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Thermobacterium salinum gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a hydrothermal zone

Beihan Chen, Haichen Zhang, Dan Lin, Jianing Ye and Kai Tang*

Abstract

A Gram-stain-negative, aerobic, non-motile and rod-shaped bacterial strain, designated as strain TK19130^T, was isolated from the Lonqi hydrothermal zone in the Southwest Indian Ridge. Growth occurred with 1–12% (w/v) NaCl (optimum, 2–4%), at 10–40°C (optimum, 30–35°C) and at pH 6.0–9.0 (optimum, pH 7.0–8.0). The genome of strain TK19130^T was 3.15 Mb, with a DNA G+C content of 41.35%. Based on the results of 16S rRNA gene sequence analysis, strain TK19130^T was affiliated with the family *Flavobacteriaceae*, in which the highest similarity was 90.54% to *Aureisphaera salina* A6D-50^T, under the genus demarcation boundary (94.50%). Average nucleotide identity values between strain TK19130^T and adjacent strains were 67.17–72.00%, lower than the recommended threshold of 73.98% for genus delineation. The predominant respiratory quinone of strain TK19130^T was menaquinone 6. Major polar lipids were phosphatidylethanolamine, three aminolipids and one unidentified polar lipid. Major fatty acids were detected as iso-$C_{15:1}$ G, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. Based on the polyphasic taxonomic evidence presented above, strain TK19130^T formed an independent branch representing a new species of a novel genus within the family *Flavobacteriaceae*, for which the name *Thermobacterium salinum* gen. nov., sp. nov. is proposed. The type strain is TK19130^T (=CGMCC 1.18993^T=JCM 35842^T=MCCC M28200^T).

INTRODUCTION

The family *Flavobacteriaceae* was validly published in 1992 [1] and was subject to three major emendations [2–4]. At the time of writing, this family contains 151 validly published and correctly named genera [5], which have been widely found in fresh water [6], seawater [7, 8] and marine sediments [9]. Members of *Flavobacteriaceae* show a rapid response to the introduction of organic matter, becoming dominant in aquatic ecosystems when these substrates are introduced [10]. It is believed that *Flavobacteriaceae* are skilled at using high molecular weight components when phytoplankton bloom occurs, thus helping in the transformation of phytoplankton-derived organic matter [11]. This process, which is driven by these prevalent heterotrophic bacteria, can convert phytoplankton-derived organic matter into CO_2 or bacterial biomass. These products then enter the marine food web or contribute to the recalcitrant carbon pool in the ocean [11]. However, limited strains of the family *Flavobacteriaceae* have been studied in the deep-sea hydrothermal vent zones [12], where special organic carbon is produced via chemosynthesis [13]. Here we report a new species of a novel genus within the family *Flavobacteriaceae* isolated from the Southwest Indian Ridge hydrothermal zone. The isolation of strain TK19130^T will help to understand the potential organic matter metabolism process of the family *Flavobacteriaceae* in deep-sea hydrothermal zone.

Author affiliations: [1]State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Science, Fujian Key Laboratory of Marine Carbon Sequestration, Xiamen University, Xiamen, PR China.
***Correspondence:** Kai Tang, tangkai@xmu.edu.cn
Keywords: *Thermobacterium salinum*; hydrothermal zone; polyphasic taxonomic approach.
Abbreviations: AL, aminolipid; ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; L, polar lipid; MK-6, menaquinone 6; ML, maximum-likelihood; NJ, neighbour-joining; PE, phosphatidylethanolamine; TEM, transmission electron microscopy; UBCG, up-to-date bacterial core gene.
The GenBank accession number of the 16S rRNA gene sequence of strain TK19130^T is OM744364. The bioSample number, Genbank accession number and GenBank assembly number of strain TK19130^T are SAMN25961263, JALAHT000000000.1 and GCA_025384855, respectively. The culture collection accession numbers of strain TK19130^T are CGMCC 1.18993^T, JCM 35842^T and MCCC M28200^T.
Three supplementary figures are available with the online version of this article.

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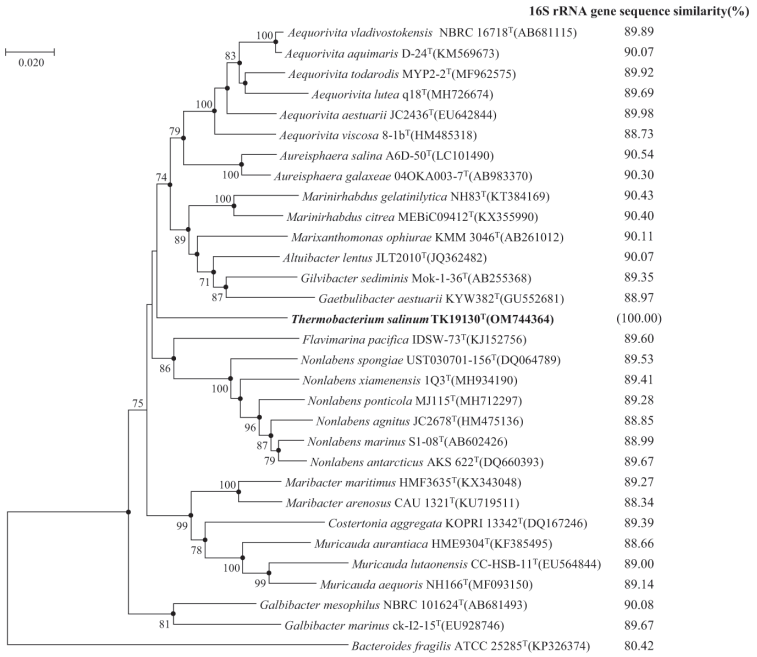


Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain TK19130^T within the family *Flavobacteriaceae*. Bootstrap values are shown at nodes as percentages of 1000 replicates. Values under 70% are hidden. Bar, 0.02 changes per nucleotide position. 16S rRNA similarities are shown next the tree. Filled circles represent nodes found in both the NJ tree and the ML tree.

ISOLATION AND CULTIVATION

Seawater samples were collected within the Lonqi hydrothermal zone of the mid-ridge of the Southwest Indian Ocean at a depth of about 3300 m and a latitude and longitude of 37.78048° S and 49.62056° E. The *in situ* temperature and salinity were 2.22°C and 34.7423 psu, in March 2019. The samples were preserved at 4°C before laboratory experiments in August 2019. One hundred microlitres of the sample was taken and spread on marine agar 2216 plates and incubated for 3 days at 28°C. After three rounds of plate streaking from a single sample, a yellow-coloured colony was considered pure and selected for the following study. The strain was preserved at −80°C with 15% (v/v) glycerol solution in 2216E liquid medium.

16S RRNA GENE PHYLOGENY

The 16S rRNA gene sequence of strain TK19130^T was amplified by PCR using universal primers: 27F (5′-AGAGTTTGATC-CTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGATCCAGCC-3′). The amplification product was subjected to Sanger sequencing. The full-length 16S rRNA gene sequence was extracted with the online tool ContEst16S (www.ezbiocloud.net/tools/contest16s) [14] and identified through the EzBioCloud database for top hit strains. The 16S rRNA gene sequences of top hit strains were downloaded from the NCBI database (www.ncbi.nlm.nih.gov). Then, 16S rRNA phylogenetic trees were reconstructed by MEGA version 7.0 [15] using the neighbour-joining (NJ) algorithm with Kimura's two-parameter method [16] and the maximum-likelihood (ML) algorithm with the K2 +G+I model, both bootstraps were tested in 1000 replications. 16S rRNA gene sequence similarities aligned with the NJ tree were recalculated via BLASTn on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), with the 16S/ITS database selected.

The amplified 16S rRNA gene sequence sequenced with the 27F-1492R primer set was 1438 bp and the extracted one was 1529 bp. Additionally, during the splicing of the amplified DNA fragment sequence, poorly sequenced regions could have been lost. Strain TK19130^T showed the highest 16S rRNA gene sequence similarity of 90.54% to *Aureisphaera salina* A6D-50^T, followed by *Marinirhabdus gelatinilytica* NH83^T and *Marinirhabdus citrea* MEBiC09412^T of 90.43 and 90.40%. These similarities are under the threshold for defining a novel genus of 94.50% [17] (Fig. 1). Furthermore, strain TK19130^T formed a deep novel branch within the family *Flavobacteriaceae* and was distinct from relative genera in the NJ and ML trees, which supports the proposal that strain TK19130^T represents a novel genus of the family *Flavobacteriaceae*and(Fig. 1 and Fig. S1) available in the online version of this article).

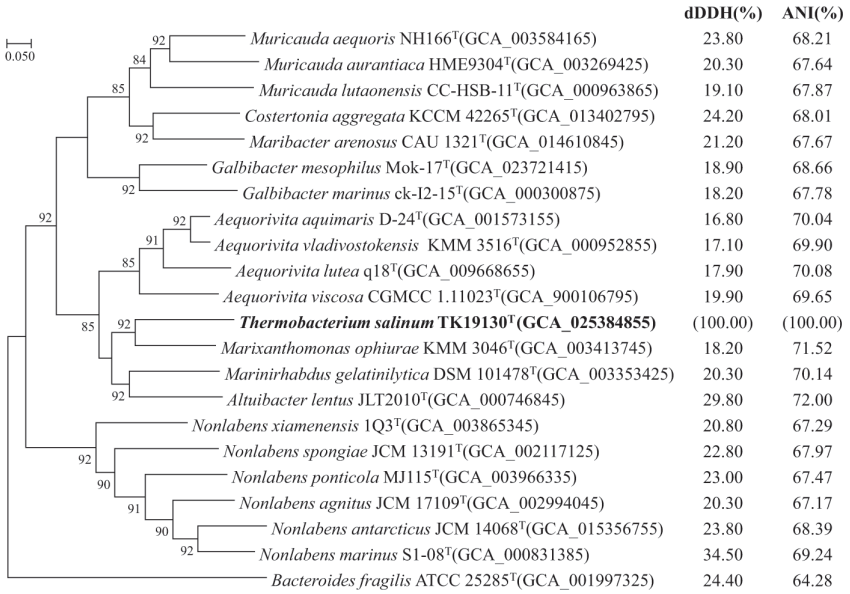


Fig. 2. The UBCG phylogenetic tree based on 92 core genes indicating the position of strain TK19130^T within the family *Flavobacteriaceae*. Bootstrap values are shown at nodes as percentages of 1000 replicates. Values under 70% are hidden. Bar, 0.05 substitutions per nucleotide base. ANI values are shown next to the UBCG tree.

GENOME FEATURES

Genomic DNA (>100 ng μl^{-1}) was extracted by a GENEray Bacteria DNA kit (Generay), split to about 500 bp, ligated with adapters, purified by gel electrophoresis and sequenced using Illumina HiSeq. After quality control, the reads were assembled by Velvet (version 1.2.10) [18, 19] and gapfilled with SSPACE (version 3.0) [20, 21] and GapFiller (version 1–10) [22]. Then, the digital DNA G+C content was analysed using EzBioCloud (www.ezbiocloud.net/tools/ani) [23] from the assembled draft genome. Twenty-two available assembled genomes of related valid hit strains were downloaded from the Assembly database on the NCBI website (www.ncbi.nlm.nih.gov/assembly). A phylogenomic tree was then reconstructed based on the up-to-date bacterial core gene (UBCG) set and pipeline, following the instructions of the manual (www.ezbiocloud.net/tools/ubcg) [24]. All parameters followed the default set up. Average nucleotide identity (ANI) [25] values were calculated using an online ANI calculator (www.ezbiocloud.net/tools/ani) [23]. Digital DNA–DNA hybridization (dDDH) estimates between strain TK19130^T and relative strains listed in the next paragraph were obtained using the online Genome-to-Genome Distance Calculator (GGDC 3.0; https://ggdc.dsmz.de/distcalc2.php) [26].

The whole genome shotgun sequence of strain TK19130^T was about 3.15 Mb on 33 contigs in total, with an N50 size of 427132 bp and complete coverage. The genome described above was predicted to have 2933 protein coding genes, 5 rRNA genes, 36 tRNA genes and 12 other non-coding genes. The digital DNA G+C content was 41.35%. The UBCG phylogenomic tree indicated that strain TK19130^T formed a deep but separate branch in the family *Flavobacteriaceae* (Fig. 2). In addition, the ANI values between strain TK19130^T and surrounding strains were 67.17–72.00%, lower than the recommended mean threshold of 73.98% (95% CI 73.34–74.62%) for genus definition [27] (Fig. 2). Moreover, dDDH estimates between strain TK19130^T and relative strains were 16.80–34.50% with all probabilities of equal or greater than 70% [28] close to zero. These results support that strain TK19130^T represents a new species in the family *Flavobacteriaceae* (Fig. 2). Combined with the 16S rRNA gene sequence analysis results, strain TK19130^T represented a novel genus within the family *Flavobacteriaceae*. Considering the 16S rRNA gene sequence similarity, phylogenetic relationship and availability, *Marinirhabdus gelatinilytica* CGMCC 1.15462^T [8], *Altuibacter lentus* JLT2010^T [7], *Galbibacter mesophilus* NBRC 101624^T [9], *Nonlabens xiamenensis* MCCC 1A14023^T [29] and *Aequorivita viscosa* CGMCC 1.11023^T [30] were chosen as type strains and tested under the same conditions for the following experiments.

PHYSIOLOGY AND CHEMOTAXONOMY

Strain TK19130^T was cultured on marine agar 2216E at a constant temperature of 28°C for morphological, physiological, biochemical and chemotaxonomic experiments. A Gram stain kit (HB8278, Hopebio) was used for the Gram stain test. Flexirubin pigments were tested using 20% (w/v) KOH. Congo red adsorption was tested by 0.01% (w/v) aqueous solution of the dye. Cell morphology was observed by transmission electron microscopy (TEM; Tecnai G2 Spirit BioTwin) after 2 days of growth. Before the TEM observation, the cells were first diffused into sterile seawater and adsorbed onto the copper mesh. Then, the cells were

gently washed with a small amount of Milli-Q water and stained with 0.5% uranyl acetate for 7 s. Finally, the cells were dried on the copper mesh at room temperature. Cell motility was examined by semi-solid puncture method with agar content of 0.5% (w/v). Swimming motility was observed by the hanging drop method [3]. Gliding motility was tested on 1% agar plates [31]. In a semi-solid puncture experiment, the colonies were picked with sterile inoculation needles, inoculated into semi-solid medium by vertical puncture, and cultured at 28°C for 3 days. Then we observed whether there was growth diffusion along the inoculation path. If so, cells of strain TK19130^T were judged to be mobile, and *vice versa*. Anaerobic growth and capnophilic metabolism were tested using an anaerobic gas pack (2.5 l; HBYY001, Hopebio) and a microaerobic gas pack (2.5 l; HBYY008, Hopebio) for a month to observe colony formation. Oxidase activity was tested using oxidase reagent (Huankai Microbial) to observe if the colony culture turned purple in 30 s. Catalase activity was determined by bubble production at 3% H_2O_2. Salt tolerance was tested in a rich organic liquid medium (KCl 0.3 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2{\cdot}2H_2O$ 0.038 g, NH_4Cl 0.3 g, K_2HPO_4 0.3 g, yeast 1.0 g, tryptone 1.0 g, CH_3COONa 1.0 g and Milli-Q water 1.0 l) at 28°C, with a range of 1–12% (with 1% intervals, w/v) and 15% NaCl. Temperature tolerance was investigated at 4, 10, 15, 20, 25, 30, 35, 40 and 45°C on marine agar 2216E. The pH range for growth was assessed in 2216E liquid medium, with pH ranging from pH 3 to 12 (in 1 pH unit increments) adjusted with 2 mol l^{-1} HCl

Table 1. Morphological, physiological, biochemical and chemotaxonomic characteristics of strain TK19130^T compared to the closest related genera within the family *Flavobacteriaceae*

Strains: 1, TK19130^T; 2, *Marinirhabdus gelatinilytica* CGMCC 1.15462^T; 3, *Galbibacter mesophilus* NBRC 101624^T; 4, *Altuibacter lentus* JLT2010^T; 5, *Nonlabens xiamenensis* MCCC 1A14023^T; 6, *Aequorivita viscosa* CGMCC 1.11023^T. +, Positive; –, negative. NR, not reported. In descending order according to 16S rRNA gene sequence similarity.

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**
Isolation source	Hydrothermal-zone seawater	Surface seawater§	Marine sediment†	Seawater‡	Coastal seawater*	seaweed¶
Cell size (μm)	0.4–0.5×1.1–1.6	0.5–0.8×2.5–4.5*	0.5–0.7×1.0–3.0†	0.4–0.6×1.1–1.8‡	1.0–6.0×0.7–1.0§	4.0–4.5×1.8–2.2¶
Genome size (Mb)	3.15	3.3‡‡	3.8**	3.2††	3.7§	3.5§§
DNA G+C content (mol%)	41.35	41‡‡	37**	42††	41.1§	36.5§§
Optimum growth:						
Temperature (°C)	30–35	30*	25–30†	25–30‡	28§	28–32¶
NaCl (%, w/v)	2.0–4.0	2.0–5.0*	3.0–5.0†	3.0‡	NR	1–3¶
pH	7.0–8.0	7.0*	NR†	7.0–8.0‡	7.0–8.0§	7.0–8.5¶
Oxidase activity	–	–*	+†	+‡	+§	–¶
Catalase activity	+	+*	+†	+‡	–§	+¶
Main respiratory quinone	MK-6	MK-6*	MK-6†	MK-6‡	MK-6§	MK-6¶
API ZYM (partial):						
Lipase (C14)	+	+	+	+	+	–
α-Chymotrypsin	+	+	+	+	+	–
α-/*β*-Glucosidase	–/–	–/–	+/+	–/–	–/+	–/–
API 20NE (partial):						
Nitrate reduction	–	–	+	–	–	–
4-Nitrophenyl-*β*-D-galactopyranoside	–	–	+	–	–	–

*Data taken from Wu *et al*. [8].
†Data taken from Khan *et al*. [9].
‡Data taken from Chen *et al*. [7].
§Data taken from Huang *et al*. [29].
¶Data taken from Liu *et al*. [30].
**Data taken from NCBI. Accession: ASM2372141v1.
††Data taken from NCBI. Accession: ASM74684v1.
‡‡Data taken from NCBI. Accession: ASM335342v1.
§§Data taken from NCBI. Accession: GCF_900106795.1.

Table 2. Cellular fatty acid composition of strain TK19130^T and related type strains of the family *Flavobacteriaceae*

Strains: 1, TK19130^T; 2, *Nonlabens xiamenensis* MCCC 1A14023^T; 3, *Galbibacter mesophilus* NBRC 101624^T; 4, *Altuibacter lentus* JLT2010^T; 5, *Marinirhabdus gelatinilytica* CGMCC 1.15462^T; 6, *Aequorivita viscosa* CGMCC 1.11023^T. All cells were harvested after 3 days of growth. All data taken from this study. –, Trace (< 1.0%) or not detected. Major fatty acids (>10%) are highlighted in bold.

Fatty acid (%)	1	2	3	4	5	6
iso-$C_{11:0}$	1.3	–	–	–	–	–
iso-$C_{15:1}$ G	**20.3**	–	**13.4**	**13.9**	8.9	4.1
anteiso-$C_{15:1}$ A	1.2	–	–	–	–	–
iso-$C_{15:0}$	**24.1**	**29.5**	**10.5**	**17.0**	**35.3**	**31.3**
anteiso-$C_{15:0}$	6.8	**12.7**	–	6.0	2.7	5.2
iso-$C_{16:1}$ G	1.1	–	–	2.1	1.9	–
iso-$C_{16:0}$	1.1	5.0	1.4	8.8	**11.9**	2.0
$C_{16:0}$	2.5	–	–	–	–	3.6
iso-$C_{15:0}$ 3-OH	2.8	3.0	5.3	3.1	3.0	3.2
iso-$C_{16:0}$ 3-OH	2.6	2.3	2.0	6.4	8.2	2.5
iso-$C_{17:0}$ 3-OH	**19.1**	**11.6**	**21.8**	**16.9**	**13.2**	**13.9**
$C_{17:0}$ 2-OH	2.6	3.6	–	2.8	–	1.0
Summed feature 3*	7.8	7.4	**16.0**	**10.8**	2.4	2.2

*Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. Summed feature 3 contains $C_{16:1}$ $\omega 6c$ and/or $C_{16:1}$ $\omega 7c$.

and NaOH at 28°C. In order to avoid a change of pH in the culture medium, the medium in the adjusted pH test was sterilized through filtration (Millex-GP, 0.22 µm) rather than autoclaving. Growth tests for salt, temperature and pH tolerance were all performed in triplicates in 2 days and detected by photometric measurement at 600 nm (OD_{600}) using a hybrid multi-mode microplate reader (H1, Biotek Synergy). Identification of the utilization of certain organic substrates was performed in Biolog GENIII MicroPlates, with cells suspended in IF-A inoculum, and cultured for 7 days at 28°C. As a supplement, sole carbon utilization experiments were performed in minimum medium (artificial seawater 1 l, trace metal 1 ml, vitamin 1 ml, carbon source 1 g, pH 7.8–8.0) for 4 days at 28°C. D-Fructose, maltose, gelation, D-glucose, D-mannose, soluble starch, sodium acetate, sodium pyruvate, and yeast, which was set up as positive control, were used as sole carbon sources. API 20NE and API ZYM tests were performed according to the manufacturer's instructions, using the recommended incubation time and an excepted temperature of 28°C. Cellular fatty acids were saponified, methylated and extracted as described by Komagata and Suzuki [32] and identified following the instructions of the Microbial Identification System (MIDI; Sherlock version 6.0). Strain TK19130^T and related type strains were examined under the same conditions in the API and fatty acid profile tests. Respiratory quinones were identified by ultra performance liquid chromatography/tandem mass spectrometry. Polar lipids of strain TK19130^T were extracted through a chloroform–methanol system, then analysed using one- and two-dimensional thin-layer chromatography.

Cells of strain TK19130^T were Gram-stain-negative, aerobic, non-motile and rod-shaped, which were approximately 0.4–0.5×1.1–1.6 µm in size (Fig. S2). Swimming motility and gliding motility were negative. Growth was not observed under microaerobic conditions. Flexirubin-type pigments and Congo red absorbtion results were negative. Large amounts of vesicles, which were about 0.05–0.1 µm in diameter and surrounded the outer membrane, were observed in the TEM image. This surface structure is similar to that of two reported *Flavobacteriaceae* strains [33, 34], and may be rich in degradative enzymes and help to utilize polysaccharide substrate. Colonies were yellow-pigmented, smooth-edged, oil-like and about 2.0–3.0 mm in diameter. Activities of catalase, oxidase and other enzymes, growth characteristics, and certain substrate utilization results are described in Table 1. Results of the Biolog GEN III MicroPlate and carbon utilization experiments indicated that acetic acid and gelatin were utilized as sole carbon sources. Iso-$C_{15:1}$ G, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH were found to be major fatty acids in strain TK19130^T. The fatty acid profile of strain TK19130^T was consistent with the phenomenon that iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH also accounted for a large proportion of fatty acids in the type strains (Table 2). However, the overall fatty acids profile of the strains showed a notable difference from those of compared strains (Table 2). The predominant respiratory quinone found in strain TK19130^T was menaquinone 6 (MK-6), which is common in the family *Flavobacteriaceae*. Phosphatidylethanolamine, three aminolipids and one unidentified polar lipid were detected as major polar lipids in strain TK19130^T (Fig. S3).

TAXONOMIC CONCLUSION

Based on the above results, strain TK19130^T represents a new species of a novel genus, for which the name *Thermobacterium salinum* gen. nov., sp. nov is proposed.

DESCRIPTION OF *THERMOBACTERIUM* GEN. NOV. NON *THERMOBACTERIUM* BUCHANAN 1918

Thermobacterium (Ther.mo.bac.te'ri.um. Gr. masc. adj. *thermos*, from hydrothermal zone; N.L. neut. n. *bacterium*, rod; N.L. masc. n. *Thermobacterium*, rod from thermophilic zone).

Cells are Gram-stain-negative, aerobic, non-motile, rod-shaped, oxidase-negative and catalase-positive. The major cellular fatty acids are iso-$C_{15:1}$ G, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. MK-6 is the predominant respiratory quinone. Major polar lipids are phosphatidylethanolamine, three aminolipids and one unidentified polar lipid. The DNA G+C content of the type species is 41.35mol%. The type species is *Thermobacterium salinum*.

DESCRIPTION OF *THERMOBACTERIUM SALINUM* SP. NOV.

Thermobacterium salinum (sa.li'num. (N.L. neut. adj. *salinum*, salted).

Cells are Gram-stain-negative, strictly aerobic, non-motile and rod-shaped, about 0.4–0.5 μm wide and 1.1–1.6 μm long. Colonies are bright yellow after 2–3 days incubation on marine agar 2216E plates. Swimming motility and gliding motility are negative. Growth occurs at 1–12% (w/v) NaCl (optimum, 2–4%), at 10–40°C (optimum, 30–35°C) and at pH 6.0–9.0 (optimum, pH 7.0–8.0). Acetic acid and gelatin are utilized as sole carbon sources. The API ZYM system shows positive results for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, a-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but negative results for *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase and *β*-fucosidase. The API 20NE test shows positive results for aesculin and gelatin hydrolysis, but negative results for nitrate reduction, arginine dihydrolase, urease and 4-nitrophenyl-β-D-galactopyranoside utilization. MK-6 is the predominant respiratory quinone. Major polar lipids contain phosphatidylethanolamine, three aminolipids and one unidentified polar lipid. Iso-$C_{15:1}$ G, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH are major fatty acids (> 10%).

The type strain is TK19130^T (=CGMCC 1.18993^T=JCM 35842^T=MCCC M28200^T), isolated from the Southwest Indian Ridge hydrothermal zone (37.78048° S 49.62056° E), at a depth of 3300 m. The draft genome of strain TK19130^T is 3.15 Mb in size, with a DNA G+C content of 41.35%. The GenBank accession number of the 16S rRNA gene sequence of strain TK19130^T is OM744364. The bioSample, GenBank accession and GenBank assembly numbers for strain TK19130^T are SAMN25961263, JALAHT000000000.1 and GCA_025384855, respectively.

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Author contributions
B.C., H.Z., D.L. and J.Y. designed and performed the experiments. B.C. analyzed the data. B.C., D.L., H.Z. and K.T. wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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