioMérieux) according to the manufacturer's instructions using API 50 CHB/E medium. Results were recorded after 24 and 48 h and 5 days of incubation at 50 °C.

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 *Rdv. sulfidophilum* DSM 1374^T 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-7^T and *Rut. flocculans* H-8^T 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

out as described previously (Rainey *et al.*, 1996). Purified reactions were electrophoresed using a model 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequence was aligned against representative reference sequences of members of the *Proteobacteria* using the ae2 editor (Cole *et al.*, 2003). The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Phylogenetic dendrograms and bootstrap analyses were generated using various algorithms contained in the PHYLIP package (Felsenstein, 1993).

Strain TU-7^T was isolated from a hot spring with a temperature of 70 °C and pH of 7.5. Only strain TU-7^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^T to represent a distinct lineage within the *Alphaproteobacteria* (Fig. 1). Strain TU-7^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^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^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^T is *Acidisphaera rubrifaciens*. This relationship is reflected in the short branch of this species, but indicates no close relationship between strain TU-7^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^T was 70.9 ± 0.6 mol%.

Strain TU-7^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^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^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 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^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.

The addition of thiosulfate to DSMZ medium 27 containing yeast extract, succinate and acetate led to an increase in the biomass of strain TU-7^T, 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 *Rdv. sulfidophilum*, we did not detect the presence of BChl *a* in strain TU-7^T 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-7^T.

The polar lipid pattern on TLC revealed the presence of phosphatidylcholine, phosphatidylethanolamine, diphosphosphatidylglycerol 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-7^T was dominated by 18:1 ω 7c, 18:0, 19:0 cyclo ω 8c and a rare fatty acid with an equivalent chain-length (ECL) of 20.195, tentatively identified as 19:0 2-OH cyclo ω 8 (Δ 11:12) (Supplementary Table S1).

Unexpectedly, strain TU-7^T 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 ω 8 (Δ 11:12) (Ralf Paisley, Microbial ID, Inc., personal communication), were found in this organism. This fatty acid, along with the less rare 19:0 cyclo ω 8c, 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-7^T 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-7^T. 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-7^T is assigned. Based on the phylogenetic analysis and the phenotypic characteristics, which are distinct from those of members of the family *Acetobacteriaceae* (Table 1), we are of the opinion that the organism designated TU-7^T represents a new genus and species of the *Alphaproteobacteria*.

Description of *Elioraea* gen. nov.

Elioraea (E.li.o'ra.e.a. N.L. fem. n. *Elioraea* named in honour of Israeli microbiologist Eliora Z. Ron).

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, diphosphosphatidylglycerol 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 μ m long and 0.5–1.0 μ m 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%), 18:1 ω 7c (19.0%), 19:0 2-OH cyclo ω 8 (Δ 11:12) (18.6%), 19:0 cyclo ω 8c (12.4%), 18:1 ω 7c 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, gluconate, citrate, proline and glutamine 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 $\pm$ 0.6 mol%.

The type strain, TU-7^T (=DSM 17972^T =CIP 109115^T), was isolated from a hot spring at Furnas on the island of São Miguel in the Azores.

Table 1. Characteristics that distinguish strain TU-7^T and the genera *Acetobacter*, *Acidocella*, *Craurococcus*, *Roseococcus* and *Rubritepida*

Data for related genera were taken from Sievers & Swings (2005) (*Acetobacter*), Hiraishi (2005) (*Acidocella*), Rathgeber & Yurkov (2005) (*Craurococcus*), Alarico *et al.* (2002) (*Craurococcus*, *Roseococcus* and *Rubritepida*) and Yurkov (2005) (*Roseococcus*). –, Negative; +, positive; (+), weakly positive; ND, no data available.

Characteristic	Strain TU-7 ^T	<i>Acetobacter</i>	<i>Acidocella</i>	<i>Craurococcus</i>	<i>Roseococcus</i>	<i>Rubritepida</i>
Cell morphology	Rods	Rods	Rods	Cocci	Cocci	Rods
Cell size (µm)	0.5–1.0 × 1.0–1.5	0.6–0.9 × 1.0–4.0	0.5–0.8 × 1.0–2.0	0.8–2.0	0.9–1.3 × 1.3–1.6	0.7–1.2 × 1.4–1.7
Pigmentation	Not pigmented	Not pigmented, brown or pink	White, cream or light brown	Pink	Pink	Red
Motility	+	+ or –	+ or –	–	+	+
Growth temperature (°C)						
Range	30–50	≤ 42	ND	18–37	ND	≤ 50
Optimum	45–50	28–30	Mesophilic	28–32	30	50
pH for growth						
Range	6.0–9.8	3.0–9.0	2.5–6.0	7.2–8.0	ND	6.5–9.5
Optimum	8.0–8.5	4.0–6.0	Acidophilic	7.5	ND	7.0–8.0
Presence of:						
BChl <i>a</i>	–	–	–	+	+	+
Oxidase	+	–	– or (+)	+	+	+
Catalase	+	+	+	+	+	+
Major quinone	Q-10	Q-9	Q-10	Q-10	Q-9	Q-9
Presence of:						
19:0 cyclo ω8c	+	ND	ND	–	–	+
19:0 2-OH cyclo ω8 (Δ11:12)	+	ND	ND	–	–	+
DNA G + C content (mol%)	70.9	50.5–60.3	58–65	70.5	70.4	70.2

*Except *Acetobacter peroxydans*.

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