

Isolation and Characterization of a Thermophilic Sulfate-Reducing Bacterium, *Desulfotomaculum thermosapovorans* sp. nov.

MARIE-LAURE FARDEAU,^{1,2,*} BERNARD OLLIVIER,² BHARAT K. C. PATEL,^{2,3} PREM DWIVEDI,³
MICHEL RAGOT,² AND JEAN-LOUIS GARCIA²

Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, 13277 Marseille cedex 9,¹ and
Laboratoire de Microbiologie ORSTOM, Université de Provence, 13331 Marseille cedex 3,² France, and
Faculty of Science and Technology, Griffith University, Brisbane, Queensland 4111, Australia³

Strain MLF^T (T = type strain), a new thermophilic, spore-forming sulfate-reducing bacterium, was characterized and was found to be phenotypically, genotypically, and phylogenetically related to the genus *Desulfotomaculum*. This organism was isolated from a butyrate enrichment culture that had been inoculated with a mixed compost containing rice hulls and peanut shells. The optimum temperature for growth was 50°C. The G+C content of the DNA was 51.2 mol%. Strain MLF^T incompletely oxidized pyruvate, butyrate, and butanol to acetate and presumably CO₂. It used long-chain fatty acids and propanediols. We observed phenotypic and phylogenetic differences between strain MLF^T and other thermophilic *Desulfotomaculum* species that also oxidize long-chain fatty acids. On the basis of our results, we propose that strain MLF^T is a member of a new species, *Desulfotomaculum thermosapovorans*.

In environments where conditions for the survival of the strictly anaerobic sulfate-reducing bacteria are not provided continuously, the sporulating species of the genus *Desulfotomaculum* are usually dominant (2). In recent years, several species of this genus have been isolated from thermal ecosystems (3, 22). Anaerobic degradation of fatty acids by sulfate-reducing bacteria has been investigated primarily by using members of the genus *Desulfotomaculum*. The following five thermophilic species oxidize these compounds: *Desulfotomaculum kuznetsovii* (15), *Desulfotomaculum geothermicum* (3), *Desulfotomaculum thermoacetoxidans* (14), *Desulfotomaculum thermobenzoicum* (22), and *Desulfotomaculum australicum* (11). *D. australicum* uses acetate. In addition to acetate, *D. thermoacetoxidans* uses propionate. *D. thermobenzoicum* oxidizes propionate and butyrate, while *D. geothermicum* and *D. kuznetsovii* use long-chain fatty acids (LCFA). In contrast to utilization of LCFA, oxidation of propanediols has been poorly studied with members of the genus *Desulfotomaculum*. *D. thermobenzoicum* has been reported to use 1,2-propanediol and 1,3-propanediol (22), but the products of metabolism have not been identified.

In this paper we describe a new spore-forming, thermophilic, sulfate-reducing bacterium (strain MLF^T [T = type strain]) which degrades LCFA and propanediols. Because of significant differences in physiological, phylogenetic, and metabolic properties between this isolate and other members of the genus *Desulfotomaculum*, we propose that strain MLF^T should be placed in a new species, *Desulfotomaculum thermosapovorans*.

MATERIALS AND METHODS

Sources of organisms. Strain MLF^T was isolated from a thermophilic anaerobic enrichment culture growing on rice hulls at 55°C. The initial inoculum was a mixed compost containing rice hulls and peanut shells.

D. geothermicum DSM 3669 was grown by using the media and conditions described by Dumas et al. (3).

Media. Growth media were prepared by using the technique of Hungate (7).

The organisms were cultivated under strictly anoxic conditions at 55°C. The basal medium contained (per liter of distilled water) 1.0 g of NH₄Cl, 0.15 g of CaCl₂ · 2H₂O, 1 g of NaCl, 0.5 g of KCl, 0.4 g of MgCl₂ · 6H₂O, 0.2 g of KH₂PO₄, 1.0 g of CH₃COONa · 3H₂O, 0.001 g of resazurin, 1 ml of a sodium selenite solution (17), and 1.5 ml of a trace mineral solution (8); the pH of this medium was 7.0. The basal medium was boiled under a stream of O₂-free N₂ and cooled to room temperature, and 5-ml portions were distributed into Hungate tubes under an N₂-CO₂ (80:20) gas mixture. After the tubes were autoclaved at 110°C for 30 min, 0.05 ml of Na₂S · 9H₂O, 0.2 ml of 10% NaHCO₃ (sterile, anaerobic solution), 0.05 ml of a filter-sterilized vitamin solution (26), and 0.05 ml of 0.1% dithionite solution were added to each tube just before inoculation. To isolate pure cultures, solid medium for roll tubes was prepared by adding 2% (wt/vol) Noble agar (Difco Laboratories, Detroit, Mich.) to the growth medium.

Culture purity. Cultures were routinely checked for purity by microscopic examination; to do this, we used cultures grown in aerobic and anaerobic liquid media containing 20 mM glucose and 0.2% yeast extract at 35 and 55°C.

Substrate utilization. Substrate utilization was determined with duplicate preparations by using the basal growth medium described above. The substrates (which were prepared as autoclaved or filter-sterilized stock solutions and stored under N₂) were added to autoclaved media so that the final substrate concentrations were between 5 and 20 mM, depending on the substrate used. Growth was determined by measuring optical density and sulfide production after 1 week of incubation (see below).

Analytical methods. Optical density at 580 nm was determined with a model UV-160A spectrophotometer (Shimadzu Corp., Kyoto, Japan). Sulfide production was determined by the method described by Cord-Ruwisch (1). Volatile fatty acid and alcohol contents were determined by gas chromatography at 170°C by using a Poraplot Q capillary column (Chrompack France, Les Ulis, France) connected to a flame ionization detector (model CP 9000; Chrompack France); N₂ was used as the carrier gas. Prior to injection, the culture supernatants were acidified by adding 50% (vol/vol) H₃PO₄. Propanediol and 3-hydroxypropionate contents were determined by high-performance liquid chromatography by using an ORH 801 column (length, 250 mm; diameter, 4.1 mm; Interaction Chemicals, Inc., Mountain View, Calif.) and a model RID-6A detector (Shimadzu).

DNA base composition. Cells were lysed with sodium dodecyl sulfate, and DNA was purified from the cell lysate by the method of Marmur (12). The buoyant density of the DNA was determined by centrifugation with a Beckman model E centrifuge in a cesium chloride density gradient, using the methods of Meselson and Stahl (13) and Szybalski (21). The density of the cesium chloride was determined with a digital precision densitometer (model DMA 02C; Anton Paar) by using the method of Stabinger (20).

16S rRNA gene sequence studies. DNAs were extracted from strain MLF^T and *D. geothermicum*, and then the 16S rRNA gene was amplified and purified as described previously (11, 18). The sequence of the purified PCR product was determined directly or after the product was cloned. The method used to clone the 16S rRNA genes has been described previously (11). Sequences were determined with a model ABI 373A automated DNA sequencer by using prism dideoxy terminator protocols (Applied Biosystems, Ltd.). The primers used for sequencing have also been described previously (18).

16S rRNA sequences were obtained from the Ribosomal RNA Database Project and from GenBank and EMBL. The 16S ribosomal DNA sequences were aligned with the sequences of various members of the bacterial phylum by using

* Corresponding author. Mailing address: Laboratoire de Microbiologie ORSTOM, Université de Provence, 3 Place Victor Hugo, 13331 Marseille cedex 3, France. Phone: (33) 91.10.64.80. Fax: (33) 91.10.64.81.

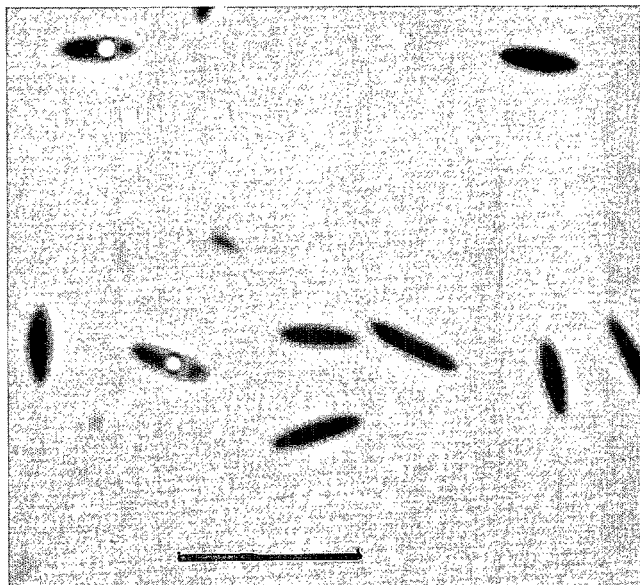


FIG. 1. Photomicrograph of strain MLF^T cells, showing the central to sub-terminal position of spores. Bar = 10 μ m.

alignment editor ae2 (16). Positions at which the sequence and/or alignment was uncertain were omitted from the analysis (positions 1 to 107, 213 to 235, 469 to 497, 526 to 573, 856 to 867, 921 to 1063, 1207 to 1248, and 1382 to 1542; *Escherichia coli* numbering of Winker and Woese [27]). Pairwise evolutionary distances were determined from percentages of similarity by using the correction factor of Jukes and Cantor (10). Dendrograms were constructed from evolutionary distances by using the program of De Soete (4). Tree topology was reexamined by using 100 bootstrapped data sets. The SEQBOOT, DNADIST, FITCH, and CONSENS programs available in the PHYLIP package (5) and other programs available in the TREECON package (23) were used for this purpose.

Nucleotide sequence accession numbers. The sequence of the 16S rRNA gene of strain MLF^T has been deposited in the EMBL database under accession number Z26315, and the sequence of the 16S rRNA gene of *D. geothermicum* has been deposited under accession number X80789. The accession numbers for the *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter brockii*, *Propionigenium modestum*, *Desulfotomaculum nigrificans*, *Desulfotomaculum orientis*, and *D. thermobenzoicum* sequences which we used were L09161, L09165, X54275, X62176, M34417, and L15628, respectively.

RESULTS

Enrichment and isolation. A mixed compost containing rice hulls and peanut shells was incubated anaerobically at 55°C. The resulting enrichment culture was transferred several times into bottles containing only rice hulls. For the last transfer we used a medium containing butyrate as the energy source and sulfate as an electron acceptor. In this enrichment culture the dominant microbial population consisted of sporulating rods. Pasteurization of the enrichment culture at 90°C for 30 min followed by dilution in agar tubes led to development of colonies within 2 weeks with incubation at 55°C. Pure cultures were obtained from single colonies after pasteurization as described above, and the agar shake dilution procedure was repeated several times. The resulting pure culture was designated MLF^T and characterized.

Morphology. The vegetative cells of strain MLF^T were straight or slightly curved motile rods (Fig. 1). Spores in central positions were also observed (Fig. 1). The cells were 1.5 to 2 μ m in diameter and 5 to 8 μ m long and occurred singly or in pairs. No gas vacuoles were observed.

Growth characteristics. Strain MLF^T grew at temperatures between 35 and 60°C. The optimum temperature was 50°C.

TABLE 1. Products formed during growth of strain MLF^T on different substrates in the presence of sulfate

Substrate	Substrate concn (mM)	Concn of acetate produced (mM)	Other product formed
Pyruvate	20	19.3	
Butanol	20	19.8	
Butyrate	20	19.2	
1-Propanol	20	0	Propionate
H ₂ -CO ₂	— ^a	0	
Ethanol	20	19.8	
Isobutanol	20	0	Isobutyrate
1,2-Propanediol	10	9.8	
1,3-Propanediol	10	0	3-Hydroxypropionate

^a —, an excess of H₂-CO₂ was present.

The optimum pH for growth was between 7.2 and 7.5. This isolate did not require complex organic nutrients such as yeast extract or Bacto Peptone and did not grow after four subcultures without added vitamins. It grew in medium containing 0 to 35 g of NaCl per liter; optimum growth occurred in medium containing 15 g of NaCl per liter. In the presence of sulfate, strain MLF^T utilized butyrate and LCFA (C₃, C₆, C₇, C₈, C₉, C₁₂, C₁₆, C₁₈, C₂₀, and C₂₂ fatty acids). It also used the following substrates as energy sources: methanol, ethanol, 1-propanol, butanol, isobutanol, pentanol, lactate, pyruvate, malate, fumarate, 1,2-propanediol, and 1,3-propanediol. It utilized formate and H₂-CO₂ as sole carbon and energy sources; it reduced sulfate to sulfide and did not produce acetate during growth on sulfate. Strain MLF^T converted butanol, pyruvate, ethanol, butyrate, and 1,2-propanediol to acetate in the presence of sulfate (Table 1). Isobutanol was oxidized to isobutyrate, while 1,3-propanediol was oxidized to 3-hydroxypropionate and 1-propanol was oxidized to propionate (Table 1).

The substrates tested but not used were acetate, propionate, succinate, isobutyrate, 2-propanol, fructose, glucose, xylose, benzoate, *p*-hydroxybenzoate, 3,5-, 2,6-, and 2,4-dihydroxybenzoates, 3,4,5-trihydroxybenzoate, and glycerol.

Lactate and pyruvate were also used without added electron acceptors.

In the presence of butyrate, strain MLF^T was able to grow by using sulfate, sulfite, and thiosulfate as electron acceptors. Sulfur was not used as an electron acceptor.

G+C content. The DNA guanine-plus-cytosine (G+C) content of the isolate, as determined by ultracentrifugation, was 51.2 mol%.

Phylogeny. We sequenced 1,529 and 1,522 bases from position 8 to position 1542 (*E. coli* numbering of Winker and Woese [27]) of the 16S rRNA genes of strain MLF^T and *D. geothermicum*, respectively. The G+C content of both 16S rRNA genes was 57.44 mol%. Sequence alignment followed by a phylogenetic analysis in which we compared these rRNA gene sequences with the sequences of representatives of the domain *Bacteria* revealed that strain MLF^T belonged to the subphylum containing gram-positive bacteria having G+C contents less than 55 mol%. Further analysis with members of the low-G+C-content branch revealed that strain MLF^T was related to the thermophilic sulfate-reducing species belonging to the genus *Desulfotomaculum*, including *D. geothermicum* (level of similarity, 93%) and *D. australicum* and *D. thermobenzoicum* (level of similarity, 89%); these three species, in turn, were related to the moderately thermophilic organism *D. nigrificans* and the mesophilic sulfate-reducing species *Desulfotomaculum ruminis* (average level of similarity, 88%). The

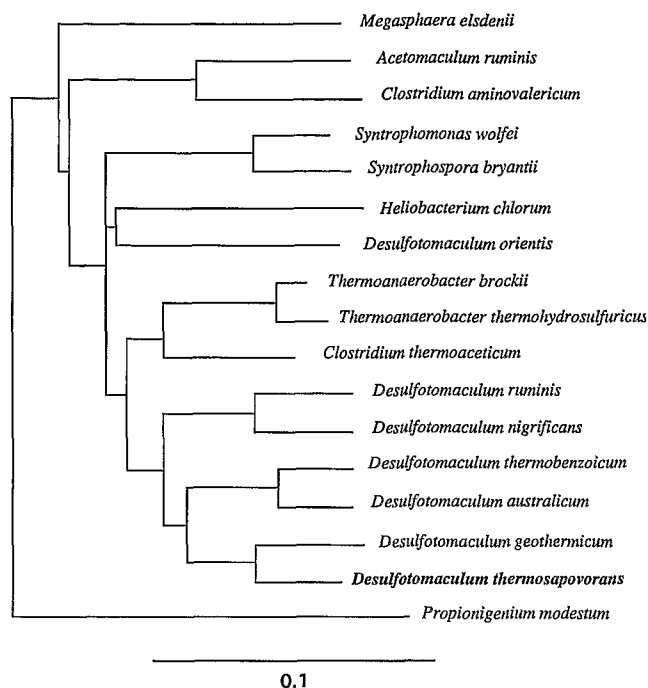


FIG. 2. Phylogenetic position of *D. thermosapovorans* within the radiation of gram-positive bacteria having DNA G+C contents less than 55 mol%.

five *Desulfotomaculum* species were peripherally related to *D. orientis* (level of similarity, 86%). Figure 2 is a dendrogram generated from the matrix in Table 2 and shows these relationships. A bootstrap analysis of the data, as implemented in the PHYLIP package (5) and TREECON package (23), gave similar results.

DISCUSSION

Strain MLF^T is unique among the spore-forming, thermophilic, sulfate-reducing bacteria in that it grows on 1,2-propanediol and 1,3-propanediol and produces acetate and 3-hydroxypropionate, respectively, from these substrates. The only other organism that grows on these compounds is *D. thermobenzoicum*, but the end products from growth on these substrates have not been studied (22).

In anoxic sewage digesters, significant amounts of neutral fats and LCFA are present, in addition to carbohydrates, proteins, and lipids (6). Therefore, LCFA oxidizers are ecologically important in such ecosystems. In the absence of sulfate, and in methanogenic environments, LCFA are oxidized to methane and carbon dioxide via β -oxidation only when this process is coupled to interspecies H_2 transfer (9, 24). For example, a syntrophic association of *Methanospirillum hungatei* with *Syntrophomonas sapovorans* is able to oxidize fatty acids (up to C_{18} fatty acid) to methane and acetate (19). In the presence of sulfate, a few *Desulfotomaculum* species have the ability to use LCFA, but only two of these organisms are thermophilic (*D. geothermicum* [3] and *D. kuznetsovii* [15]). However, strain MLF^T, *D. geothermicum*, and *D. kuznetsovii*

TABLE 2. Evolutionary similarity matrix for various gram-positive bacteria obtained by using the method of Jukes and Cantor^a

Species	% Sequence similarity to:																
	<i>Desulfotomaculum thermosapovorans</i>	<i>Desulfotomaculum geothermicum</i>	<i>Desulfotomaculum australicum</i>	<i>Desulfotomaculum thermobenzoicum</i>	<i>Desulfotomaculum nigrificans</i>	<i>Desulfotomaculum ruminis</i>	<i>Desulfotomaculum orientis</i>	<i>Heliobacterium chlorum</i>	<i>Syntrophospora bryantii</i>	<i>Syntrophomonas wolfei</i>	<i>Thermoanaerobacter thermohydrosulfuricus</i>	<i>Thermoanaerobacter brockii</i>	<i>Clostridium thermoaceticum</i>	<i>Acetomaculum ruminis</i>	<i>Clostridium aminovalericum</i>	<i>Megasphaera elsdenii</i>	
<i>Desulfotomaculum geothermicum</i>	93.4																
<i>Desulfotomaculum australicum</i>	89.6	88.8															
<i>Desulfotomaculum thermobenzoicum</i>	89.5	89.4	95.3														
<i>Desulfotomaculum nigrificans</i>	88.2	87.4	87.7	88.2													
<i>Desulfotomaculum ruminis</i>	88.4	87.8	86.9	87.0	93.5												
<i>Desulfotomaculum orientis</i>	86.4	85.3	85.6	84.6	84.8	85.0											
<i>Heliobacterium chlorum</i>	85.6	84.0	83.7	83.7	84.9	85.8	85.0										
<i>Syntrophospora bryantii</i>	82.5	81.8	85.9	84.8	82.8	83.5	84.4	84.4									
<i>Syntrophomonas wolfei</i>	83.9	82.9	86.1	85.0	83.5	83.6	85.0	84.1	94.3								
<i>Thermoanaerobacter thermohydrosulfuricus</i>	86.5	86.5	87.8	87.9	85.0	85.6	85.3	84.5	86.4	86.8							
<i>Thermoanaerobacter brockii</i>	87.2	86.8	88.5	88.5	85.4	86.3	85.4	85.1	86.7	87.2	97.5						
<i>Clostridium thermoaceticum</i>	88.8	88.5	88.8	89.8	87.4	85.7	86.3	83.8	85.9	87.0	90.5	90.9					
<i>Acetomaculum ruminis</i>	82.7	83.4	82.0	82.5	82.3	81.9	81.9	81.9	82.1	82.5	83.2	84.4	84.2				
<i>Clostridium aminovalericum</i>	81.7	81.0	81.0	81.9	83.1	82.2	82.0	81.1	84.5	83.7	82.7	83.5	83.9	89.5			
<i>Megasphaera elsdenii</i>	83.5	82.7	81.9	82.2	82.6	82.3	82.7	80.8	81.1	81.8	84.2	84.6	85.2	83.0	81.8		
<i>Propionigenium modestum</i>	77.7	77.0	76.9	77.0	77.2	78.2	78.5	78.0	77.8	78.2	78.0	78.3	78.8	77.7	77.0	78.3	

^a Some of the sequences used in this analysis were obtained from the Ribosomal RNA Database Project, version 2 (16). Additional sequences not available in this database were obtained from GenBank or EMBL, including the *T. thermohydrosulfuricus*, *T. brockii*, *P. modestum*, *D. nigrificans*, *D. orientis*, and *D. thermobenzoicum* sequences. Only 895 unambiguous nucleotides were used in the analysis. See text.

differ markedly phenotypically. In contrast to strain MLF^T, *D. kuznetsovii* uses acetate and propionate and has a higher optimum temperature for growth (65°C). We were not able to compare the phylogenetic position of *D. kuznetsovii* as a culture of this organism was not available. However, the physiological data provide evidence that strain MLF^T is different from *D. kuznetsovii*. Strain MLF^T differs from *D. geothermicum* in that it does not utilize propionate or sugars (fructose) but uses methanol and heptanoate, does not possess gas vacuoles, and grows in the presence of a different range of NaCl concentrations (0 to 35 g/liter for strain MLF^T and 2 to 50 g/liter for *D. geothermicum*). Phylogenetic evidence also indicates that strain MLF^T and *D. geothermicum* are distinct species. There are also considerable phylogenetic differences between strain MLF^T and the other physiologically distinct non-LCFA-degrading thermophilic *Desulfotomaculum* species, including *D. thermobenzoicum* (22), *D. nigrificans* (25), and *D. australicum* (11). In conclusion, strain MLF^T is not a member of a previously described *Desulfotomaculum* species. We propose that this organism belongs to new species, *Desulfotomaculum thermosapovorans*.

Description of *Desulfotomaculum thermosapovorans* sp. nov. *Desulfotomaculum thermosapovorans* (ther.mo.sa.po.vo'rans. Gr. adj. *thermos*, hot; M. L. neut. n. *sapo*, soap; L. v. *voro*, to devour; M.L. part. adj. *sapovorans*, soap devouring; M. L. part. adj. *thermosapovorans*, thermophilic and soap devouring). Rod-shaped cells are 1.5 to 2 µm in diameter and 5 to 8 µm long and occur singly or in pairs. The cells are slightly motile. The spore position is central to subterminal. No gas vacuoles are observed. The following substrates are utilized as electron donors in the presence of sulfate: formate, butyrate, valerate, caproate, LCFA (see Results), lactate, pyruvate, malate, fumarate, ethanol, methanol, propanol, butanol, isobutanol, pentanol, and H₂. Pyruvate and butyrate are incompletely oxidized to acetate and presumably CO₂. Acetate and propionate are not used. Pyruvate and lactate are fermented in the absence of sulfate. The electron acceptors include sulfate, sulfite, and thiosulfate. Sulfur is not used as an electron acceptor.

Vitamins are required for growth. Addition of NaCl is not necessary, but strain MLF^T tolerates up to 35 g of NaCl per liter in the medium; optimal growth occurs in the presence of 15 g of NaCl per liter. The temperature range for growth is 35 to 60°C; the optimum temperature is 50°C. The optimum pH for growth is 7.2 to 7.5.

The G+C content of the DNA is 51.2 mol% (as determined by ultracentrifugation).

Isolated from a thermophilic anaerobic enrichment culture containing rice hulls grown at 55°C. The type strain is strain MLF (DSM 6562).

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