Thermosyntropho tengcongensis sp. nov., a thermophilic bacterium that degrades long-chain fatty acids syntrophically

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A novel anaerobic, thermophilic, syntrophic, fatty-acid-oxidizing bacterium, strain L-60^T, was isolated from a Chinese hot spring. Cells of the strain were non-motile, non-spore-forming, slightly curved rods. Growth occurred between 55 and 70 °C (optimum about 60 °C) and at pH 7.0–9.3 (optimum about pH 8.2). Crotonate was the only tested carbon source that supported the growth of the strain in pure culture. In co-culture with the thermophilic, hydrogenotrophic *Methanothermobacter thermautotrophicus* DSM 1053^T, the isolate could oxidize saturated straight-chain fatty acids with 4–18 carbon atoms, and also unsaturated fatty acids such as oleate, syntrophically. The strain was unable to utilize sulfate, sulfite, thiosulfate, nitrate, fumarate or Fe(III) as electron acceptors. The major cellular fatty acids were C_{14:0} (35.0%), C_{16:0} (20.3%) and iso-C_{17:1}/anteiso-C_{17:1} B (30.9%). The genomic DNA G + C content was 40.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain was affiliated to the family *Syntrophomonadaceae* and was most closely related to *Thermosyntropho lipolytica* DSM 11003^T (96.7% similarity). On the basis of phylogenetic and phenotypic evidence, it is proposed that strain L-60^T represents a novel species, for which the name *Thermosyntropho tengcongensis* sp. nov. is proposed. The type strain is L-60^T (=CGMCC 1.5161^T =JCM 17260^T).

Fatty acids such as butyrate are important intermediates in the anaerobic degradation of organic matter. As the reactions involved have unfavourable energetics, the fatty acids have to be degraded through a synergetic reaction that involves a partnership between syntrophic fatty-acid-oxidizing bacteria and hydrogen scavengers such as methanogens (Schink, 1992, 1997). To date, 11 syntrophic bacterial species have been described that, when associated with hydrogen-utilizing partners, are able to degrade fatty acids that have 4–18 carbon atoms (McInerney *et al.*, 1979, 1981; Stieb & Schink, 1985; Roy *et al.*, 1986; Beaty & McInerney, 1987; Lorowitz *et al.*, 1989; Zhao *et al.*, 1990; Svetlitsnyi *et al.*, 1996; Jackson *et al.*, 1999; Liu *et al.*, 1999; Sekiguchi *et al.*, 2000; Zhang *et al.*, 2004, 2005; Hatamoto *et al.*, 2007; Sousa *et al.*, 2007). The results of physiological characterization and phylogenetic analysis have led to these syntrophic bacteria being considered as two lineages: the family *Syntrophomonadaceae* (in the phylum *Firmicutes*) and the family *Syntrophaceae* (in the class *Deltaproteobacteria* of the phylum *Proteobacteria*). Only two thermophilic species of syntrophic

bacteria, *Thermosyntropho lipolytica* (Svetlitsnyi *et al.*, 1996) and *Syntrophothermus lipocalidus* (Sekiguchi *et al.*, 2000), have been described so far.

During a survey of the bacterial diversity in a hot spring in Yunnan province, China, we isolated 12 thermophilic bacterial strains, all of which grew well at 60 °C in pre-reduced basal medium (McInerney *et al.*, 1979), with a variety of carbon sources. By using butyrate as the sole carbon source, one of these isolates, strain L-60^T, was identified as a thermophilic syntrophic butyrate-degrading bacterium. Phenotypic and phylogenetic characterization indicated that strain L-60^T represented a novel species of *Thermosyntropho*.

Throughout the present study, strain L-60^T and *Methanothermobacter thermautotrophicus* DSM 1053^T, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, were routinely maintained in the pre-reduced basal medium described by McInerney *et al.* (1979). *Thermosyntropho lipolytica* DSM 11003^T (also from the DSMZ) was cultured as described by Svetlitsnyi *et al.* (1996). The gas phase was H₂/CO₂ (80:20, v/v; 1.25 × 10⁵ Pa) for *M. thermautotrophicus* DSM 1053^T and N₂ (1.01 × 10⁵ Pa) for the other

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene of *Thermosyntropho tengcongensis* L-60^T is HM807481.

A supplementary table is available with the online version of this paper.

strains. All inoculations and transfers were made with syringes and needles and, unless indicated otherwise, incubations were at 60 °C in the dark.

In the initial isolation of strain L-60^T, a sediment sample from a hot spring in Tengcong was preserved in a sterile serum bottle that was sealed with a butyl-rubber stopper and then transported to a laboratory. There, the sediment was inoculated into the pre-reduced basal medium with 20 mM butyrate as sole carbon source and incubated at 60 °C. After 10 days, methane production and butyrate degradation were detected in the culture. The culture was subcultured a few times to give an enriched butyrate-degrading mixed culture that was then inoculated into Hungate agar roll tubes (Hungate, 1969) with fresh butyrate-supplemented basal medium. After incubation at 60 °C for 2 weeks, the tubes were found to contain single colonies that fluoresced under light at a wavelength of 420 nm, indicating the presence of a methanogen. The cultures were found to be mixtures of two types of bacterial cell: a *M. thermautotrophicus*-like rod and a slightly curved rod. One of these co-cultures was then inoculated into basal medium with 20 mM crotonate as sole substrate (instead of butyrate) and 10 mM 2-bromoethanesulfonic acid (BrES) added to inhibit the growth of the methanogen. After several subcultures in the crotonate-BrES liquid medium, no methane production could be detected, butyrate and acetate were being produced, and only the slightly curved rods could be observed. After purification of the slightly curved rods, by repeated picking of isolated colonies from the Hungate agar roll tubes, they were named strain L-60^T. The purity of this novel strain was further confirmed by light microscopy and by investigation of the strain's 16S rRNA gene sequence in a constructed clone library. The bacteria-specific primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), the archaea-specific primer 83F (5'-ACKGCTCAGTAACAC-3') and the prokaryotic universal primer 1492R (5'-TACGGYTACCTGTTACGACTT-3') were used to amplify the 16S rRNA gene.

The morphology of the cells of strain L-60^T in the exponential growth phase was studied under a light microscope (BH-2; Olympus) and a transmission electron microscope (H-600A; Hitachi), as described by Zhang *et al.* (2005). For electron microscopy, some cells were negatively stained with uranyl acetate while others were fixed, embedded, cut into ultrathin sections and then stained with uranyl acetate and lead citrate (Reynolds, 1963). The cells were confirmed to be slightly curved rods, with rounded ends, 0.3–0.4 µm wide and 4.5–5.0 µm long. They occurred singly and in pairs and chains. Although whole cells stained Gram-negative, their cell walls were found to be Gram-type-positive when examined by transmission electron microscopy (Fig. 1). Spore formation was not observed.

To test the substrate range of strain L-60^T in pure culture, various compounds including fatty acids and saccharides were added individually to the basal medium, with *T. lipolytica* DSM 11003^T similarly tested in parallel experiments.

Growth was monitored by determination of the OD₆₀₀ of the cultures, with similarly inoculated basal medium used as the blank control. Fermentation products were detected by GC (GC-14B; Shimadzu), as described by Zhang *et al.* (2004). Crotonate was the only tested substrate that supported the growth of strain L-60^T in pure culture (Table 1). In contrast, *T. lipolytica* DSM 11003^T could utilize many more substrates (Table 1), confirming the observations of Svetlitsnyi *et al.* (1996). Stoichiometrically, strain L-60^T converted 17.5 mM crotonate to 5.7 mM butyrate and 18.6 mM acetate in 4 days, indicating electron and carbon recoveries of 83.4 and 85.7 %, respectively. Although neither tryptone (0.05 %) nor peptone (0.05 %) were required, each stimulated the growth of strain L-60^T. Growth and acid formation were not observed on the following substrates after 2 months: yeast extract (5 g l⁻¹), peptone (5 g l⁻¹), Casamino acids (5 g l⁻¹), betaine (10 mM), pyruvate (15 mM), glucose (5 g l⁻¹), ribose (2.5 g l⁻¹), xylose (2.5 g l⁻¹), glycerol (20 mM), H₂/CO₂ (80:20, v/v), straight-chain fatty acids from C₂ to C₄ (20 mM), straight-chain fatty acids from C₅ to C₈ (5 mM), pelargonate or caprate (5 mM each, with 5 mM CaCl₂), laurate, myristate, palmitate, stearate or oleate (1 mM each, with 1 mM CaCl₂), olive oil (5 ml l⁻¹), tributyrin, trilaurin, tripalmitin, tristearin or triolein (1 mM each, with 3 mM CaCl₂), or isobutyrate (20 mM). None of the following compounds was used as an electron acceptor in the oxidation of 20 mM butyrate: sulfate (20 mM), nitrate (20 mM), sulfite (20 mM), thiosulfate (20 mM), fumarate (20 mM) and ferric nitrilotriacetate (5 mM). The growth of strain L-60^T was strictly anaerobic; no growth occurred in the presence of air.

To determine the substrate utilization of strain L-60^T in co-culture, a culture of the strain grown in basal medium with crotonate was mixed with a H₂/CO₂-grown culture of *M. thermautotrophicus* DSM 1053^T at a volume ratio of 1:1. A

Fig. 1. Transmission electron micrograph of an ultrathin section of cells of strain L-60^T. Bar, 0.2 µm.

Table 1. Characteristics of strain L-60^T and related type strains in the family Syntrophomonadaceae

Strains: 1, L-60^T (this study); 2, *Thermosyntropha lipolytica* DSM 11003^T (unless indicated, data from Svetlitsnyi *et al.*, 1996); 3, *Syntrophothermus lipocalidus* DSM 12680^T (Sekiguchi *et al.*, 2000); 4, *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245^T (McInerney *et al.*, 1979, 1981; Beaty & McInerney, 1987); 5, *Syntrophomonas wolfei* subsp. *saponavida* DSM 4212^T (Lorowitz *et al.*, 1989); 6, *Syntrophomonas sapovorans* DSM 3441^T (Roy *et al.*, 1986); 7, *Syntrophospora bryantii* DSM 3014^T (Stieb & Schink, 1985; Zhao *et al.*, 1990). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Cell width (µm)	0.3–0.4	0.3–0.4	0.4–0.5	0.5–1.0	0.4–0.6	0.5	0.4
Cell length (µm)	4.5–5.0	2.0–3.5	2.0–4.0	2.0–7.0	2.0–4.0	2.5	4.5–6.0
Gram reaction	–	–	–	–	–	–	+ / –
Motility	–	–	+	+	+	+	–
DNA G + C content (mol%)	40.3	45.2*	51.0	ND	ND	ND	37.6
Optimum pH	8.2	8.1–8.9	6.5–7.0	ND	ND	7.3	6.5–7.5
Optimum temperature (°C)	60	60–66	55	ND	ND	35	28–34
Spore formation	–	–	–	–	–	–	+
Utilization in pure culture of:							
Yeast extract	–	+	–	–	ND	ND	–
Tryptone	–	+	–	–	–	ND	ND
Casamino acids	–	+	–	+	ND	ND	ND
Crotonate	+	+	–	ND	+	–	+
Betaine	–	+	–	–	ND	ND	ND
Pyruvate	–	+	–	–	ND	–	–
Glucose	–	–*	–	–	ND	ND	–
Ribose	–	+	–	–	ND	ND	–
Xylose	–	+	–	–	ND	–	ND
Glycerol	–	–*	ND	ND	ND	ND	ND
H ₂ /CO ₂	–	–*	ND	ND	ND	ND	ND
Utilization in co-culture with methanogen of:							
Acetate (C _{2:0})	–	–*	ND	–	ND	–	ND
Propionate (C _{3:0})	–	–*	ND	–	–	–	–
Butyrate to caprylate (C _{4:0} –C _{8:0})	+	+	ND	+	+	+	+
Pelargonate (C _{9:0})	+	+	ND	ND	+	+	+
Caprate (C _{10:0})	+	+	ND	ND	+	+	+
Laurate (C _{12:0})	+	+	ND	ND	+	+	–
Myristate (C _{14:0})	+	+	ND	ND	+	+	–
Palmitate (C _{16:0})	+	+	ND	–	+	+	–
Stearate (C _{18:0})	+	+	ND	–	+	+	–
Oleate (C _{18:1})	+	+	ND	–	–	+	ND
Isobutyrate	–	–*	ND	–	–	–	–
Triacylglycerides	–	+	ND	ND	–	–	ND

*Data from this study.

similar co-culture of *T. lipolytica* DSM 11003^T with *M. thermotrophicus* DSM 1053^T was constructed and tested in a parallel experiment. With the co-cultures containing strain L-60^T, methane production was only detected, after 2 months of cultivation, when butyrate, a straight-chain fatty acid from C₅ to C₈, pelargonate, caprate, laurate, myristate, palmitate, stearate or oleate was added to the basal medium, as substrate (Table 1). When cultured with *M. thermotrophicus* DSM 1053^T, strain L-60^T converted 20 mM butyrate into 38 mM acetate and 4.6 mM methane within 20 days. While fatty acids with even numbers of carbon atoms were degraded into just acetate and methane, those with odd numbers of carbon atoms were converted

into acetate, propionate and methane, indicating that strain L-60^T subjected fatty acids to β -oxidation.

Cultures in basal medium containing 20 mM crotonate, each set up using a 2% (v/v) inoculum, were used to investigate the pH and temperature ranges for the growth of strain L-60^T. Growth was again evaluated, after various periods of incubation, as OD₆₀₀. To explore the effects of pH, the initial pH of medium was adjusted to 4.3–10.0 by addition of HCl or NaOH under an atmosphere of pure N₂ at room temperature, before the medium was inoculated with the novel strain and incubated at 60 °C. To determine the optimum temperature for growth, cultures were

incubated at 18, 37, 45, 55, 60, 65, 70, 75 and 80 °C. Growth occurred at pH 7.0–9.3 (optimum about pH 8.2) and between 55 and 70 °C (optimum about 60 °C). Under optimal conditions (pH 8.2 and 60 °C), the OD₆₀₀ of a pure culture of strain L-60^T in the basal medium with 20 mM crotonate increased by about 0.3 h⁻¹. The corresponding rate for *T. lipolytica* DSM 11003^T (about 0.7 h⁻¹) was of the same order of magnitude.

The effects of antibiotics on growth of strain L-60^T were tested at pH 8.2 and 60 °C, using 10 mM crotonate as substrate and a 2 % (v/v) inoculum. As growth was inhibited completely by ampicillin, chloramphenicol, kanamycin, neomycin, rifampicin or vancomycin (each at 50 µg ml⁻¹), the cells of the strain probably have a Gram-type-positive cell wall.

The cellular fatty acids of strain L-60^T and *T. lipolytica* DSM 11003^T were extracted, methylated and analysed as described in the standard protocol of the Sherlock Microbial Identification System (Sasser, 2001), except that both strains were grown in crotonate-supplemented liquid medium and incubated at 60 °C until they reached the late-exponential phase of growth. The major cellular fatty acids of strain L-60^T were C_{14:0} (35.0%), C_{16:0} (20.3%) and iso-C_{17:1} I/anteiso-C_{17:1} B (30.9%), whereas C_{14:0} (26.5%), C_{16:0} (21.0%) and iso-C_{15:1} H/C_{13:0} 3-OH (12.4%) were the predominant cellular fatty acids of *T. lipolytica* DSM 11003^T (Table S1, available in IJSEM Online).

The method described by Marmur (1961) was used to extract genomic DNA from the cells of strain L-60^T and *T. lipolytica* DSM 11003^T that had been grown in crotonate-supplemented basal medium. By using HPLC (Mesbah *et al.*, 1989), with *Escherichia coli* K-12 as a reference, the G+C contents of the genomic DNA of strain L-60^T and *T. lipolytica* DSM 11003^T were determined to be 40.3 and 45.2 mol%, respectively.

The 16S rRNA gene of strain L-60^T was amplified by PCR, using the 27F and 1492R primers, and then sequenced by the BioSune Company (Beijing, China). The BLAST algorithm was used to search GenBank for similar sequences, before CLUSTAL_X (Thompson *et al.*, 1994) was employed to align the best matching sequences and evaluate their levels of similarity. Based on a consensus length of the 16S rRNA gene sequences (1410 nt), a neighbour-joining phylogenetic

tree (Fig. 2) rooted with *Clostridium butyricum* ATCC 43755 was constructed, using the MEGA4 package (Tamura *et al.*, 2007). This tree indicated that *Thermosyntropho lipolytica* DSM 11003^T was most closely related to strain L-60^T (96.7% sequence similarity).

Using a spectrophotometer (UV800; Beckman) and the initial reassociation rates at 61–65 °C, as described by Owen & Pitcher (1985), the level of DNA–DNA relatedness between strain L-60^T and *T. lipolytica* DSM 11003^T was determined to be 44.0%.

Although the phenotypic and phylogenetic data indicate that strain L-60^T should be assigned to the genus *Thermosyntropho*, the novel strain can be distinguished from the only species currently recognized in this genus (*T. lipolytica*). Strain L-60^T can only grow on crotonate in pure culture, whereas *T. lipolytica* can use several substrates, including yeast extract and tryptone. Although *T. lipolytica* can hydrolyse lipids, forming fatty acids and glycerol, strain L-60^T lacks this ability. Finally, the DNA G+C content of strain L-60^T is lower by 5 mol% than that of the type strain of *T. lipolytica* grown under the same conditions. Based on the phylogenetic and phenotypic evidence, we propose that strain L-60^T represents a novel species of *Thermosyntropho*, for which the name *Thermosyntropho tengcongensis* sp. nov. is proposed.

Description of *Thermosyntropho tengcongensis* sp. nov.

Thermosyntropho tengcongensis (teng.con.gen'sis. N.L. fem. adj. *tengcongensis* referring or pertaining to Tengcong, China, where the type strain was isolated).

Cells are non-motile, Gram-staining-negative, slightly curved rods, 0.3–0.4 µm wide and 4.5–5.0 µm long, that occur singly or in pairs or chains. Spores were not observed. Grows on crotonate in pure culture but not on yeast extract, tryptone, Casamino acids, betaine, pyruvate, ribose or xylose. In syntrophic association with hydrogenotrophic methanogens, the organism can utilize saturated straight-chain fatty acids with 4–18 carbon atoms as well as unsaturated fatty acids such as oleate, but not triglycerides. Growth occurs between 55 and 70 °C (optimum about 60 °C) and at pH 7.0–9.3 (optimum about pH 8.2). The major cellular fatty acids are C_{14:0}, C_{16:0} and iso-C_{17:1} I/anteiso-C_{17:1} B.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (a consensus length of 1410 nt), showing the relationships of strain L-60^T and related taxa. Numbers at nodes indicate percentages of bootstrap support, based on analysis of 1000 resampled datasets. The sequence of *Clostridium butyricum* ATCC 43755 was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

The type strain, L-60^T (=CGMCC 1.5161^T =JCM 17260^T), was isolated from a sediment sample collected from a hot spring in the Chinese town of Tengcong. The DNA G+C content of the type strain is 40.3 mol%.

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