Tepidicella xavieri gen. nov., sp. nov., a betaproteobacterium isolated from a hot spring runoff

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Strains TU-16^T and TU-18, two non-pigmented bacterial isolates with an optimum growth temperature of about 45 °C and an optimum pH of about 8·5–9·0, were recovered from the Furnas geothermal area on the Island of São Miguel in the Azores. Phylogenetic analysis of the 16S rRNA gene sequence of these strains indicated that they represent a novel species in a new genus of the phylum *Betaproteobacteria*. The major fatty acids of strains TU-16^T and TU-18 were 16 : 0 and 18 : 1ω7*c*. Ubiquinone 8 was the major respiratory quinone and the major polar lipids were phosphatidylethanolamine and phosphatidylglycerol. The novel isolates were aerobic; thiosulfate was oxidized to sulfate in the presence of a metabolizable carbon source. The organism assimilated organic acids and amino acids, but did not assimilate carbohydrates or polyols. Based on phylogenetic analyses and physiological and biochemical characteristics, it is proposed that strain TU-16^T (=LMG 23030^T=CIP 108724^T) represents the type strain of a novel species in a new genus, *Tepidicella xavieri* gen. nov., sp. nov.

Three slightly thermophilic species of betaproteobacteria named *Tepidimonas ignava* (Moreira *et al.*, 2000), *Tepidimonas aquatica* (Freitas *et al.*, 2003) and '*Tepidimonas taiwanensis*' (Chen *et al.*, 2006) have been described recently. Two related strains, designated AA-1 and AA-2, have also been isolated recently from Aachen in Germany (Albuquerque *et al.*, 2006). These organisms were all isolated from hot springs and an industrial hot water tank. A strain designated SMC-6271 and named '*Tepidimonas arfidensis*', which represents an additional lineage within the genus *Tepidimonas*, was isolated from the bone marrow of a person with leukaemia, but this organism appears to represent a contaminant of the sample (Ko *et al.*, 2005). The species of this genus have optimum growth temperatures of about 50 °C, do not assimilate carbohydrates or polyols, do not grow at pH below 6·0 or above 9·5 and oxidize thiosulfate to sulfate in the presence of an assimilable carbon source.

Two strains, TU-16^T and TU-18, with identical 16S rRNA gene sequences, were isolated recently from the Furnas geothermal area on the Island of São Miguel in the Azores; they represent a new lineage closely related to the genus *Tepidimonas*. The organisms share many physiological and biochemical characteristics with species of the genus *Tepidimonas*, but have a distinctly lower temperature range and a higher pH range for growth. Based on these characteristics and phylogenetic analysis, it is proposed that these strains represent a novel species in a new genus for which the name *Tepidicella xavieri* gen. nov., sp. nov. is recommended.

Strains TU-16^T and TU-18 were isolated from a hot spring runoff at Furnas (temperature of 70 °C and pH 7·5). Water samples were transported without temperature control and filtered through membrane filters. The filters were placed on the surface of agar-solidified *Thermus* medium (Williams & da Costa, 1992), wrapped in plastic bags and incubated at 50 °C for up to 4 days. Cultures were maintained as described previously (Moreira *et al.*, 2000). Culturing in Degryse 162 medium (Degryse *et al.*, 1978) was later adopted because it resulted in higher growth yields. *Tepidimonas ignava* SPS-1037^T (=DSM 12034^T), *Tepidimonas aquatica* CLN-1^T (=DSM 14833^T=ATCC BAA-469^T), '*Tepidimonas taiwanensis*' I1-1 (=BCRC 17406=LMG 22826) and strain AA-1 (=LMG 23094=CIP 108777) were used as controls.

Unless otherwise stated, all morphological examinations and biochemical and tolerance tests were performed as described previously (Santos *et al.*, 1989; Nunes *et al.*, 1992) in Degryse 162 liquid medium or Degryse 162 agar at pH 8·0 and at 45 °C for up to 5 days. The growth temperature range of the strains in liquid Degryse 162 medium was examined

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in a reciprocal water-bath shaker between 20 and 60 °C. The NaCl range for growth of the organisms was determined at 45 °C. The range for growth was determined at 45 °C in the same medium between pH 6 and 11 using 50 mM MES, HEPES, TAPS CAPSO and CAPS.

Single-carbon source assimilation tests were performed in a minimal medium composed of Degryse 162 basal salts with yeast extract (0·1 g l^{-1}) to which filter-sterilized ammonium chloride (0·5 g l^{-1}), a vitamin, nucleotide and amino acid mixture (Sharp & Williams, 1988), and the carbon source (2·0 g l^{-1}) were added. Growth of the strains was examined by measuring the turbidity of cultures incubated at 45 °C in 20 ml screw-capped tubes containing 10 ml medium for up to 5 days. Fermentation was tested using the API 50 CHL system (bioMérieux), as recommended by the manufacturer, incubated at 45 °C for up to 5 days. The ability of the strains to grow with several electron acceptors was examined in a defined basal medium described previously (Kieft *et al.*, 1999) at 45 °C and pH 8·5. Lactate (30 mM) was used as the electron donor to examine growth under an N_2 atmosphere coupled to the reduction of nitrate, nitrite, Fe(III)–NTA, fumarate, sulfate and thiosulfate (each at 10 mM, except nitrite, which was at 1·0 mM). Control cultures lacking an electron acceptor were also tested for growth.

Aerobic growth on reduced sulfur compounds was tested in modified medium 69 (www.dsmz.de/media/med069.htm) containing the following components (l^{-1}): $Na_2HPO_4.12H_2O$, 10·6 g; KH_2PO_4, 1·5 g; NH_4Cl, 0·3 g; yeast extract, 1·0 g; $MgCl_2$, 0·1 g; and trace element solution of medium 27 (www.dsmz.de/media/med027.htm). Cysteine, thiosulfate and tetrathionate were added to the medium at concentrations of 0·1–1·0 g l^{-1} (Moreira *et al.*, 2000; Freitas *et al.*, 2003). Levels of thiosulfate and sulfate in the supernatants were determined using the methods described by Westley (1987) and Sörbo (1987), respectively.

Isolates TU-16^T and TU-18 formed round, creamy-white colonies. Cells were rod-shaped, 0·5–1·0 μm in width by 1·0–2·0 μm in length and were motile by one polar flagellum. The optimum growth temperature was about 45 °C; growth was observed between about 25 and 55 °C (Table 1). Type strains of species of the genus *Tepidimonas* had higher optimum growth temperatures (around 50 °C), did not grow at 25 °C and, with the exception of *Tepidimonas ignava*, did not grow above 60 °C. The optimum pH for growth of the novel isolates was 8·5–9·0; growth was not observed below pH 6·5 or above 10·5. Type strains of species of the genus *Tepidimonas* had lower pH ranges for growth, i.e. between about pH 6·0 and 9·5 (Moreira *et al.*, 2000; Freitas *et al.*, 2003; Albuquerque *et al.*, 2006). DNase and urease activities were detected, but amylase and xylanase were not detected. Aesculin, hippurate and Tween 20 were hydrolysed.

The novel isolates were unable to grow on any of the carbohydrates and polyols tested. On the other hand, they utilized the majority of the organic acids and amino acids tested. Fermentation of carbohydrates was not observed. Nitrate, nitrite, Fe(III)–NTA, fumarate, sulfate and thiosulfate were not used as electron acceptors under anaerobic conditions. L-Cysteine, thiosulfate and tetrathionate did not lead to an increase in the biomass of strains TU-16^T and TU-18, but were oxidized to sulfate.

For polar lipid analysis, cells were grown in 1 l Erlenmeyer flasks containing 200 ml Degryse 162 medium at 45 °C in a water-bath shaker until the exponential phase of growth. Harvesting of the cultures, extraction of lipids and their separation were performed as described previously (Prado *et al.*, 1988; Donato *et al.*, 1990). Lipoquinones were extracted from freeze-dried cells and purified by TLC as described previously (Tindall, 1989; Moreira *et al.*, 2000; Freitas *et al.*, 2003). Cultures for fatty acid analysis were grown on plates of Degryse 162 medium in sealed plastic bags submerged in a water-bath at 50 °C for 24 h. Fatty acid methyl esters were obtained from fresh wet biomass; their identification and quantification, as well as numerical analysis of the fatty acid profiles, were performed using the standard MIS library Generation Software (Microbial ID).

Phosphatidylethanolamine and phosphatidylglycerol dominated the polar lipid profiles of strains TU-16^T and TU-18, which were similar to those of the type strains of members of the genus *Tepidimonas*. Ubiquinone 8 was the major respiratory quinone. The fatty acid composition of strains TU-16^T and TU-18 was characterized by very large relative proportions of 16 : 0, which reached about 45 % of the total, and 18 : 1ω7*c*, which reached levels of about 20 % (Table 2).

DNA for the determination of the G+C content was isolated as described by Nielsen *et al.* (1995). The DNA G+C content 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 purified PCR products were carried out as described previously (Rainey *et al.*, 1996). Purified reactions were electrophoresed using a model 310 Genetic Analyser (Applied Biosystems). The 16S rRNA gene sequences were aligned against representative reference sequences of members of the *Betaproteobacteria* lineage using MEGA version 3.1 (Kumar *et al.*, 2004). 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).

The DNA G+C contents of strains TU-16^T and TU-18 were 64·9 and 65·5 mol%, respectively. These values are about 3 mol% lower than those of species of the genus *Tepidimonas*. Strains TU-16^T and TU-18 had identical 16S rRNA gene sequences over the 1480 nt that were determined and compared. A comparative analysis of 1393 nt positions of the 16S rRNA gene sequence of strain TU-16^T with those of other members of the *Betaproteobacteria* lineage showed that strain TU-16^T was closely related (99·7 and 99·5 % similarity) to sequences of two environmental clones

Table 1. Characteristics that distinguish strains TU-16^T and TU-18 from related strains/species

Strains: 1, *Tepidicella xavieri* sp. nov. strains TU-16^T and TU-18; 2, strain AA-1; 3, *Tepidimonas ignava* SPS-1037^T; 4, *Tepidimonas aquatica* CLN-1^T; 5, '*Tepidimonas taiwanensis*' I1-1; 6, '*Tepidimonas arfidensis*' SMC-6271. −, Negative; +, positive; W, weakly positive; ND, not determined. Strains TU-16^T, TU-18, AA-1, SPS-1037^T and CLN-1^T hydrolysed hippurate; none of these strains hydrolysed arbutin or xylan. All organisms were oxidase-positive. Strains TU-16^T, TU-18, AA-1, SPS-1037^T, CLN-1^T and I1-1 were positive for alkaline phosphatase, esterase (C4), lipase (C8), leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase; these strains were all negative for cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Strains TU-16^T, TU-18, AA-1, SPS-1037^T, CLN-1^T and I1-1 assimilated succinate, acetate, lactate, pyruvate, L-ornithine, L-proline, L-alanine, L-asparagine, L-glutamate and L-glutamine; none of them assimilated lactose, D-arabinose, D-ribose, glycerol, D-mannitol, ribitol, xylitol, sorbitol, *myo*-inositol, benzoate, glycine, L-valine, L-methionine or L-threonine.

Characteristic	1	2	3	4	5*	6†
Temperature (°C) for growth:						
Range	25–55	30–60	30–65	30–60	35–60	ND
Optimum	45	50–55	50–55	50	55	50
pH for growth:						
Range	6·5–10·5	6·0–9·5	6·0–9·5	6·0–9·5	6·0–8·0	ND
Optimum	8·5–9·0	7·5–8·0	7·5–8·0	7·5–8·0	7·0	ND
NaCl range (%, w/v) for growth	0–2	0–1	0–1	0–2	ND	ND
Presence of:						
Catalase	+	+	+	+	− (W)	ND
DNase	+	+	+	+	ND	ND
Urease	+	ND	+	−	−	−
Lipase (C14)	W/+	−	−	−	+	ND
Acid phosphatase	+	+	+	+	−	ND
Degradation of:						
Aesculin	+	−	−	−	+ (−)	−
Casein	−	−	−	−	+ (+)	ND
Starch	−	−	−	−	+ (−)	ND
Tween 20	+	+	−	+	ND	ND
Tween 40	−	−	−	+	ND	ND
Tween 60	−	+	−	+	−	ND
Tween 80	−	−	−	+	−	ND
Assimilation of:						
D-Glucose	−	−	−	−	+ (+)	−
D-Fructose	−	−	−	−	− (+)	ND
Citrate	+	−	−	−	+ (+)	−
Malate	+	−	+	−	+ (+)	−
2-Oxoglutarate	+	−	+	+	+ (+)	ND
Aspartate	+	−	+	+	+ (+)	ND
L-Phenylalanine	+	−	−	−	ND	ND
L-Lysine	+	+	−	−	ND (+)	ND
L-Leucine	+	−	+	+	ND	ND
L-Serine	W/+	−	−	−	ND	ND
L-Histidine	+	−	−	−	− (+)	−
L-Tryptophan	+	−	−	−	ND	ND
L-Tyrosine	+	ND	−	−	ND	ND
L-Isoleucine	−	+	+	+	ND (−)	ND
L-Arginine	−	−	−	−	ND (+)	ND
L-Cysteine	−	−	−	−	ND	ND
Reduction of NO_3^- to NO_2^-	−	−	−	+	+	+
G+C content (mol%)	64·9/65·5	67·8	69·7	68·6	68·1	69·2

*Data from Chen *et al.* (2006) are shown; data from this study are given in parentheses.
†Data from Ko *et al.* (2005).

Table 2. Mean fatty acid content (%) of strains TU-16^T and TU-18 and related strains/species grown at 50 °C

Strains: 1, *Tepidicella xavieri* strains TU-16^T and TU-18; 2, strain AA-1; 3, *Tepidimonas ignava* SPS-1037^T; 4, *Tepidimonas aquatica* CLN-1^T; 5, '*Tepidimonas taiwanensis*' I1-1; 6, '*Tepidimonas arfidensis*' SMC-6271. −, Fatty acids present at levels of less than 0·5 % or not detected. ECL, equivalent chain-length.

Fatty acid	1	2	3	4	5	6*
8 : 0 3-OH	0·9±0·1	0·5±0·1	0·7±0·1	−	−	−
10 : 0	0·6±0·1	−	−	−	−	−
12 : 0	1·5±0·1	−	−	−	−	−
15 : 1ω6*c*	−	3·0±0·3	3·7±1·5	−	0·6±0·1	−
15 : 0	0·7±0·1	5·1±0·6	6·2±0·7	0·5±0·1	0·9±0·1	1·0
16 : 0 iso	1·6±0·1	−	−	0·8±0·1	−	−
16 : 1ω7*c*	6·8±0·2	27·4±1·1	24·3±0·5	11·1±5·1	22·2±0·1	16·7
16 : 0	44·5±0·1	32·9±0·6	23·2±3·4	42·5±0·6	42·8±0·2	51·3
Unknown (ECL 16·090)	−	−	−	0·8±0·3	−	−
Unknown (ECL 16·559)	1·1±0·1	−	−	−	−	−
17 : 1ω8*c*	−	−	1·0±0·1	−	−	−
17 : 1ω6*c*	−	−	3·1±0·7	−	−	−
17 : 0 cyclo	11·9±0·1	7·0±3·2	−	33·2±3·5	7·0±0·1	17·2
17 : 0	1·8±0·3	8·5±1·1	19·9±3·9	3·2±0·4	4·5±0·3	2·7
18 : 1ω7*c*	20·8±0·9	10·0±1·2	11·9±1·8	−	18·4±0·2	6·6
18 : 0	2·4±0·1	1·2±0·1	2·2±0·8	4·9±0·2	2·5±0·1	1·6
Unknown (ECL 18·082)	0·7±0·2	1·3±0·6	3·2±1·7	−	−	−
Unknown (ECL 18·422)	0·9±0·2	−	−	1·0±0·3	−	−
Unknown (ECL 18·824)	0·7±0·3	−	0·7±0·2	−	−	−
19 : 0 cyclo ω8*c*	4·6±0·9	−	−	−	0·5±0·1	1·1

*Data from Ko *et al.* (2005).

recovered from a deep terrestrial fracture system (AY768824) and a gold mine borehole (AY796039) (Fig. 1). Of cultured taxa with validly described names, species of the genus *Tepidimonas* had highest 16S rRNA gene sequence similarity (94·6–95·5 %; Fig. 1) to strain TU-16^T. The relationship to species of other genera of the *Betaproteobacteria* lineage was below 95 %. The phylogenetic relationship between strain TU-16^T and species of the genus *Tepidimonas* is supported by a bootstrap value of 97 %, whereas the individual lineages comprising strain TU-16^T and related clone sequences and that of the *Tepidimonas* species cluster are both supported by 100 % bootstrap values, thus indicating their distinctiveness. This is in contrast to the stability of the branching order within the *Tepidimonas* species cluster, where some branching points have low statistical support.

Species of the genus *Tepidimonas* and strains TU-16^T and TU-18 have rather homogeneous phenotypic characteristics indicating a close relationship. Strains TU-16^T and TU-18 can be distinguished from the type strains of *Tepidimonas* species by differences in carbon source assimilation and fatty acid composition. These differences could, by themselves, indicate that strains TU-16^T and TU-18 represent a novel species of the genus *Tepidimonas*. However, strains TU-16^T and TU-18 can be distinguished from species of the genus *Tepidimonas* by their lower temperature range and higher pH range for growth. Strain SMC-6271 named '*Tepidimonas arfidensis*', in common with other members of the genus, also has an optimum growth temperature of about 50 °C, a pH optimum for growth of about 7·0 and does not utilize glucose; however, few characteristics of this strain have been reported, making it very difficult to compare phenotypic characteristics of this organism with those of other species of the genus *Tepidimonas* or strains TU-16^T and TU-18 (Ko *et al.*, 2005).

The ecological significance of the isolation of strains TU-16^T and TU-18 from a neutral pH hot spring and their close phylogenetic relationship to environmental 16S rRNA gene clones from deep gold mines or fractures cannot be explained at this time. Perhaps environments with similar physico-chemical parameters exist at these subsurface sites. The lower DNA G+C contents, grouping within a distinct phylogenetic lineage, the lower growth temperature range and the higher pH range of strains TU-16^T and TU-18 indicate that these strains belong to a novel species in a new genus for which the name *Tepidicella xavieri* gen. nov., sp. nov. is proposed.

Description of *Tepidicella* gen. nov.

Tepidicella (Te.pi.di.cel′la. L. adj. *tepidus* warm; L. fem. n. *cella* chamber/cell; N.L. fem. n. *Tepidicella* a cell living in a warm environment).

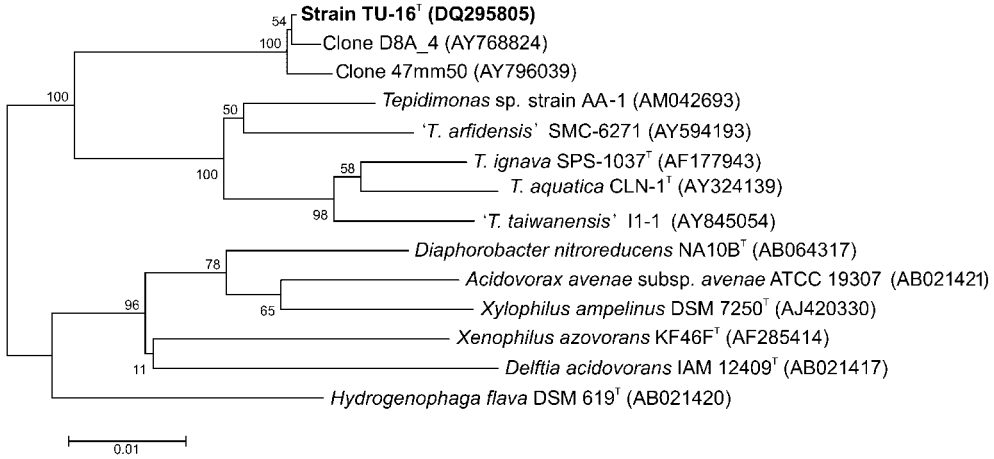

Fig. 1. Phylogeny based on 16S rRNA gene sequences showing the relationships of strain TU-16^T and related taxa. The dendrogram was constructed from distance matrices using the neighbour-joining method. Numbers at branching points represent bootstrap values from 1000 replicates. Bar, 1 inferred nucleotide substitution per 100 nucleotides.

Forms motile rod-shaped cells that stain Gram-negative. Endospores are not formed. Slightly thermophilic and slightly alkaliphilic. Strictly aerobic; oxidase- and catalase-positive. Fatty acids are straight-chained; major phospholipids are phosphatidylethanolamine and phosphatidylglycerol; ubiquinone 8 is the major respiratory quinone. Reduced sulfur compounds are oxidized to sulfate. Organic acids and amino acids are used as carbon and energy sources, but sugars and polyols are not assimilated. The genus *Tepidicella* belongs to the *Betaproteobacteria*. The type species is *Tepidicella xavieri*.

Description of *Tepidicella xavieri* sp. nov.

Tepidicella xavieri (xa.vi.e′ri. N.L. gen. n. *xavieri* of Xavier, in honour of the Portuguese biochemist António V. Xavier).

Forms short rod-shaped cells, 0·5–1·0 × 1·0–2·0 μm. Gram stain is negative. Motile by one polar flagellum. Colonies on Degryse 162 medium are creamy-white. Growth occurs between 25 and 55 °C; the optimum growth temperature for the type strain is about 45 °C. Optimum pH for growth is 8·5–9·0; growth does not occur below pH 6·5 or above pH 10·5. Major fatty acids are 16:0 and 18:1ω7*c*. Ubiquinone 8 is the major respiratory quinone. Strain TU-16^T is strictly aerobic and chemo-organotrophic. Strain TU-16^T does not reduce nitrate to nitrite. Thiosulfate is oxidized to sulfate in the presence of a carbon source. Positive for cytochrome oxidase, catalase, urease and DNase. Aesculin, hippurate and Tween 20 are degraded. Several amino acids and organic acids are utilized for growth, but the type strain does not utilize hexoses, disaccharides, pentoses or polyols.

The type strain is TU-16^T (=LMG 23030^T=CIP 108724^T), isolated from a hot spring runoff in Furnas, the Azores; it has a DNA G+C content of 64·9 mol%.

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