Tepidibacter mesophilus sp. nov., a mesophilic fermentative anaerobe isolated from soil polluted by crude oil, and emended description of the genus *Tepidibacter*

Hai-Qin Tan, Xiao-Yue Wu, Xin-Qi Zhang, Min Wu and Xu-Fen Zhu

College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

Correspondence
Xu-Fen Zhu
xufenzhu@zju.edu.cn

A mesophilic, aerotolerant, endospore-forming, fermentative bacterium, designated strain B1^T, was isolated from soil polluted by crude oil in the Karamay Oil Field, China. Cells were Gram-positive, rod-shaped, 1.1–1.6 μm wide and 2.3–4.7 μm long, and were motile by means of peritrichous flagella. Growth occurred at 10–40 °C and pH 6.0–8.9; optimal growth occurred at 28–32 °C and pH 7.3. The optimal concentrations of NaCl and sea salts for growth were 0.5 and 1 % (w/v), respectively. The strain was halotolerant and grew in the presence of NaCl or sea salts up to a concentration of 9 % (w/v). Substrates utilized as sole carbon sources were beef extract, yeast extract, peptone, tryptone, casein, D-glucose, D-fructose, D-xylose, D-ribose, D-galactose, maltose, L-rhamnose, trehalose, L-valine, DL-alanine plus L-proline and DL-alanine plus L-glycine. The main products of glucose fermentation were ethanol and acetate. iso-$C_{15:0}$, iso-$C_{14:0}$, $C_{16:0}$ and iso-$C_{13:0}$ were the major fatty acids. 16S rRNA gene sequence analysis revealed that the isolate belongs to the genus *Tepidibacter*, showing 94.7 and 94.1 % similarity to the type strains of *Tepidibacter formicigenes* and *Tepidibacter thalassicus*, respectively. The genomic DNA G+C content of strain B1^T was 29.8 mol%. On the basis of its phenotypic and genotypic properties, strain B1^T is suggested to represent a novel species of the genus *Tepidibacter*, for which the name *Tepidibacter mesophilus* sp. nov. is proposed. The type strain is B1^T (=CGMCC 1.5148^T =JCM 16806^T).

The genus *Tepidibacter* was proposed by Slobodkin *et al.* (2003) with *Tepidibacter thalassicus* as the type species. *Tepidibacter formicigenes* was subsequently described by Urios *et al.* (2004). The two species were isolated from hydrothermal vents, and are thermophilic. Here we describe a novel fermentative, aerotolerant bacterium isolated from an oilfield as representing a third species of the genus. It was isolated from warm soil polluted by crude oil, and was mesophilic.

Strain B1^T was isolated from soil polluted by crude oil in the Karamay Oil Field (45° 36′ N 84° 57′ E) in north-western China in June 2009. For initial enrichment we used medium YC (pH 7.0), which contains (per litre distilled water) 20 g NaCl, 0.9 g $MgCl_2.6H_2O$, 0.33 g KCl, 1.4 g $MgSO_4.7H_2O$, 0.14 g $CaCl_2.2H_2O$, 0.25 g NH_4Cl, 0.45 g K_2HPO_4, 1 g yeast extract (Difco), 3 g Casamino acids, 1 ml trace element solution SL-10 (Widdel *et al.*, 1983), 0.4 g L-cysteine, 0.5 g $Na_2S.9H_2O$ and 0.001 g resazurin. Serum bottles (50 ml) containing a liquid phase of YC medium under a gas phase of O_2-free N_2 were sealed and autoclaved for 20 min at 121 °C. Bottles were anaerobically inoculated with approximately 2 g soil sample and incubated at 32 °C for 2 days. The Hungate roll-tube technique (Hungate, 1969; Bryant, 1972) was used to isolate strains from the turbid enrichment cultures. Strains were purified at least twice before being preserved in 25 % glycerol at −80 °C. One of the isolates obtained, designated strain B1^T, was phylogenetically affiliated with the genus *Tepidibacter*, showing 94.7 and 94.1 % 16S rRNA gene sequence similarity to the type strains of *T. formicigenes* and *T. thalassicus*, respectively. Strain B1^T also grew in aerobic medium.

DSMZ medium 985, in which the vitamin solution was replaced by 1 g yeast extract l^{-1}, was used as the basal medium for cultivation of strains B1^T, *T. formicigenes* DSM 15518^T and *T. thalassicus* DSM 15285^T. Unless stated otherwise, the medium was adjusted to pH 7.0, before sterilization for 20 min at 121 °C, and cultures were grown under a gas phase of O_2-free N_2. Cell morphology and motility were examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) during the late-exponential or stationary growth phases. Colonies of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B1^T is GQ231514.

One supplementary figure is available with the online version of this paper.

strain B1^T were white and 0.2–0.5 mm in diameter after growing on modified medium 985 for 20 h. Cells of strain B1^T were rods (1.1–1.6 × 2.3–4.7 μm) with peritrichous flagella. Round terminal endospores were also observed. Thin-section electron micrographs revealed a Gram-positive cell-wall structure and a lamellar structure in its endospores (Supplementary Fig. S1 available in IJSEM Online).

To determine the optimal conditions for growth, we incubated the isolate at 4, 10, 13, 15, 18, 21, 25, 28, 32, 35, 37, 40 and 45 °C. To test the pH range of growth, we adjusted the pH of the medium with the following buffers: MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CAPSO (pH 9.0–10.0), at a concentration of 25 mM. To examine the effect of salt concentration on growth, different concentrations of NaCl or sea salts were added [0–10 % (w/v), in increments of 0.5 %]. Strain B1^T showed optimal growth at 28–32 °C and pH 7.3. No growth was observed at 4 or 45 °C after incubation for 10 days. The pH range for growth was 6.0–8.9. Optimal growth was observed

Table 1. Differential phenotypic, physiological and genotypic characteristics between strain B1^T and the type strains of recognized species of the genus *Tepidibacter*

Strains: 1, B1^T; 2, *T. formicigenes* DSM 15518^T; 3, *T. thalassicus* DSM 15285^T. Beef extract, peptone, tryptone, yeast extract, D-glucose, D-arabinose, cellulose, maltose and DL-alanine plus L-proline were used by all three strains. None of the strains used chitin, filter paper, olive oil, lactose, mannose, DL-alanine, L-glycine, acetate, betaine, butyrate, formate, fumarate, glycerol, lactate, methanol, D-sorbitol, succinate or urea. All strains were negative for the use of sodium thiosulfate, sodium sulfite, sodium sulfate, sodium nitrite, sodium nitrate and $FeCl_3$ as electron acceptors. Oxidase and catalase activity were negative for all strains.

Characteristic	1	2	3
Growth			
Optimum temperature (°C)	28–32	45	50
Temperature range (°C)	10–40	25–55	28–60
Optimum pH	7.3	6.5–6.8	6.5–7.0
pH range	6.0–8.9	5.0–8.5	5.0–8.5
Optimum NaCl concentration (%, w/v)	0.5	2	2
Optimum sea salts concentration (%, w/v)	1	3	2.5
S^0 as electron acceptor	+	−	+
Utilization of:			
Albumin	−	−	+
Casein	+	−	+
Gelatin	−	+*	−
Starch	+	+*	+
D-Fructose	+	−	−
D-Galactose	+	−	−
D-Ribose	+	−	−
L-Rhamnose	+	−	−
Sucrose	−	+	−
Trehalose	+	−	−
D-Xylose	+	−	−
L-Arginine	−	−	+
L-Valine	+	+	−†
DL-Alanine + L-glycine	+	+	+‡
D-Mannitol	−	+	−
Ethanol	−	+	−
Pyruvate	−	+	+
Fermentation products from glucose	Acetate, ethanol	Formate, acetate, ethanol	Ethanol, acetate, H_2, CO_2
DNA G+C content (mol%)	29.8	28.3	24.9

*Urios *et al.* (2004) reported a negative reaction.
†Slobodkin *et al.* (2003) reported a positive reaction.
‡Slobodkin *et al.* (2003) reported a negative reaction.

in the presence of 0.5–1 % NaCl (w/v). No growth occurred with 9.5 % NaCl or sea salts. Under optimal growth conditions, the generation time of strain B1^T was 1.2 h.

Assimilation of carbon sources was tested under a gas phase of O_2-free N_2, by using the basal salts of the modified medium 985, supplemented with 0.01 % yeast extract (BD). Complex proteinaceous substrates (10 g l^{-1}) such as tryptone (BD), yeast extract, casein (BD), peptone (BD) and beef extract, polysaccharides (10 g l^{-1}) such as filter paper, chitin and starch, and organic acids (20 mM), olive oil (10 g l^{-1}), methanol (20 mM), ethanol (20 mM), glycerol (25 mM), sorbitol (25 mM), glycine betaine (5 mM) and urea (0.5 %) were autoclaved for 20 min at 121 °C. Sugars (added at a concentration of 20 mM), amino acids (20 mM) and albumin (10 g l^{-1}) were sterilized under UV light. All tests were performed in triplicate. To analyse the use of accessory electron acceptors, elemental sulfur (150 meq l^{-1}), sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM), sodium nitrate (20 mM) or $FeCl_3$ (20 mM) was added from autoclaved stock solutions to medium YC (lacking the reductant Na_2S, L-cysteine and Casamino acids). Oxygen was removed as described by Grishchenkov *et al.* (2000). Reduction of the electron acceptors was tested by using the method described by Ogg & Patel (2009). Production of H_2S was tested in sulfate-free medium by using lead acetate paper, as indicated by Alain *et al.* (2002). The results of these phenotypic tests are given in Table 1 and in the species description below.

Fatty acid methyl esters were obtained from freeze-dried cells of strain B1^T and from the type strains of *T. formicigenes* and *T. thalassicus* as described by Kuykendall *et al.* (1988). The identification and quantification of the fatty acid methyl esters as well as the numerical analysis of the fatty acid profiles were examined as described by Zhang *et al.* (2010), matching the results with the ANAEROBE BHIBLA 3.80 library. The results are presented in Table 2. The major fatty acid in the three strains was iso-$C_{15:0}$, but its content was somewhat lower in strain B1^T than in the two reference strains, while the content of $C_{14:0}$, $C_{16:0}$, iso-$C_{13:0}$ and $C_{16:1}$ cis9 was significantly higher in strain B1^T than in the two other strains. Moreover, $C_{12:0}$, $C_{15:0}$ and $C_{16:1}$ cis7 were found in strain B1^T but not in *T. thalassicus* DSM 15285^T or *T. formicigenes* DSM 15518^T.

Genomic DNA was extracted and purified by using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR by using universal bacterial primers 27F and 1492R. Sequence similarity analysis and multiple sequence alignment were performed with the EzTaxon service (Chun *et al.*, 2007) and CLUSTAL W 1.8 (Thompson *et al.*, 1994), respectively. Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Fitch, 1971) with the MEGA4 program package (Tamura *et al.*, 2007). Evolutionary distances were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method. Consequently, the almost-complete 16S rRNA gene sequence (1438 nt) was obtained for strain B1^T. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the new isolate clustered with the two recognized members of the genus *Tepidibacter* (Fig. 1).

Table 2. Fatty acid profiles of strain B1^T and recognized members of the genus *Tepidibacter*

Strains: 1, B1^T; 2, *T. formicigenes* DSM 15518^T; 3, *T. thalassicus* DSM 15285^T. Values are percentages of the total fatty acids. −, Not detected.

Fatty acid	1	2	3
Unbranched saturated			
$C_{14:0}$	20.9	2.4	−
$C_{16:0}$	9.3	5.7	−
$C_{18:0}$	1.9	3.1	2.2
$C_{12:0}$	0.5	−	−
$C_{15:0}$	0.4	−	−
Branched saturated			
iso-$C_{15:0}$	51.8	77.7	97.0
iso-$C_{13:0}$	4.0	1.9	−
anteiso-$C_{15:0}$	1.5	4.0	−
iso-$C_{17:0}$	1.2	1.0	−
iso-$C_{14:0}$	0.6	0.7	0.8
Unsaturated			
$C_{16:1}$ cis9	6.6	2.1	−
$C_{16:1}$ cis7	0.7	−	−
$C_{18:1}$ cis9	0.4	1.0	−

The DNA G+C content was determined by HPLC, as described by Mesbah *et al.* (1989), with *Escherichia coli* K-12 DNA as the calibration standard. The genomic DNA G+C content of strain B1^T was 29.8 mol%.

Based on the phenotypic and genotypic characteristics described above, differences between strain B1^T and the type strains of the two related species of the genus *Tepidibacter* were evident. Strain B1^T utilized proteinaceous substrates and trace amounts of amino acids, but also some sugars such as D-fructose, D-galactose, D-ribose, trehalose, L-rhamnose and D-xylose (Table 1). It was able to assimilate more and different carbon sources than the recognized species of the genus *Tepidibacter*. Moreover, strain B1^T was mesophilic, being able to grow at 10–40 °C (optimally at 28–32 °C), while the two recognized species of the genus *Tepidibacter* were moderately thermophilic with optimal growth at 45 and 50 °C. Table 1 shows additional differences, such as the requirement for NaCl or sea salts for growth, and the main products of glucose fermentation. The major distinguishing chemotaxonomic characteristics were relatively larger amounts of $C_{14:0}$, $C_{16:0}$, iso-$C_{13:0}$, $C_{16:1}$ cis9 and iso-$C_{15:0}$, and a lower level of iso-$C_{15:0}$ in strain B1^T.

On the basis of data from the present polyphasic taxonomic characterization, we suggest that strain B1^T represents a novel species of the genus *Tepidibacter*, for which the name *Tepidibacter mesophilus* sp. nov. is proposed.

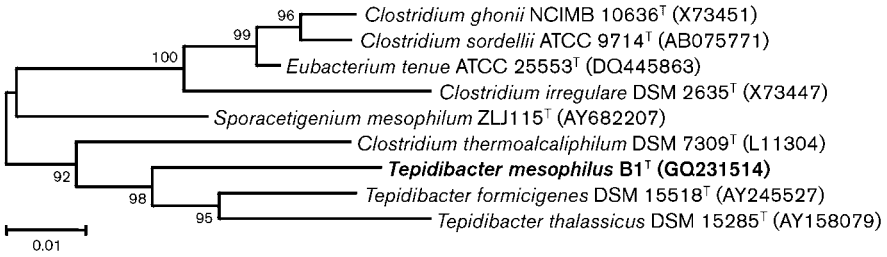

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain B1^T and related taxa. Numbers at nodes are bootstrap values based on 1000 replicates. Bar, 1 substitution per 100 nt positions.

Emended description of the genus *Tepidibacter*

Anaerobic or aerotolerant, and moderately thermophilic or mesophilic. Other properties are as given by Slobodkin *et al.* (2003).

Description of *Tepidibacter mesophilus* sp. nov.

Tepidibacter mesophilus [me.so.phi'lus. Gr. adj. *mesos* middle; Gr. adj. *philos* loving; N.L. masc. adj. *mesophilus* middle (temperature)-loving, mesophilic].

Cells are Gram-positive, aerotolerant, rod-shaped (1.1–1.6 × 2.3–4.7 μm) and motile by means of peritrichous flagella. Terminal spores are observed. Colonies are white, circular and convex with entire margins. Optimal growth occurs at 28–32 °C and pH 7.3. Growth occurs at 10–40 °C and pH 6.0–8.9. Grows in the presence of 0–9.0 % (w/v) (optimally at 0.5–1 %) NaCl or sea salts. Oxidase- and catalase-negative. H_2S is produced from cysteine or proteinaceous substrates. Utilizes beef extract, casein, peptone, tryptone, yeast extract, D-glucose, D-fructose, D-galactose, maltose, L-rhamnose, D-ribose, starch, trehalose, xylose, L-valine, DL-alanine plus L-proline and DL-alanine plus L-glycine, but not chitin, filter paper, olive oil, L-arabinose, cellulose, D-lactose, mannose, D-sorbitol, acetate, DL-alanine, L-glycine, betaine, butyrate, formate, fumarate, glycerol, lactate, methanol, succinate or urea. The main products of glucose fermentation are ethanol and acetate. Elemental sulfur is reduced to hydrogen sulfide. Sodium thiosulfate, sodium sulfite, sodium sulfate, sodium nitrite, sodium nitrate and $FeCl_3$ are not used as electron acceptors. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{14:0}$, $C_{16:0}$ and iso-$C_{13:0}$. The DNA G+C content of the type strain is 29.8 mol%.

The type strain, B1^T (=CGMCC 1.5148^T =JCM 16806^T), was isolated from an oil-polluted soil of the Karamay Oil Field in north-western China.

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