33 The Family *Thermodesulfobiaceae*

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Abstract
The family *Thermodesulfobiaceae* is a polyphyletic family within the order *Thermoanaerobacterales*, class *Clostridia*; it embraces the type genus *Thermodesulfobium* which contains one species, *T. narugense* and the genus *Coprothermobacter* which includes two species: *C. platensis* and *C. proteolyticus.* Members of the family are defined by a wide range of morphological and chemotaxonomic properties, such as fatty acids, quinones, etc. They are all strictly anaerobic. Members of the family are found in anaerobic digesters, but they have been isolated from aquatic environment as well.

Taxonomy: Historical and Current

Short Description of the Family

Ther.mo.de.sul.fo.bi.a'ce.ae. M. L. fem.n. *Thermodesulfobium* type genus of the family; -aceae ending to denote a family; M. L. fern. pl.n. *Thermodesulfobiaceae*, the family of *Thermodesulfobium* (Modified from *Bergey's Manual*). The description is an emended version of the description given in *Bergey's Manual*, 2nd edition (Mori and Hanada 2009).

The family *Caldicoprobacteraceae* is a member of the order *Thermoanaerobacterales*, phylum Firmicutes. It contains the type genus *Thermodesulfobium* (Mori et al. 2003) and *Coprothermobacter* (Rainey and Stackebrandt 1993a). Gram-negative. Cells are rods of varying lengths. Nonmotile. Strictly anaerobic. Thermophilic or moderately thermophilic.

$C_{16:0}$ is the prominent fatty acid; $C_{15:0}$, iso- $C_{14:0}$ 3 OH, iso-$C_{17:0}$ may also occur. Menaquinone MK-7 (H2) and MK-7 are the predominant quinones (when mentioned). Polar lipids were not analyzed. G+C values of DNA range between 35 and 45 mol%. Isolated from microbial mats in a hot spring, and from anaerobic mesothermic or hot digesters.

Phylogenetic Structure of the Family and Its Genera

According to the phylogenetic branching of *Firmicutes* type strains in the RaxML 16S rRNA gene tree of the Living Tree Project (Yarza et al. 2008), the family is moderately related to the families *Synergistetes* and *Aquificae* (Fig. 33.1).

The family *Thermodesulfobiaceae* contains two genera *Thermodesulfobium* and *Coprothermobacter.* The genus *Coprothermobacter* contains two species: *C. proteolyticus* and *C. platensis. C. proteolyticus* was first described by Ollivier et al. (1985) as *Thermobacteroides proteolyticus*, but, later, phylogenetic studies (Rainey and Stackebrandt 1993a; Rainey et al. 1993) of anaerobic thermophilic bacteria demonstrated that some of them had to be reclassified. It was the case for members of the genus *Thermobacteroides* (Ben-Bassat and Zeikus 1981; Ollivier et al. 1985) which belonged to phylogenetically very diverse taxa (Rainey and Stackebrandt 1993b). Within this genus, *Thermobacteroides proteolyticus* represented a deep root adjacent to members of the order *Thermotogales*, showing only 81.9 % sequence similarity with *Thermobacteroides acetoethylicus* over the stretch of about 1,200 analyzed nucleotides (Rainey and Stackebrandt 1993a). On the basis of these phylogenetic findings, supported by phenotypic characteristics, the reclassification of the species investigated was evident. In consequence, the genus *Thermobacteroides* was invalidated, and the description of the genus *Coprothermobacter* and the assignment of *Thermobacteroides proteolyticus* as the type species *Coprothermobacter proteolyticus* was proposed (Rainey and Stackebrandt 1993a).

Another strain of *C. proteolyticus* was also isolated by Kersters et al. (1994), showing the same metabolic properties of the type strain of the species isolated by Ollivier et al. (1985).

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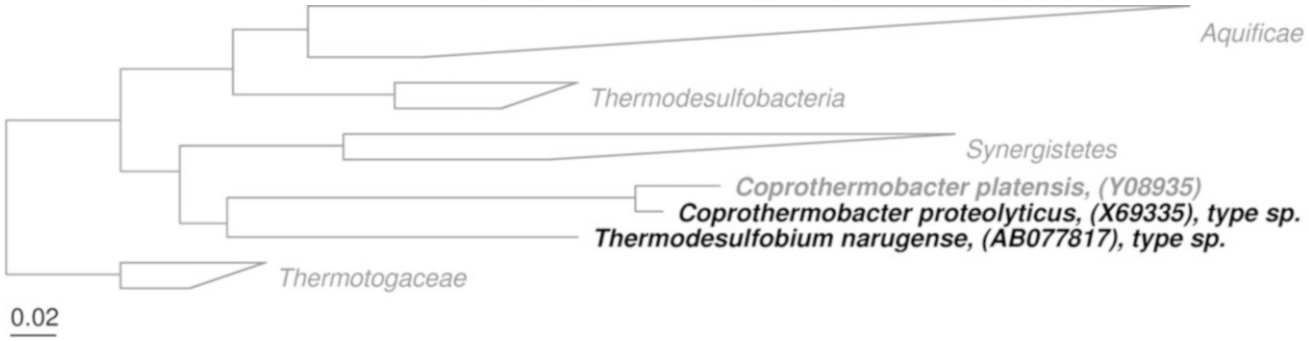

Fig. 33.1
Phylogenetic reconstruction of the family *Thermodesulfobiaceae* based on 16S rRNA and created using the maximum likelihood algorithm RAxML (Stamatakis 2006). The sequence datasets and alignments were used according to the All-Species Living Tree Project (LTP) database (Yarza et al. 2008; http://www.arb-silva.de/projects/living-tree). Representative sequences from closely related taxa were used as outgroups. In addition, a 40 % maximum frequency filter was applied in order to remove hypervariable positions and potentially misplaced bases from the alignment. Scale bar indicates estimated sequence divergence

Molecular Analyses

DNA-DNA Hybridization Studies

Results of DNA-DNA hybridization studies is only reported for one species of *Coprothermobacter* and shows less than 12 % similarity between the chromosomal DNAs of the two species of *Coprothermobacter*, *C. proteolyticus* and *C. platensis* (Etchebehere et al. 1998).

Genome Comparison

The genome of one species has been released. The genome of the type strain *Thermodesulfobium narugense* Na82, DSM 14796 T is 1,898,865 bp long, contains 1,807 protein genes, including 56 RNA genes, and the mol% G+C of DNA is 33.8 %. The latter value falls in the range of 96.3 % determined for the species (*Table 33.1*) by HPLC. http://www.genome.jp/kegg-bin/show_organism?org, http://genome.jgi-psf.org/thenr/thenr.info.html.

Phenotypic Analyses

The main features of members of *Thermodesulfobiaceae* are listed in *Tables 33.1* and *33.2*.

Thermodesulfobium Mori et al. 2003, 288

Ther.mo.de.sul.fo'bi.um. Gr. adj. thermos hot; L. pref. de from; L. n. sulfur sulfur; Gr. n. bios life; L. neut. n. *Thermodesulfobium* a thermophilic organism that reduces a sulfur compound.

Table 33.1
Morphological and chemotaxonomic characteristics of genera of *Thermodesulfobiaceae*

	Thermodesulfobium	*Coprothermobacter*
Morphology	Rods	Rods
Gram stain	Negative	Negative
Metabolism	Anaerobic growth	Anaerobic growth
Motility	–	–
Major fatty acids	C16 :0	iso-C15 : 0 and C16 :0
Menaquinone	MK-7 (H2) and MK-7	nd
G+C content	35.1	43–45

As the genus *Thermodesulfobium* contains only one species, the description of the genus is also the description of the type species, Na82^T *Thermodesulfobium narugense*^{T}.

Cells of *Thermodesulfobium narugense*^{T} are rod-shaped (0.5 · 2–4 µm diameter-length). This bacterium shows no motility under the microscope. Spore formation is not observed. Gram staining is negative with cell wall having an outer membrane. Neither storage compounds nor extensive internal membranes are observed. The polymyxin B-LPS test (Wiegel and Quandt 1982) also suggests that it possesses the typical Gram-negative cell wall, since the fibrous structure and blebs of lipopolysaccharides are observed around the surface of polymyxin-B-treated cells.

Growth occurs only in strictly anaerobic conditions under an H_2/CO_2 atmosphere and cannot occur under aerobic conditions. Growth occurs between 37 °C and 65 °C, with an optimum at 50–55 °C. Anaerobic growth is always coupled to sulfate reduction. In the presence of sulfate, the bacterium also uses formate as electron donor, but growth on formate is clearly

Table 33.2
Comparison of selected characteristics of members of the genus *Coprothermobacter*

Characteristics	*C. proteolyticus*[a]	*C. proteolyticus*[b]	*C. platensis*[c]
Dimensions (μm)	0.5 × 1–6	0.5 × 1–6	0.5 × 1.5–2.0
Optimum temperature (°C)	63	63–70	55
Growth on			
Fructose	+	+	+
Sucrose	+	+	+
Melibiose	–	–	ND[d]
Xylose	+	+	–
Resistance to			
Penicillin G (20 U/ml)	–	–	+
Polymixin (20 mg/l)	+	+	–
G+C %	45	43–44	43
Major fatty acid	Iso-$C_{15:0}$ and $C_{16:0}$	Iso-$C_{15:0}$ and $C_{16:0}$	ND[d]

[a]Data from Ollivier et al. (1985)
[b]Data from Kersters et al. (1994)
[c]Data from Etchebbehere et al. (1998)
[d]*ND* Not determined

lower than that on H_2. When H_2 and CO_2 are provided as energy and carbon sources, thiosulfate, nitrate, or nitrite also serves as an electron acceptor. The isolate, however, does not use sulfite, elemental sulfur, Fe (III), citrate, fumarate, dimethyl sulfoxide, or O_2 as electron acceptor. No growth occurs in the presence of glucose, acetate, lactate, pyruvate, malate, propionate, butyrate, fumarate, succinate, citrate, ethanol, propanol, or methanol (Mori et al. 2003). Menaquinone (MK)-7(H2) and MK-7 (53.6 % and 35.8 %, respectively, of total quinones) are the major quinones. MK-7(H4) (5.1 %) and MK-8 (5.5 %) are detected as minor fractions. Hexadecanoic (C16:0) acid is the dominant component of fatty acid pattern (45.7 % of the total fatty acids). The following fatty acids are also detected: cyclo-$C_{19:0}$(9,10)cis (15.2 %), $C_{18:0}$ (14.8 %), $C_{18:1}$(M9)cis (13.9 %), $C_{20:0}$ (3.8 %), $C_{14:0}$ (3.2 %), $C_{12:0}$-3OH (2.6 %), and $C_{12:0}$ (0.7 %). No data on the analysis of polar lipids are available.

Coprothermobacter Rainey and Stackebrandt. 1993a, 857

Co pro. ther mo-bac'ter. Gr. fem. n. kopros manure; Gr. adj. thermos warm; Gr. hyp. mas. n. bakter rod; N.L. mas. n. Coprothermobacter, because it is a thermophilic rod-shaped bacterium isolated from cattle manure.

Cells of *Coprothermobacter* are rod-shaped ranging from 1 to 6 μm in length, occurring singly or in pairs in young cultures. Colonies are white, circular (diameter 1–2 mm), convex, smooth with entire edges. Gram staining is negative. Electron micrographs of thin sections of the two species of the genus reveal a thin inner wall layer and a heavy outer wall (Rainey and Stackebrandt 1993a). Thermophilic temperature range for growth was between 35 °C and 75 °C, with optimum at 55–70 °C, 55 °C for *C. platensis* and 65–70 °C for *C. proteolyticus*.

Cells are strictly anaerobic. They are proteolytic using gelatin and peptones. Sugars are used poorly unless yeast extract or rumen fluid is added. *C. proteolyticus* ferments peptone, gelatin, casein, and Trypticase peptone in the presence of 0.1 % yeast extract. It grows on the following sugars when yeast extract and either rumen fluid or Trypticase is added: glucose, fructose, maltose, sucrose, and mannose. The fermentation products from gelatin or glucose in the presence of yeast extract are acetate, H_2, and CO_2 along with smaller quantities of isobutyrate, isovalerate, and propionate (Ollivier et al. 1985, Rainey and Stackebrandt 1993b). *Coprothermobacter platensis* ferments peptone, gelatin, casein, bovine albumin, and yeast extract. The addition of 0.02 % of yeast extract in the culture medium is necessary to stimulate growth on sugars. Glucose, fructose, sucrose, maltose, and starch are poorly fermented. Fermentation products from glucose are acetate, H_2, and CO_2. The major fermentation products from gelatin are acetate, propionate, H_2, and CO_2 (Etchebehere et al. 1998).

Both species reduce thiosulfate, but not sulfate, to sulfide with a concomitant increase in growth and glucose utilization (Etchebehere et al. 1998).

An extracellular protease activity is observed for cells grown on gelatin (Etchebehere et al. 1998; Kersters et al. 1994).

The major polyamines synthesized by *Coprothermobacter proteolyticus* are putrescine, spermidine, and spermine (Hamana et al. 1996).

The main features of members of *Thermodesulfobiaceae* are listed in Tables 33.1 and 33.2.

Growth of *C. proteolyticus* is inhibited by neomycin (0.15 g/l) and penicillin G (20 U/ml); vancomycin, polymyxin B, sodium azide, and kanamycin are not effective inhibitors (Kersters et al. 1994). Vancomycin (2.5 mg/l), neomycin (0.15 g/l), and polymyxin (20 mg/l) inhibit growth of *Coprothermobacter platensis* (Etchebehere et al. 1998).

Biochemical Characteristics

Protease Assays

Protease activity is assayed using the azocasein method under anaerobic conditions as described by Brock et al. (1982), (Etchebehere et al. 1998) or by the method of Twining (1984), (Kersters et al. 1994).

Coprothermobacter proteolyticus possesses a thermostable protease with optimal temperature of 85 °C and optimal pH of 9.5. The protease retains about 90 % of its activity at pH 10.0 and appears quite specific as compared to enzymes from other thermophilic or hyperthermophilic proteolytic microorganisms (Klingeberg et al. 1991).

Isolation, Enrichment, and Maintenance Procedures

Members of the family *Thermodesulfobiaceae* are mainly isolated on two types of culture medium under anaerobic conditions.

For enrichment of *Thermodesulfobium narugense*, the sulfate is used as terminal electron acceptor as described by Mori et al. (2003), with H_2 used as the energy source. The enrichment culture is transferred several times to new culture medium of the same composition. Single colonies are formed after 2 weeks of incubation at 55 °C on the culture medium solidified with 2 % agar in vials. After a second purification step on agar, a uniformly shaped axenic culture is obtained.

T. narugense is maintained in the enrichment medium under a H_2/CO_2 (4:1, v/v) atmosphere at 55 °C. The culture should be transferred every 2 weeks. After growth, the culture can also be stored at room temperature for several weeks. For long-term storage, it can be preserved in liquid nitrogen (−196 °C) under strictly anaerobic conditions with 5 % dimethylsulfoxide or 10 % glycerol. Liquid drying is also successful with a protective medium composed of 0.1 M potassium buffer (pH 7.0), 3 % sodium glutamate, 1.5 % ribitol, and 0.05 % cysteine hydrochloride monohydrate.

Coprothermobacter species can be enriched using culture media and procedures similar to those described by Ollivier et al. (1985) with gelatin as the energy source and Na_2S and cysteine as the reductive agents. In parallel, peptone-yeast medium (Holdeman et al. 1977) may be used for the enrichment of *Coprothermobacter proteolyticus* (Kersters et al. 1994). At least, three subcultures in the same growth conditions, at temperature from 55 °C up to 70 °C, are needed before isolation.

After several transfers, the enrichment cultures are serially diluted using the method of Hungate (1969), with roll tubes containing the basal medium, gelatin as the energy source, and purified agar at a concentration of 2 %. For isolation, agar medium can also be poured into plates within an anaerobic chamber. In order to detect selectively proteolytic colonies during the first step of isolation, casein can be used as substrate. Colonies, surrounded with large clearing zones, are picked and re-streaked on gelatin agar plates or roll tubes. At least two colonies are picked and the process of serial dilution in roll tubes is repeated in order to purify the cultures.

Stock cultures can be maintained on the medium described by Ollivier et al. (1985) and transferred at least monthly. Liquid cultures retain viability after several weeks of storage at room temperature. Cultures have also to be refrigerated. Because of lysis of cells, it is recommended to stock cultures before the end of the exponential phase.

Ecology

Habitat

The members of *Thermodesulfobiaceae* family were mainly isolated from digesters or hot spring.

Thermodesulfobium narugense was isolated from a vent of Narugo hot spring located in the prefecture of Miyagi in Japan (Mori et al. 2003). This vent harbored white microbial mats, mainly formed by sulfur-oxidizing bacteria. A mat sample was taken at a site presenting a temperature and a pH of 58 °C and 6.9, respectively (Mori et al. 2003). Several clones close to *Thermodesulfobium* were also detected across the planet. For example, clones were retrieved from anaerobic sediments at Rio Tinto, located in the core of the Iberian Pyritic Belt having a constant acidic pH (Sanchez-Andrea et al. 2011). Other clones were detected from hot springs in Yellowstone National Park (USA) (unpublished), from thermal pools in the uzon caldera, Kamchatka, Russia (Burgess et al. 2012), and mud and water from Los Azufres geothermic belt, Michoacan, Mexico (unpublished) (Table 33.3).

Strains of *Coprothermobacter proteolyticus* were isolated from anaerobic hot digesters. The type strain BT (ATTCC 35245 = OCM 4 = DSMZ 5265 = LMG 11567) was isolated from a digester fermenting tannery wastes and cattle manure. Strain 18 was isolated from a biokitchen waste digester. Strain 18 has been deposited in the LMG culture Collection (LGM 14268).

Coprothermobacter platensis was isolated from a methanogenic mesothermic reactor treating a protein-rich wastewater (Kersters et al. 1994). Clones close to *Coprothermobacter* were also retrieved mainly in anaerobic digesters (Riviere et al. 2009; Tandishabo et al. 2012). Some clones were indigenous to petroleum reservoirs (Kobayashi et al. 2012).

Table 33.3
Clones close to *Thermodesulfobium* or *Coprothermobacter*

GenBank accession #	Sequences origin	Reference	Closest genus
	DNA from environmental samples		
HQ730662.1	Anaerobic zones of Tinto River, Iberian Pyritic Belt	Sanchez-Andrea et al. (2011)	*Thermodesulfobium*
JQ815731.1			
JQ420033.1			
DQ834002.1	Hot Springs in Yellowstone National Park	Unpublished	
GQ328297.1	Thermal pools in the uzon caldera, kamchatka, Russia	Burgess et al. (2012)	
GQ328292.1			
GQ328235.1			
GQ328409.1			
GQ328239.1			
GQ328359.1			
GQ328317.1			
GQ328359.1			
HF677523.1	Mud and water from Los Azufres geothermic belt, Michoacan, Mexico	Unpublished	*Coprothermobacter*
JF808034.1	Depleted oil reservoir	Kobayashi, H.,	
GU363592.1	Anaerobic digesters	Tandishabo et al. (2012)	
CU924340.1	Anaerobic digesters	Riviere et al. (2009)	

Other clones were found in microbial mats, mud, and water from Los Azufres geothermic belt, Michoacan, Mexico (unpublished) (*Table 33.3*).

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