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Tepidimonas thermarum sp. nov., a new slightly thermophilic betaproteobacterium isolated from the Elisenquelle in Aachen and emended description of the genus *Tepidimonas* ☆

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Abstract

Several non-pigmented bacterial isolates, with an optimum growth temperature of about 50–55 °C, were recovered from the Elisenquelle at Aachen, Germany. Phylogenetic analysis of the 16S rRNA gene sequence of strains AA-1^T and AA-2 indicated that these organisms represent a new species of the genus *Tepidimonas*. The major fatty acids of strains AA-1^T and AA-2 are 16:0 and 16:1 $\omega 7c$. Ubiquinone 8 is the major respiratory quinone, the major polar lipids are phosphotidylethanolamine and phosphotidylglycerol. The new isolates are aerobic; thiosulfate is 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. On the basis of the phylogenetic analyses, physiological and biochemical characteristics, we propose that strains AA-1^T (= LMG 23094^T; = CIP 108777^T) and AA-2 (= LMG 23095; = CIP 108778) represents a new species for which we recommend the name *Tepidimonas thermarum*.

Keywords: *Tepidimonas thermarum*; *β*-subclass; *Proteobacteria*; Slightly thermophilic

Introduction

The species of the genus *Tepidimonas*, namely *Tepidimonas ignava*, *Tepidimonas aquatica* and "*Tepidimonas taiwanensis*", were isolated from hot springs and from an industrial hot water tank, respectively [6,3,13]. These *Betaproteobacteria* have optimum growth temperatures around 50 °C, two of these three species do not utilize carbohydrates as single carbon sources, oxidize reduced sulfur compounds to sulfate and are strictly aerobic. One strain named "*Tepidimonas arfidensis*" was isolated from the bone marrow of a patient with leukemia, but the isolation of this organism may have been due to contamination of the sample [10], which also appears not utilize carbohydrates.

We recently recovered several isolates from the Elisenquelle (Elisa Spring) at Aachen (Aix-la-Chapelle) in Germany. Two of the isolates, AA-1^T and AA-2, were characterized in detail and possessed many

☆ *Nucleotide sequence accession numbers.* The 16S rRNA gene sequences determined in this study were deposited in EMBL data library under the accession numbers: AA-1^T (AM042693) and AA-2 (AM042694).

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characteristics similar to the type strains of *T. ignava, T. aquatica* and "*T. taiwanensis*". However, differences in physiological, biochemical and chemotaxonomic characteristics allied to the phylogenetic analysis show that strains AA-1^T and AA-2 belong to a novel species of the genus *Tepidimonas* for which we propose the name *Tepidimonas thermarum* sp.nov.

Material and methods

Isolation and bacterial strains

Strains AA-1^T (T = type strain) and AA-2 were isolated from the Elisenquelle at Aachen, Germany. 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 agar-solidified Degryse medium 162 [4], wrapped in plastic bags and incubated at 50 °C for up to 4 days. Cultures were purified by sub-culturing and the isolates stored at −70 °C in Degryse medium 162 with 15% (w/v) glycerol. The type strains of *Tepidimonas ignava* SPS-1037^T (= DSM 12034^T), *T. aquatica* CLN-1^T (= DSM 14833^T) and "*T. taiwanensis*" I1-1^T (= LMG 22826^T) were used for comparative purposes.

Morphology, growth, biochemical and physiological characteristics

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 [8]. The growth temperature range of the strains was examined by measuring the turbidity (610 nm) of cultures incubated in 300 ml metal-capped Erlenmeyer flasks, containing 100 ml Degryse medium in a reciprocal water-bath shaker [13]. The pH range for growth was examined at 50 °C in the same medium by using 50 mM MES, HEPES, TAPS, CAPSO and CAPS over a pH range from 5.5 to 11.0 [13]. Unless otherwise stated, all biochemical and tolerance tests were performed, as described previously [6], in Degryse liquid medium or Degryse agar at 50 °C for up 6 days. Catalase, oxidase and DNAse activities were examined as described previously [13]. Additional enzymatic activities were obtained using the API ZYM system (bioMérieux) at 50 °C. Anaerobic growth was assessed in cultures in Degryse medium containing KNO_3 (1.0 g/l) incubated in anaerobic chambers (GENbox anaer, bioMérieux). Single-carbon source assimilation tests were performed in a minimal medium composed of Degryse basal salts to which filter-sterilized ammonium sulfate (0.5 g/l), vitamin and nucleotide solution [17] and the carbon source (2.0 g/l) 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 of medium for up to 6 days. Growth on reduced sulfur compounds was assessed on modified 69 medium (www.dsmz.de/media/med069.htm) containing the following components per liter: $Na_2HPO_4 \cdot 12H_2O$, 10.6 g; KH_2PO_4, 1.5 g; NH_4Cl, 0.3 g; yeast extract, 1.0 g; $MgCl_2$, 0.1 g; trace elements solution of medium 27 (www.dsmz.de/media/med027.htm) without sulfate, 1 ml and containing the vitamin and nucleotide solution [17]. Thiosulfate was added to this media at concentrations that varied between 0.1 and 5.0 g/l. At appropriate intervals, the turbidity of the cultures was measured, the cells were harvested by centrifugation and the levels of thiosulfate and sulfate in the supernatants were determined using the methods described by Westley [22] and Sörbo [18], respectively.

Polar lipid, lipoquinone and fatty acid composition

The cultures used for polar lipid analysis were grown in 11 Erlenmeyer flasks containing 250 ml of Degryse medium at 50 °C in a reciprocal water bath shaker until late exponential phase of growth. Harvesting of the cultures, extraction of the lipids and single dimensional thin-layer chromatography were performed as described previously [1]. Lipoquinones were extracted, purified by thin-layer chromatography and separated by high performance liquid chromatography [21]. Cultures for fatty acid analysis were grown on solidified Degryse medium, in sealed plastic bags submerged in a water bath at 50 °C for 24 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 [2].

Determination of G + C content of DNA and 16S rRNA gene sequence determination and phylogenetic analyses

The DNA for the determination of the G + C content of the DNA was isolated as described previously [14]. The G + C content of DNA was determined by high-performance liquid chromatography as described by Mesbah et al. [12].

The 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 [15]. Purified reactions were electrophoresed using a model 310 Genetic Analyzer (Applied Biosystems, Foster City,

CA, USA). The quality of 16S rRNA gene sequences was checked manually using the BioEdit sequence editor [7] and aligned against representative reference sequences of members of the class *Betaproteobacteria*, obtained from EMBL, using the multiple-alignment CLUSTAL X software package [20]. The method of Jukes and Cantor [9] was used to calculate evolutionary distances; phylogenetic dendrograms were constructed using the neighbor-joining method [16], and trees topologies were evaluated by performing bootstrap analysis [5] of 1000 data sets using the MEGA2 package [11].

Results

Morphology, growth, biochemical and physiological characteristics

Strains AA-1^T and AA-2 were isolated from the Elisenquelle, with a temperature of 45 °C, at Aachen (Aix-la-Chapelle) in Germany. Strains AA-1^T and AA-2 formed very short, Gram-negative rod-shaped cells and were motile by means of one polar flagellum. Colonies were translucent and non-pigmented. The organisms had an optimum growth temperature between 50 and 55 °C and did not grow at 25 or 60 °C (results not shown). The optimum pH of strain AA-1^T and AA-2 was between 7.5 and 8.5 and no growth was detected at pH 5.5 or 9.8 (results not shown). Of the proteinaceous substrates examined only gelatine was hydrolyzed. Vitamins and nucleotide solution was required for growth on single carbon sources. These organisms were unable to utilize any of carbohydrates or polyols tested. Several organic acids and amino acids were used as a single carbon and energy sources. On the other hand, we confirmed that the type strain of *T. taiwanensis* utilized glucose and also fructose [3]. Strains AA-1^T and AA-2 produced sulfate from thiosulfate without an overt increase in the final biomass [6,13]. Unlike *T. aquatica*, strains AA-1^T and AA-2 do not grow in medium containing 2.0% NaCl, although the strains that represent the new species grow in medium containing 0.05% potassium tellurite which the type strains of the other two species of the genus do not tolerate (Table 1).

Polar lipids, respiratory quinone and fatty acids composition

The polar lipid pattern on TLC revealed the presence of phosphotidylethanolamine and phosphotidylglycerol among other minor phospholipids. The major respiratory lipoquinone was ubiquinone 8. The fatty acid composition of strains AA-1^T and AA-2 was dominated by 16:0 and 16:1 $\omega 7c$ (Table 2). The strains of the new species could be distinguished from the type strains of the other species of this genus by the levels of 16:0, 16:1 $\omega 7c$, 17:0 cyclo, 17:0 and 18:1 $\omega 7c$.

16S. rRNA gene sequence comparison and G + C content of DNA

Partial 16S rRNA gene sequences comprising 1512 and 1507 nucleotides were determined for strains AA-1^T and AA-2, respectively. Comparison of these sequences with representatives of the main lines of descent within the domain *Bacteria* indicated that these strains were members of the class *Betaproteobacteria* (Fig. 1). The pairwise 16S rRNA gene sequence similarity determined between strains AA-1^T and AA-2 was 100% with each other and of 96.9% with the type strain of *T. ignava* and 95.5% with the type strain of *T. aquatica* (Fig. 1). The new strains from Aachen had a pairwise 16S rDNA sequence similarity of 97.2% with the type strain of "*T. arfidensis*" (AY594193), 97.3% with strain ac-15, 96.7% with the type strain of "*T. taiwanensis*" and 95.4% with strain DhA-73. The G + C content of the DNA of strain AA-1^T and AA-2 was 67.9 mol%.

Discussion

The species of the genus *Tepidimonas*, like strains AA-1^T and AA-2, have been primarily isolated from natural geothermally heated aquatic environments [3,6,13,19]. *T. aquatica* was isolated from an industrial hot water tank, while strain DhA-73, which shares 99.8% sequence similarity with the *T. aquatica*, was isolated from a heated fermentor containing paper pulp effluent [23]. Unexpectedly, one strain, with an optimum growth temperature of about 50 °C, designated "*T. arfidensis*", was isolated from bone marrow of a person with leukemia [10]. The species *T. aquatica*, *T. ignava* and strain DhA-73 are unable to utilize carbohydrates, but the type strain of "*T. taiwanensis*" assimilates glucose, fructose and was reported to degrade starch, although we were unable to confirm this result. The carbohydrate utilization of "*T. arfidensis*" was not reported, but only nitrate and cytochrome oxidase were considered to be positive in the API 20NE system, which includes the assimilation of glucose, among other carbohydrates [10]. We, therefore, assume that this strain, like other strains of the genus does not utilize carbohydrates.

The new species of the genus *Tepidimonas* represented by strains AA-1^T and AA-2 shares many of the physiological and biochemical characteristics of *T. ignava*, *T. aquatica* and "*T. taiwanensis*", clearly indicating that these organisms are closely related to each other and form a rather homogeneous group of organisms.

Table 1. Characteristics that distinguish strains AA-1^T, AA-2, SPS-1037^T (*T. ignava*), CLN-1^T (*T. aquatica*), I1-1^T (*"T. taiwanensis"*) and SMC-6271^T (*"T. arfidensis"*)

Characteristics	Strains				
	AA-1^T/AA-2	SPS-1037^T	CLN-1^T	I1-1^{Ta,b}	SMC-6271^{Tc}
Growth in 2% NaCl	−	−	+	nd	nd
Growth in 0.05% potassium tellurite	+	−	−	nd	nd
Reduction of NO_3^- to NO_2^-	−	−	+	+	+
Presence of					
Catalase	+	+	+	− (w)	nd
Lipase (C14)	−	−	−	+	nd
Hydrolysis of					
Gelatin	w	−	−	+ (+)	−
Casein	−	−	−	+ (+)	nd
Starch	−	−	−	+ (−)	nd
Esculin	−	−	−	+ (−)	−
Assimilation of					
D-Glucose	−	−	−	+ (+)	−
D-Fructose	−	−	−	− (+)	nd
Aspartate	−	+	+	+ (+)	nd
Fumarate	−	+	+	nd (+)	nd
Malate	−	+	−	+ (+)	−
α-ketoglutarate	−	+	+	+ (+)	nd
Citrate	−	−	−	+ (+)	−
L-lysine	+	−	−	nd (+)	nd
L-histidine	−	−	−	− (+)	−
L-isoleucine	+	+	+	nd (−)	nd
L-arginine	−	−	−	nd (+)	nd
Mol% G+C	67.9	69.7	68.6	68.1	69.2

−, negative result; +, positive result; w, weakly positive result.
Strains AA-1^T, AA-2, SPS-1037^T and CLN-1^T did not hydrolyse arbutin, elastin and xylan. These strains hydrolysed hippurate. These strains did not possess lipase (C14), cistine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acethyl-β-glucosaminidase, α-mannosidase and α-fucosidase. These strains possess oxidase, DNAse, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Strains AA-1^T, AA-2, SPS-1037^T, CLN-1^T and I1-1^T assimilate succinate, lactate, pyruvate, acetate, L-glutamate, L-alanine, L-aspargine, L-glutamine, L-ornithine, and proline. These strains did not assimilate D-galactose, D-mannose, D-trehalose, D-cellobiose, D-melibiose, D-raffinose, D-ribose, D-xylose, D-arabinose, L-arabinose, L-rhamnose, L-fucose, L-sorbose, sucrose, lactose, maltose, ribitol, xylitol, sorbitol, erytritol, D-mannitol, *myo*-inositol, glycerol, benzoate, formate, glycine, L-methionine, L-serine, L-threonine and valine.
[a]Results from Chen et al. [3].
[b]Our results on strain I1-1^T are in parenthesis.
[c]Results from Ko et al. [10].

Despite similar physiological and tolerance characteristics, some characteristics distinguish the new species from the other four species of the genus, namely the inability of strains AA-1^T and AA-2 to assimilate several of the carbon sources which the other strains are able to assimilate. The original description of the genus *Tepidimonas* did not foresee that some strains could utilize carbohydrates. It seems, therefore, necessary to emend the description of the genus to accommodate carbohydrate-assimilating strains. On the basis of the 16S rRNA gene sequence analysis, physiological and biochemical characteristics we are of the opinion that strains AA-1^T and AA-2 represent a new species of the genus *Tepidimonas* for which we propose the name *Tepidimonas thermarum*.

Emended description of the genus *Tepidimonas* [13]

The characteristics of the genus *Tepidimonas* are those described by Moreira et al. [13]. Addition results of Chen et al. [3] and of this study show that some strains of this genus assimilate carbohydrates, namely glucose and fructose. The type species of the genus is *Tepidimonas ignava*, strain SPS-1037^T (= DSM 12034^T).

Table 2. Mean fatty acid composition of strains AA-1^T, AA-2, SPS-1037^T (*T. ignava*), CLN-1^T (*T. aquatica*), I1-1^T (*"T. taiwanensis"*) and SMC-6271^T (*"T. arfidensis"*) grown at 50 °C

Fatty acids[a]	% of the total in				
	AA-1^T/AA-2	SPS-1037^T	CLN-1^T	I1-1^T	SMC-6271^{Tc}
8:0 3OH	0.6±0.07	0.7±0.1	—[b]	—	—
15:1 *ω6c*	2.6±0.5	3.7±1.5	—	0.6±0.1	—
15:0	4.4±1.0	6.2±0.7	0.5±0.1	0.9±0.07	1.0
16:0 iso	—	—	0.8±0.1	—	—
16:1 *ω7c*	26.9±0.7	24.3±0.5	11.1±5.1	22.2±0.01	16.7
16:0	34.8±2.7	23.2±3.4	42.5±0.6	42.8±0.2	51.3
Unknown (ECL[d] 16.090)	—	—	0.8±0.3	—	—
17:0 *ω9c* iso	0.5±0.05	—	—	—	—
17:1 *ω8c*	—	1.0±0.1	—	—	—
17:1 *ω6c*	—	3.1±0.7	—	—	—
17:0 cyclo	7.4±0.5	—	33.2±3.5	7.0±0.1	17.2
17:0	7.6±1.3	19.9±3.9	3.2±0.4	4.5± 0.3	2.7
18:1 *ω7c*	10.0±0.2	11.9±1.8	—	18.4±0.2	6.6
18:0	1.3±0.3	2.2±0.8	4.9±0.2	2.5±0.01	1.6
Unknown (ECL 18.082)	1.5±0.2	3.2±1.7	—	—	—
Unknown (ECL 18.422)	—	—	1.0±0.3	—	—
Unknown (ECL 18.824)	—	0.7±0.2	—	—	—
19:0 cyclo *ω8c*	—	—	—	0.5±0.1	1.1

[a]Values for fatty acids present at levels of less than 0.5% are not shown.
[b]Not detected.
[c]Results from Ko et al. [10].
[d]ECL, equivalent chain length.

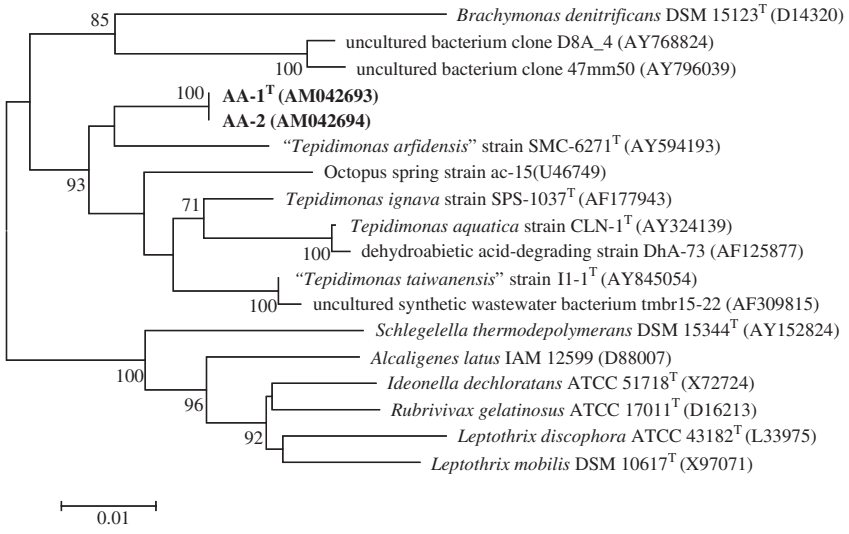

Fig. 1. Phylogenetic dendrogram based on a comparison of the 16S rDNA sequences of strains AA-1^T and AA-2, and the closest phylogenetic relatives. Scale bar, 10 inferred nucleotide substitutions per 100 nucleotides.

Description of *Tepidimonas thermarum* sp. nov.

Tepidimonas thermarum (ther.ma'rum; L. fem. pl. n. *thermae*, public baths; L. gen. pl. n. *thermarum* of public baths, because the organism was isolated from the Elisenquelle spa in Aachen, Germany). *Tepidimonas thermarum* forms short rod-shaped cells 1.0–2.0 μm in length and 0.5–1.0 μm in width. Gram stain is negative. The cells are motile by one polar flagellum. Colonies on

Degryse 162 medium are not pigmented and are 1–2 mm in diameter after 48 h of growth. The optimum growth temperature is about 50–55 °C; growth does not occur at 25 and 60 °C. The optimum pH is between 7.5 and 8.5; growth does not occur at pH 5.5 and 9.8. The major fatty acids are 16:0 and 16:1 $\omega 7c$. Ubiquinone 8 is the major respiratory quinone. Strictly aerobic, cytochrome oxidase and catalase positive. Yeast extract or growth factors are required for growth. Do not reduce nitrate to nitrite. Thiosulfate is oxidized to sulfate. Gelatin was degraded; esculin, arbutin, casein, elastin, starch and xylan were not degraded. Several organic acids and amino acids are utilized for growth, namely succinate, pyruvate, acetate, glutamate, lactate, proline, L-aspargine, L-glutamine, L-alanine, L-lysine, L-isoleucine and L-ornithine, but the strains do not utilize carbohydrates and polyols. The mole G+C ratio of the DNA is 67.9%. This bacterium was isolated from the Elisenquelle at Aachen in Germany. The type strain, AA-1^T, has been deposited in the BCCM/LMG Bacteria Collection, Ghent, Belgium as strain LMG 23094^T and in the Collection of the Institute Pasteur, Paris, France as strain CIP 108777^T. Strain AA-2 (= LMG 23095; = CIP 108778) is an additional strain of this species.

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