Schleiferia thermophila gen. nov., sp. nov., a slightly thermophilic bacterium of the phylum 'Bacteroidetes' and the proposal of *Schleiferiaceae* fam. nov.

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A bacterial isolate, with an optimum growth temperature of about 50 °C and an optimum pH for growth between 7.5 and 8.5, was recovered from a hot spring in the Furnas area of the Island of São Miguel in the Azores. The novel isolate is orange-pigmented, forms non-motile, rod-shaped cells that stain Gram-negative, is strictly aerobic, oxidase-positive and catalase-negative. The major fatty acids of strain TU-20^T are 16 : 1 ω 6c, 17 : 0 iso 3-OH and 15 : 0 iso. Menaquinone 6 is the major respiratory quinone. Based on 16S rRNA gene sequence analysis, physiological and biochemical characteristics, we describe a novel species of a novel genus represented by strain TU-20^T (=DSM 21410^T =LMG 24594^T) for which we propose the name *Schleiferia thermophila* gen. nov., sp. nov. We also propose the family *Schleiferiaceae* fam. nov. to accommodate this new genus.

Most species of the phylum 'Bacteroidetes' have optimum growth temperatures that range from about 25 to 45 °C. Several organisms are psychrophilic with very low growth temperatures; these include *Psychroserpens burtonensis*, and members of the genera *Gelidibacter* and *Maribacter* (Bowman *et al.*, 1997; Nedashkovskaya *et al.*, 2004). However, within this phylum, thermophilic or slightly thermophilic species are very rare. Some of these organisms have slightly elevated growth temperatures of around 40–45 °C, such as *Pseudozobellia thermophila* (Nedashkovskaya *et al.*, 2009) and *Lutaonella thermophila* (Arun *et al.*, 2009), or are slightly thermophilic, namely *Anaerophaga thermohalophila* (Denger *et al.*, 2002), with an optimum growth temperature around 50 °C but unable to grow at 60 °C. The species of two other genera of the phylum 'Bacteroidetes' are thermophilic; these are *Thermonema lapsus* (Hudson *et al.*, 1989) and *Thermonema rossianum* (Tenreiro *et al.*, 1997), with optimum growth temperatures of about 60 °C and maximum growth temperatures around 65 °C, and the two species of the genus *Rhodothermus*, namely *Rhodothermus marinus* and the recently described *Rhodothermus profundus* (Alfredsson *et al.*, 1988; Nunes *et al.*, 1995; Silva *et al.*, 2000;

Marteinsson *et al.*, 2010). The two species of the genus *Rhodothermus* have optimum growth temperatures of over 65 °C and maximum growth temperatures just below 80 °C. The species of the genus *Rhodothermus* have the deepest branches of the phylum 'Bacteroidetes'.

We recently isolated several slightly thermophilic organisms from the Furnas area, such as *Eliaorea tepidiphila* (Albuquerque *et al.*, 2008), *Tepidicella xavieri* (França *et al.*, 2006), *Truepera radiovictrix* (Albuquerque *et al.*, 2005), *Meiothermus timidus* (Pires *et al.*, 2005) and one undescribed slightly thermophilic organism with an optimum growth temperature around 50–55 °C and a maximum growth temperature of 60 °C. Phylogenetic analysis of the 16S rRNA gene sequence showed that this organism represents a distinct lineage within the phylum 'Bacteroidetes'. Based on phylogenetic, physiological and biochemical parameters, we are of the opinion that strain TU-20^T represents a novel genus and species for which we propose the name *Schleiferia thermophila* gen. nov., sp. nov. We are also of the opinion that this organism represents a new family for which we propose the name *Schleiferiaceae* fam. nov.

Strain TU-20^T was isolated from a hot spring in the Furnas area of the Island of São Miguel in the Azores. Water samples were maintained without temperature control for 6 days, and then 10 to 100 ml were filtered through

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TU-20^T is HQ172900.

Two supplementary figures are available with the online version of this paper.

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), the plates were wrapped in plastic to prevent evaporation and incubated at 50 °C for up to 4 days. *Thermus* medium contained the following components per litre of media: yeast extract (Difco), 1.0 g; tryptone (Difco), 1.0 g; agar (Difco), 20.0 g; 100 ml 10 × concentrated macronutrients solution; 10 ml 100 × concentrated trace elements solution; and 10 ml 0.17 mM FeCl₃·6H₂O. The 10 × concentrated macronutrients solution contained per litre: nitrilotriacetic acid, 1.0 g; CaSO₄·2H₂O, 0.6 g; MgSO₄·7H₂O, 1.0 g; NaCl, 0.08 g; KNO₃, 1.03 g; NaNO₃, 6.89 g; and NaHPO₄, 1.11 g. The 100 × concentrated trace elements solution contained per litre: MnSO₄·H₂O, 0.22 g; ZnSO₄·7H₂O, 0.05 g; H₃BO₃, 0.05 g; CuSO₄·5H₂O, 0.0025 g; Na₂MoO₄·2H₂O, 0.0025 g; and CoCl₂·6H₂O, 0.0046 g. Cultures were purified by subculturing and the isolates stored at -70 °C in *Thermus* medium with 15 % (w/v) glycerol. Culture in Degryse medium 162 (Degryse *et al.*, 1978), containing 0.25 % yeast extract and 0.25 % tryptone, was later adopted because this medium resulted in higher growth yields. Degryse medium contained the following components per litre of media: yeast extract (Difco), 2.5 g; tryptone (Difco), 2.5 g; agar (Difco), 20.0 g; 100 ml 10 × concentrated macronutrients solution; 5 ml 100 × concentrated trace elements solution; 0.5 ml 0.01 M C₆H₅O₇Fe·5H₂O, 10 ml 0.2 M KH₂PO₄ and 15 ml 0.2 M Na₂HPO₄·12H₂O. The 10 × concentrated macronutrients solution contained per litre: nitrilotriacetic acid, 1.0 g; CaSO₄·2H₂O, 0.4 g; and MgSO₄·7H₂O, 2.0 g. The 100 × concentrated trace elements solution contained per litre: MnSO₄·H₂O, 0.22 g; ZnSO₄·7H₂O, 0.05 g; H₃BO₃, 0.05 g; CuSO₄·5H₂O, 0.0025 g; Na₂MoO₄·2H₂O, 0.0025 g; CoCl₂·6H₂O, 0.0046 g.

The type strain of *Fluviicola taffensis* (DSM 16823^T) was used for comparative purposes.

Unless otherwise stated, all biochemical and tolerance tests were performed as described by Smibert & Krieg (1981) and Albuquerque *et al.* (2010) in Degryse medium 162 or Degryse agar 162 (Degryse *et al.*, 1978) at 50 °C for up to 6 days. Cell morphology and motility were examined by phase-contrast microscopy during the exponential growth phase. Gliding motility was examined as described by Bernardet *et al.* (2002). The growth temperature range of the strains was examined in 5 °C increments between 25 and 65 °C by measuring the turbidity (610 nm) of cultures incubated in 300 ml metal-capped Erlenmeyer flasks containing 100 ml of medium in a rotary water-bath shaker at 150 r.p.m. The pH range for growth was examined in liquid medium by using 50 mM MES, HEPES, TAPS or CAPSO over a pH range from 6.0 to 10.0 in a rotary water-bath shaker. The salt tolerance of the organisms was determined in liquid medium, with NaCl ranging between 0.0 and 4.0 %. Catalase, oxidase and DNase activities were examined as described by Smibert & Krieg (1981) and Albuquerque *et al.* (2010). Additional enzymic activities were obtained using the API ZYM

system (bioMérieux) at 50 °C. Anaerobic growth was assessed in cultures in the same medium, containing KNO₃ (1.0 g l⁻¹), incubated in anaerobic chambers (GENbox anaer; bioMérieux). Single-carbon-source assimilation tests were performed in a medium composed of Degryse basal salts (Degryse *et al.*, 1978) to which filter-sterilized ammonium sulfate (0.5 g l⁻¹), yeast extract (0.2 g l⁻¹), vitamin B12 (40 µg l⁻¹) and the carbon source (2.0 g l⁻¹) were added. Growth of the 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 48 h and 6 days of incubation at 50 °C.

Metabolic products were determined by growing the cultures in Degryse liquid medium overnight. The cultures were washed by centrifugation and incubated at 50 °C in 50 mM phosphate buffer, pH 7.5, containing glucose (11.1 mmol) or fructose (11.1 mmol) at a turbidity of 7–8 (OD 610 nm) for 24 h with periodic samplings and centrifuged. The samples were analysed using standard proton-nuclear magnetic resonance (¹H-NMR) on a Bruker AMX300 spectrometer (Antunes *et al.*, 2002).

Chemolithoorganotrophic growth on sodium thiosulfate was assessed under aerobic conditions at the optimum growth temperature in DSMZ medium 27 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium27.pdf) lacking ethanol and sulphate, and containing yeast extract (1.0 g l⁻¹), succinate (1.0 g l⁻¹) and 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. The levels of thiosulfate and sulfate in the supernatants were determined using the methods described by Westley (1987) and Sörbo (1987), respectively.

The cultures for polar lipid analysis were grown in 1 litre Erlenmeyer flasks containing 250 ml medium at 50 °C in a rotary water-bath shaker until the late exponential phase of growth. Harvesting of the cultures and extraction of lipids was as described by da Costa *et al.* (2006). Individual polar lipids were separated by two-dimensional thin-layer chromatography and visualized as described previously (da Costa *et al.*, 2006). Lipoquinones were extracted from freeze-dried cells and purified by thin-layer chromatography as described previously (da Costa *et al.*, 2006). The lipoquinones were separated by high performance liquid chromatography with a Gilson HPLC by using a reverse-phase Hichrom 5 C18 column and methanol/heptane (10:2, v/v) as the mobile phase and were detected at 269 nm. Cultures for fatty acid analysis were grown on solidified Degryse medium 162 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) as described previously (da Costa *et al.*, 2006).

DNA for the determination of G + C content was isolated as described by Nielsen *et al.* (1995). The G + C content of DNA was determined by high-performance liquid chromatography as described by Mesbah *et al.* (1989).

A 16S rRNA gene sequence comprising 1499 nt was determined for strain TU-20^T as previously described (Rainey *et al.*, 1996). The 16S rRNA gene was amplified in a number of fragments using the primer combinations 27f-519r, 27f-1385r, 357f-1525r and 536f-1525r (Rainey *et al.*, 1996). The taxonomic affiliation of strain TU-20^T, including the designation of its closest relatives based on the 16S rRNA gene sequence, was determined using the EzTaxon facility (Chun *et al.*, 2007).

Strain TU-20^T formed very short, Gram-stain-negative, rod-shaped cells (0.5 to 0.7 µm in width and 0.5 to 1.5 µm in length) without flagella and without visible gliding motility. Colonies were orange-pigmented on Degryse medium 162. The organism had an optimum growth temperature of about 50 °C and did not grow at 25 or 62.5 °C. This organism was oxidase-positive and catalase-negative and hydrolysed hippurate, starch, gelatin and casein. Aesculin, arbutin and xylan were not hydrolysed. Other enzyme activities are listed in the species description.

Small amounts of yeast extract (0.2 g l⁻¹) and vitamin B12 (40 µg l⁻¹) were added to the minimal medium to assess assimilation of single carbon sources. The organism produced acid from several carbohydrates, leading us to determine the acids produced. However, only very low levels of succinate were detected. Nitrate was reduced to nitrogen, but anaerobic growth with nitrate as electron acceptor was not observed (Table 1). Thiosulfate was completely oxidized to sulphate in media containing 0.5, 1.0 and 2.0 g thiosulfate l⁻¹ but there was no measurable increase in biomass.

The polar lipid pattern produced by thin-layer chromatography revealed the presence of one aminophospholipid, two aminolipids and three unidentified lipids. We also examined the polar lipids of *F. taffensis*, where we encountered one phospholipid, one aminophospholipid, one aminolipid and two unidentified lipids (Supplementary Fig S1, available in IJSEM Online), but only two lipids, an aminophospholipid and an aminolipid seem to be shared by both organisms. Moreover, strain TU-20^T possesses a major aminophospholipid which is not found in *F. taffensis*. The major respiratory lipoquinone was menaquinone 6, as in all members of the families *Cryomorphaceae* and *Flavobacteriaceae* examined. The fatty acid composition of strain TU-20^T was dominated by 16:1ω6c, 17:0 iso 3-OH and 15:0 iso (Table 2). However, many other organisms within the phylum 'Bacteroidetes' have large amounts of branched-chain fatty acids and 2- and 3-OH fatty acids.

Table 1. Differential characteristics of strain TU-20^T and the type species of the genera *Owenweeksia*, *Cryomorpha* and *Fluviicola* of the family *Cryomorphaceae*

–, Negative; +, positive; (+), weakly positive.

Characteristics	Strain TU-20 ^T	<i>Owenweeksia</i> ^{a*}	<i>Cryomorpha</i> ^b	<i>Fluviicola</i> ^c
Gliding motility	–	+	–	+
Habitat	Freshwater	Marine	Marine	Freshwater
Growth temperature range (°C)	30–60	4–37	–2–25	4–25
Optimum growth temperature (°C)	50	25–33	15	20
Growth on:				
0 % NaCl	+	–	–	+
3 % NaCl	+	+	+	–
6 % NaCl	–	+	+	–
Utilization of glucose	+	–	–	–
Acid production from glucose	+	–	–	–
Nitrate reduction	+	–	–	–
Presence of:				
Oxidase	+	+	–	–
Catalase	–	+	+	+
Hydrolysis of:				
DNA	+	–	–	+
Gelatin	+	+	–	(+)
Presence of 17:0 iso 3-OH	+	+	–	+
DNA G + C content (mol%)	42.8	39.6	36–37	37.2

*Data from: a, Lau *et al.* (2005); b, Bowman *et al.* (2003); c, O'Sullivan *et al.* (2005).

Table 2. Mean fatty acid composition of strain TU-20^T grown at 50 °C and 35 °C, and *Fluviicola taffensis* grown at 25 °C

Data are percentages of total fatty acids and are presented as the mean $\pm$ standard deviation of two to four analyses. tr, Trace (<0.2 %); –, not detected.

Fatty acid	TU-20 ^T		<i>Fluviicola taffensis</i>
	50 °C	35 °C	25 °C
11:0 iso	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	–
13:0 iso	1.8 $\pm$ 0.1	1.8 $\pm$ 0.2	tr
13:0	–	tr	0.5 $\pm$ 0.0
Unknown ECL* 13.565	0.5 $\pm$ 0.2	0.5 $\pm$ 0.0	tr
14:0	–	tr	2.0 $\pm$ 0.0
15:1 iso G	3.1 $\pm$ 0.1	14.7 $\pm$ 0.7	17.9 $\pm$ 0.5
15:0 iso	18.8 $\pm$ 1.2	34.8 $\pm$ 2.0	36.9 $\pm$ 0.5
15:0 anteiso	tr	tr	0.9 $\pm$ 0.1
Unknown ECL 14.815	–	1.6 $\pm$ 0.1	3.3 $\pm$ 0.0
15:0	6.4 $\pm$ 0.1	6.5 $\pm$ 0.8	6.8 $\pm$ 0.4
16:1 ω 6c	23.2 $\pm$ 1.5	9.0 $\pm$ 1.4	12.3 $\pm$ 0.5
16:0	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	0.7 $\pm$ 0.0
15:0 iso 3-OH	2.6 $\pm$ 0.1	4.0 $\pm$ 0.4	tr
15:0 2-OH	8.4 $\pm$ 1.3	1.8 $\pm$ 0.2	3.3 $\pm$ 0.1
15:0 3-OH	0.8 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
Unknown ECL 16.580	0.8 $\pm$ 0.2	0.8 $\pm$ 0.2	1.0 $\pm$ 0.1
17:1 ω 6c	1.1 $\pm$ 0.1	tr	–
16:0 3-OH	0.5 $\pm$ 0.0	tr	2.3 $\pm$ 0.1
17:0 iso 2-OH	0.7 $\pm$ 0.0	tr	–
17:0 iso 3-OH	22.3 $\pm$ 1.3	17.5 $\pm$ 0.3	8.4 $\pm$ 0.2
17:0 2-OH	0.7 $\pm$ 0.0	–	–
17:0 3-OH	5.5 $\pm$ 0.1	1.5 $\pm$ 0.1	tr

*ECL, equivalent chain-length.

Phylogenetic analyses using a dataset that included the 16S rRNA gene sequence of the type strain of all genera of the phylum ‘*Bacteroidetes*’ showed strain TU-20^T to represent a distinct family-level line of descent. The TU-20^T lineage falls outside of all of the described and proposed (Bergey’s Taxonomic Outline; http://www.bergeys.org/outlines/bergeys_vol_4_outline_linked.pdf) families of the phylum ‘*Bacteroidetes*’ (Supplementary Fig S2). The highest sequence similarity was approximately 86 % with the species of the genera *Owenweeksia*, *Cryomorpha*, *Lishizhenia*, *Brumimicrobium*, *Wandonia*, *Fluviicola* and *Crocinitomix* (Bowman *et al.*, 2003; Lau *et al.*, 2005, 2006; Lee *et al.*, 2010; O’Sullivan *et al.*, 2005), which have been classified as members of the family *Cryomorphaceae* (Bowman *et al.*, 2003). The next closest relatives, based on pairwise similarity values (82–83 %) and phylogenetic position, are the species of the family *Flavobacteriaceae* (Fig. 1 and Supplementary Fig. S2). Fig 1 and Supplementary Fig. S2 clearly demonstrate the lack of relationship of strain TU-20^T with any of the lineages within the phylum ‘*Bacteroidetes*’ and support its description as a novel taxon at the family level. On the basis of the results, we propose that strain TU-20^T

represents a novel species of a novel genus for which we recommend the name *Schleiferia thermophila* gen. nov., sp. nov. We also propose that this organism represents a new family of the phylum ‘*Bacteroidetes*’ for which we recommend the name *Schleiferiaceae* fam. nov.

Description of *Schleiferia* gen. nov.

Schleiferia (Schlei.fe’ri.a. N.L. fem. n. *Schleiferia* named in honour of the German microbiologist Karl-Heinz Schleifer).

Forms non-motile, rod-shaped cells that stain Gram-negative. Endospores are not formed. Strictly aerobic, oxidase-positive and catalase-negative. Slightly thermophilic. Major fatty acids are 16:1 ω 6c, 17:0 iso 3-OH and 15:0 iso; menaquinone 6 is the major respiratory quinone. The G+C content of the DNA of the type species is 42.8 mol%. The type species is *Schleiferia thermophila*.

Description of *Schleiferia thermophila* sp. nov.

Schleiferia thermophila (ther.mo’phi.la. Gr. adj. *thermos* hot; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving).

In addition to the characteristics reported for the genus, forms very short rod-shaped cells, 0.5 to 1.5 μ m in length and 0.5 to 0.7 μ m in width, without motility. Colonies on Degryse medium 162 are orange-pigmented and irregular. Growth occurs between 30 and 60 °C. The optimum growth temperature is about 50 °C; growth does not occur at 25 or 62.5 °C. The optimum pH for growth is between 7.5 and 8.5; growth does not occur at pH 6.0 or pH 10.0. Grows in media with up to 3 % salt; however, optimum growth occurs in medium without added NaCl. Yeast extract and vitamin B12 are required for growth. Nitrate is reduced to nitrogen. The polar lipid profile is composed of one aminophospholipid, two aminolipids and three unidentified lipids. Gelatin, casein and hippurate are degraded; starch, aesculin, arbutin and xylan are not degraded. DNase-positive. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive in the API ZYM system; other activities are negative. Glucose, fructose, galactose, mannose, D-arabinose, sucrose, maltose, lactose, trehalose, acetate, succinate, fumarate, aspartate, glutamate, proline, L-glutamine, L-arginine, L-ornithine and threonine are assimilated. L-Rhamnose, L-fucose, L-sorbose, D-ribose, D-xylose, L-arabinose, cellobiose, raffinose, melezitose, glycerol, ribitol, xylitol, sorbitol, mannitol, *myo*-inositol, erythritol, D-arabitol, L-arabitol, α -ketoglutarate, pyruvate, malate, citrate, benzoate, formate, D-gluconate, D-glucuronate, L-alanine, L-asparagine, glycine, L-histidine, L-lysine, L-serine, valine, L-phenylalanine, L-leucine, L-isoleucine and methionine are not assimilated. Acid is produced from the following carbohydrates using API 50 CH: D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, *N*-acetylglucosamine, maltose, starch, turanose, D-lyxose,

Fig. 1. 16S rRNA gene sequence based phylogeny demonstrating the relationship of strain TU-20^T with taxa of the phylum '*Bacteroidetes*'. The tree was reconstructed by using the neighbour-joining method using the MEGA 4.0 software package. Cluster I contains representatives of the family *Flavobacteriaceae*, cluster II the type species of the genera currently assigned to the family *Cryomorphaceae* (Bergey's Taxonomic Outline; http://www.bergeys.org/outlines/bergeys_vol_4_outline_linked.pdf), and cluster III comprises representatives of other family-level lineages within the phylum. Bar, 2 substitutions per 100 nucleotides. Numbers at branching points represent bootstrap values (based on 1000 resamplings).

D-tagatose and potassium 5-ketogluconate. The DNA of the type strain has a G + C content of 42.8 ± 0.1 mol% (HPLC method).

The type strain, TU-20^T (=DSM 21410^T =LMG 24594^T), was isolated from a hot spring at Furnas on the Island of São Miguel in the Azores.

Description of *Schleiferiaceae* fam. nov.

Schleiferiaceae (Schlei.fe.ri.a.ce' a.e. N.L. fem. n. *Schleiferia* type genus of the family; suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Schleiferiaceae* family of the genus *Schleiferia*).

Cells are Gram-negative and very short rod-shaped. Endospores are not formed. Slightly thermophilic. Major respiratory quinone is menaquinone 6 (MK-6). The family *Schleiferiaceae* belongs to the Phylum '*Bacteroidetes*'. The type genus of this family is *Schleiferia*.

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