INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, Oct. 1985, p. 425–428 Vol. 35, No. 4
0020-7713/85/0400425-04$02.00/0

Emendation of the Genus *Thermobacteroides*: *Thermobacteroides proteolyticus* sp. nov., a Proteolytic Acetogen from a Methanogenic Enrichment

BERNARD M. OLLIVIER,[1] ROBERT A. MAH,[2]* THOMAS J. FERGUSON,[2] DAVID R. BOONE,[2] J. L. GARCIA,[1] AND RALPH ROBINSON[3]

Office de la Recherche Scientifique et Technique Outre-Mer, Laboratoire de Microbiologie, Université de Provence, Marseille 13331 Cedex 3, France[1]; Division of Enviromental and Occupational Health Sciences, School of Public Health, University of California, Los Angeles, California 90024[2]; and Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611[3]

***Thermobacteroides proteolyticus* sp. nov. was isolated from a methanogenic enrichment culture inoculated from a thermophilic digestor (55°C) that was fermenting tannery wastes and cattle manure. The cells were anaerobic, gram-negative, nonsporeforming, nonmotile rods that were 0.5 μm wide and 1 to 6 μm long. At the end of logarithmic growth, they were pleomorphic, with some filamentous cells. The deoxyribonucleic acid base composition was 45 mol% guanine plus cytosine. The temperature optimum was 63°C (growth range 35, to 75°C); the pH optimum was 7.5 (growth range, pH 5.0 to 8.5). The growth substrates used included yeast extract, peptone, casein, gelatin, and Trypticase peptone. Fructose, glucose, maltose, sucrose, and mannose were weakly used as growth substrates; however, addition of yeast extract and either rumen fluid or Trypticase peptone stimulated utilization of these carbohydrates. Acetate, H_2, and CO_2 were the major products of growth in medium containing gelatin or glucose. The cells were resistant to kanamycin. The type strain is strain BT (= ATCC 35242).**

We report the isolation and characterization of a new thermophilic anaerobic species obtained from a methanogenic enrichment inoculated from a thermophilic digestor that was operated on tannery wastes and cattle manure. This organism stains gram negative; it is a nonsporulating bacterium which uses proteins and sugars as substrates. We propose the name *Thermobacteroides proteolyticus* sp. nov. for this organism.

MATERIALS AND METHODS

Inoculum and enrichment. Tannery wastes and cattle manure were digested under thermophilic conditions (55°C) at the Institut de Recherche de Chimie Appliquée, Vert le Petit, France. Digestor samples were inoculated into a 50% rumen fluid medium made up in the salt solution of Balch et al. (1) with formate and H_2-CO_2 as methanogenic substrates. When methanogenesis was complete, the culture was maintained by inoculation of fresh medium with 5% (vol/vol) transfers. The most numerous methanogenic bacterium obtained from the enrichment was isolated and tentatively identified as *Methanobacterium thermoautotrophicum*.

Isolation procedures. After several transfers of the thermophilic methanogenic enrichment culture, agar plates were incubated for isolation of methanogenic and non-methanogenic bacteria. One predominant non-methanogenic colony type appeared at high dilutions on agar medium having the same composition as the enrichment medium. This colony type was picked and isolated in axenic culture by inoculation into agar medium.

Culture techniques. The anaerobic culture techniques of Hungate (5) with the modifications of Balch et al. (1) were used throughout this work. Culture media and reagents were prepared in an anaerobic glove box (Coy Lab Products, Ann Arbor, Mich.).

Culture media. The culture medium used contained 1 g of NH_4Cl, 0.4 g of $K_2HPO_4 \cdot 3H_2O$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.5 g of L-cysteine hydrochloride, 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.001 g of resazurin, 25 ml of a mineral solution (1), 5 ml of a trace mineral solution (1), and 1,000 ml of Milli-Q-deionized water (conductivity, 5.9 $\mu S \cdot m^{-1}$). The medium was adjusted to pH 7.0 with 10 M KOH and boiled under O_2-free N_2 until the resazurin was reduced. Medium was cooled and dispensed into serum tubes (10 ml/tube) that were stoppered with butyl rubber stoppers (Bellco Glass, Inc., Vineland, N.J.) and sealed with aluminum crimp closures (Wheaton Scientific, Millville, N.J.). After removal from the anaerobic chamber, the tubes were flushed with 100% N_2 for 2 min. After sterilization and just before inoculation, 0.2 ml of 1% $Na_2S \cdot 9H_2O$ and 0.05 ml of 10% $NaHCO_3$ were dispensed into each tube. Agar medium (2% agar [Difco]) was autoclaved in sealed, round-bottom flasks and plates were poured within the anaerobic chamber. Na_2S and $NaHCO_3$ were added at the same concentrations as in the liquid media. The final pH was 7.0. Agar plates were incubated in closed cylinders containing an N_2-CO_2 gas mixture (4:1) at 1 atmosphere (101 kPa). Additions to the media from sterile, anaerobic stock solutions were made as indicated below. pH adjustments to media were made by adding 1 N NaOH or 1 N HCl. Rumen fluid was centrifuged at 7,000 × *g* for 30 min and sterilized under 100% N_2 by autoclaving. Blood agar for testing hemolysis was prepared anaerobically. Physiological and metabolic tests were performed at 65°C and pH 7.5. Optimum temperature and pH values for growth were determined in medium containing 0.2% yeast extract as the energy source.

Analytical techniques. Culture headspace gases were analyzed by gas chromatography as previously described (2). Bacterial growth was quantified by measuring the optical density at 580 nm. Volatile fatty acids were measured by gas chromatography of acidified samples. Nonvolatile organic acids and alcohols were measured as described by Garcia et al. (4). Glucose concentration was determined by using an

* Corresponding author.

TABLE 1. Effect of yeast extract and rumen fluid on H_2 production from various substrates and growth of *Thermobacteroides proteolyticus*

Substrate[a]	Yeast extract[b]		Yeast extract + rumen fluid[b]	
	Optical density[c]	H_2 concn (μmol/10 ml of medium)	Optical density	H_2 concn (μmol/10 ml of medium)
None	0.08	14.2	0.10	16.7
Glucose	0.11	56.3	0.27	125.1
Fructose	0.09	45.1	0.25	104.9
Maltose	0.10	43.6	0.23	45.3
Sucrose	0.09	51.5	0.26	95.6
Mannose	0.11	52.4	0.20	57.6
Trypticase	0.17	29.2	0.19	27.6
Yeast extract	0.27	40.6	0.29	43.2
Peptone	0.38	24.0	0.48	26.4
Gelatin	0.32	24.7	0.36	22.6
Casein	0.21	23.2	0.21	24.3

[a] Sugar substrates were tested at concentrations of 10 mM; other compounds were tested at concentrations of 3 g/liter. We also tested the following compounds which did not support growth: arabinose, rhamnose, xylose, galactose, cellobiose, lactose, trehalose, glycerol, mannitol, and Casamino Acids.

[b] Yeast extract and rumen fluid were added at concentrations of 0.1 and 2.0%, respectively.

[c] Growth was measured by determining absorbancy at 580 nm after 40 h at 65°C. The inoculum used was a 40-h culture grown on medium containing yeast extract and gelatin (3 g/liter).

ortho-toluidine glucose test kit (Standbio Laboratory, Inc., San Antonio, Tex.). The presence of flagella was determined by the Rhodes technique (11) and electron microscopy, and motility was determined by microscopic (phase-contrast) observation of wet mounts.

Microscopy. Cells examined by electron microscopy were fixed with glutaraldehyde and osmium tetroxide and were treated with Spurr plastic.

Deoxyribonucleic acid preparation. Deoxyribonucleic acid was isolated and purified by the method of Price et al. (10). The guanine-plus-cytosine content was determined by ultracentrifugation in a CsCl density gradient (9, 14).

RESULTS AND DISCUSSION

A non-methanogenic bacterium, strain BT^T (T = type strain), was isolated from a thermophilic, methanogenic enrichment culture. Table 1 shows that this organism grew well on peptides; fermentation of sugars in medium containing 0.1% yeast extract was poor unless rumen fluid was added. Trypticase peptone also stimulated the fermentation of glucose, but Casamino Acids did not (Table 2). Acetate was the major volatile acid produced (Table 3), and no significant quantities of ethanol, lactate, or succinate were formed.

TABLE 2. Effect of addition of organic compounds on glucose utilization[a]

Organic compound added	% of glucose used	Optical density	Optical density of control without glucose
None	20	0.14	0.08
Trypticase peptone (1 g/liter)	32	0.38	0.11
Casamino Acids (1 g/liter)	17	0.09	0.09
Rumen fluid (2%)	36	0.29	0.09
Methylbutyrate (0.02%)	17	0.15	0.08

[a] *Thermobacteroides proteolyticus* was grown in basal medium containing 1 g of yeast extract per liter and 10 mM glucose. Controls lacked glucose. Growth was measured by determining absorbancy at 580 nm after 40 h at 65°C.

TABLE 3. Volatile acid production in gelatin or glucose medium[a]

Volatile fatty acid	Concn produced (mM) in:	
	Yeast extract-gelatin medium	Yeast extract-glucose medium
Acetic acid	7.50	3.10
Propionic acid	0.03	0
i-Butyric acid	0.15	Tr[b]
Butyric acid	0	0
i-Valeric acid	0.37	Tr
Valeric acid	0	0
i-Caproic acid	0.01	0
Caproic acid	0	0

[a] Cultures were grown on medium containing 3 g of gelatin per liter or 10 mM glucose and 1 g of yeast extract per liter. Products were examined after 40 h at 65°C.

[b] <0.01 mM.

Strain BT^T stained gram negative. Electron micrographs of thin sections revealed a thin inner wall layer and a heavy outer wall (Fig. 1).

Strain BT^T was resistant to kanamycin. In the presence of 300 ng/liter growth was reduced by 50%.

Strain BT^T differed from *Thermoanaerobium brockii* (17) and *Thermoanaerobacter ethanolicus* (16) by its Gram reaction, its substrate range, and its inability to use sugars readily in the presence of 0.1% yeast extract.

Strain BT^T might belong in the genus *Bacteroides* because it ferments proteins more strongly than sugars and does not use Casamino Acids. This latter physiological property is shared by two mesophilic *Bacteroides* species, *Bacteroides asaccharolyticus* (15) and *Bacteroides melaninogenicus* (13). *B. asaccharolyticus* grows on peptides but has a limited ability to ferment free amino acids (15); however, free amino

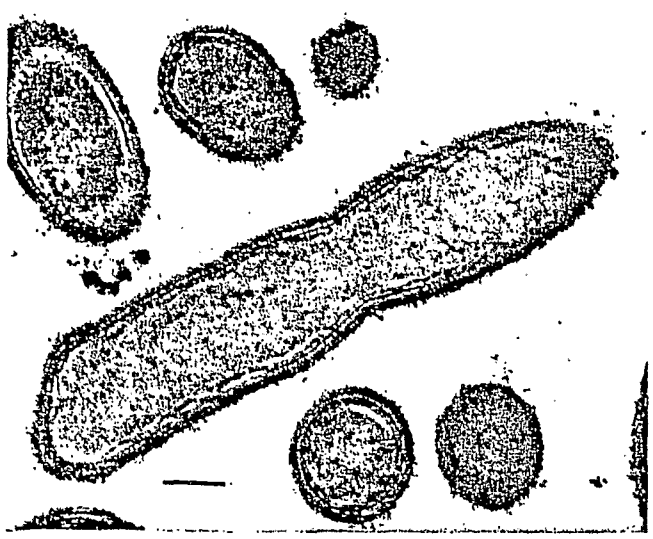

FIG. 1. Electron micrograph of a thin section showing a dense outer wall layer. Bar = 0.2 μm.

acids stimulate growth on peptides (8). Rumen fluid or Trypticase peptone stimulated the growth of strain BT^T on sugars, but Casamino Acids or 2-methylbutyrate did not. Trypticase peptone also stimulates glucose utilization in *Bacteroides ruminicola* (12), but synthesis of cell material is not always proportional to the amount of carbon and energy source utilized. A similar lack of proportionality was also observed when strain BT^T was grown on maltose and mannose. Regardless of these physiological similarities, strain BT^T cannot be placed within a recognized species of the genus *Bacteroides* because of its high growth temperature.

Recently, another thermophilic, nonsporeforming, gram-negative rod-shaped organism was described (3, 6). This organism was placed in a newly described genus, *Thermobacteroides*, as the type species, *Thermobacteroides acetoethylicus*. *Thermobacteroides acetoethylicus* would have been placed in the genus *Bacteroides* except for its thermophilic nature. The high growth temperature was the primary reason for creating a new genus for this isolate. Thus, any new thermophilic isolates with the generic characteristics of *Bacteroides* should also be placed in the genus *Thermobacteroides*. For these reasons, we propose placing strain BT^T in the recently created genus *Thermobacteroides*. Strain BT^T differs from *Thermobacteroides acetoethylicus* (3) by its ability to use peptones and by its lack of motility. Thus, strain BT^T should be placed in the genus *Thermobacteroides* as a separate species. No formal genus description has been given for *Thermobacteroides*. Therefore, we propose the emended genus description given below.

Thermobacteroides **Ben-Bassat and Zeikus 1983, 673.** (Ther.mo.bac.te.roi'des. Gr.n. *thermus* heat; M.L.n. *bacter* masc. equivalent of Gr.neut.n. *bacterium* a staff or rod; Gr.n. *idus* form, shape; *Thermobacteroides* rodlike thermophile) cells are gram-negative, nonsporeforming rods. Nonmotile or motile with peritrichous flagella.

Chemoorganotrophs which metabolize carbohydrates or peptone. The major metabolic end products include acetate, ethanol, CO_2, and H_2.

Obligately anaerobic. Thermophilic: optimum growth temperature, 55 to 70°C; no growth occurs below 35°C.

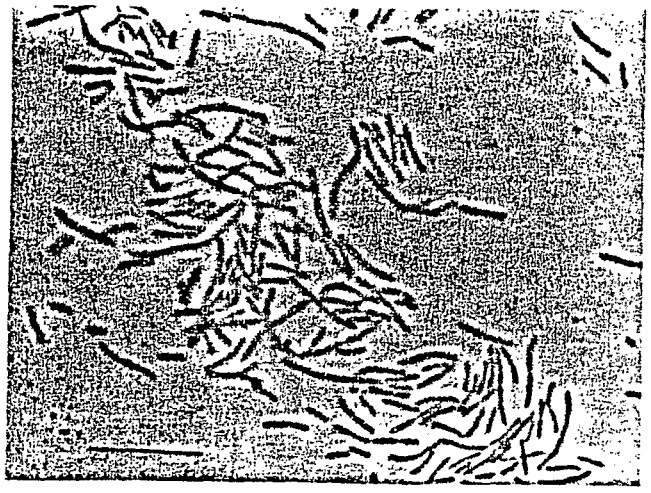

FIG. 2. Phase-contrast photomicrograph of young cells grown on gelatin at 65°C. Bar = 10 μm.

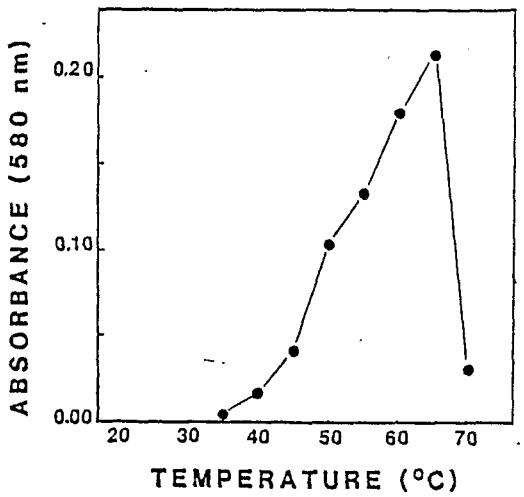

FIG. 3. Effect of temperature on growth, as determined by measuring absorbancy at 580 nm after 40 h on 0.2% yeast extract medium.

The deoxyribonucleic acid guanine-plus-cystosine content ranges from 30 to 46 mol%.

Found in thermophilic anaerobic digestors and natural thermophilic environments where organic matter is being vigorously decomposed.

Type species: *Thermobacteroides acetoethylicus* Ben-Bassat and Zeikus 1983, 673.

We propose the species description below for *Thermobacteroides proteolyticus*.

Thermobacteroides proteolyticus **sp. nov.** *Thermobacteroides proteolyticus* (pro.te.o.ly'ti.cus. Gr. adj. *protos* first; Gr. adj. *lyticus* loosening, dissolving; N.L. masc.adj. *proteolyticus* proteolytic) cells are rod shaped and 0.5 by 1 to 6 μm and occur singly or in pairs in young cultures (Fig. 2); pleomorphic in old cultures. No lysis is observed in the stationary phase. Colonies in roll tubes are 1

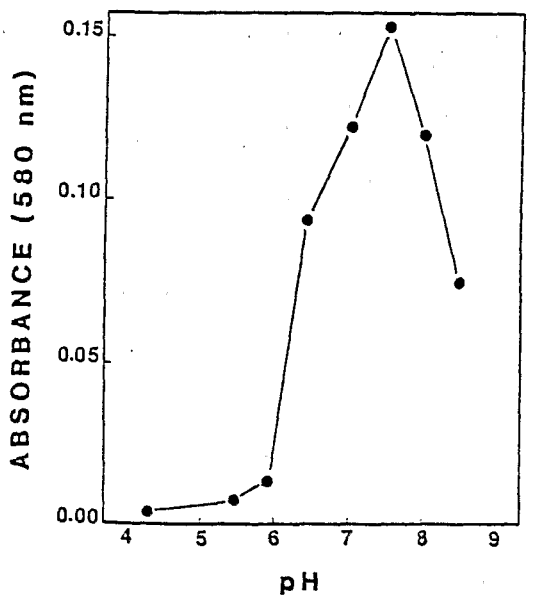

FIG. 4. Effect of pH on growth of cells on 0.2% yeast extract. Absorbancy at 580 nm was determined after 24 h.

to 2 mm in diameter after 3 to 4 days; they are white, entire, circular, smooth, and convex. Gram negative. Nonmotile. Nonsporeforming.

Obligately anaerobic. Ferments peptone, gelatin, casein, and Trypticase peptone in the presence of 0.1% yeast extract. Grows on the following sugars when yeast extract and either rumen fluid or Trypticase peptone are added: glucose, fructose, maltose, sucrose, and mannose. The fermentation products from gelatin or glucose in the presence of yeast extract are acetic acid, H_2, and CO_2, along with smaller quantities of isobutyric, isovaleric, and propionic acids.

Optimum temperature, 63°C (range, 35 to 70°C) (Fig. 3). Optimum pH, 7.5 (range, pH 5.0 to 8.5) (Fig. 4).

Yeast extract is required for growth on protein substrates. Sugars are used poorly unless yeast extract and either rumen fluid or Trypticase peptone are added.

The guanine-plus-cytosine content of deoxyribonucleic acid is 45 mol%, as determined by buoyant density.

Isolated from a themophilic (55°C) digestor that was fermenting tannery wastes and cattle manure.

The type strain is strain BT (= ATCC 35242).

ACKNOWLEDGMENTS

Thermophilic digestor samples were kindly supplied by the Institut de Recherche de Chimie Appliquée, Vert le Petit, France. We thank R. Sleat, I. Mathrani, and Y. Liu for helpful discussions.

This study was supported by grants 5080-323-0423 and IFAS-GRI-FIA-MCS 2171 from the Gas Research Institute and by research grant DE-AT03-80ER10684 from the U.S. Department of Energy.

LITERATURE CITED

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. 43:260–296.
2. **Baresi, L., R. A. Mah, D. M. Ward, and I. R. Kaplan.** 1978. Methanogenesis from acetate: enrichment studies. Appl. Environ. Microbiol. 36:186–197.
3. **Ben-Bassat, A., and J. G. Zeikus.** 1981. *Thermobacteroides acetoethylicus* gen. nov. and spec. nov., a new chemoorganotrophic, anaerobic, thermophilic bacterium. Arch. Microbiol. 128:365–370.
4. **Garcia, J. L., J. P. Guyot, B. Ollivier, M. Trad, and C. Paychenc.** 1982. Ecologie microbienne de la digestion anaerobie: techniques de numération et d'isolement. Cah. ORSTOM Ser. Biol. 45:3–15.
5. **Hungate, R. E.** 1969. A roll-tube method for cultivation of strict anaerobes, p. 117–132. *In* R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3b. Academic Press, Inc., New York.
6. **International Journal of Systematic Bacteriology.** 1982. Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List no. 9. Int. J. Syst. Bacteriol. 32:384.
7. **International Journal of Systematic Bacteriology.** 1983. Validation of the publication of new names and new combinations previously effectively published outside the IJSB. List no. 11. Int. J. Syst. Bacteriol. 33:673.
8. **Lev, M.** 1977. Casamino Acids enhance growth of *Bacteroides melaninogenicus*. J. Bacteriol. 129:562–563.
9. **Preston, J. P., and D. R. Boone.** 1973. Analytical determination of the buoyant density of DNA in acrylamide gel after preparative CsCl gradient centrifugation. FEBS Lett. 37:321–324.
10. **Price, C. W., G. B. Fuson, and H. J. Phaff.** 1978. Genome comparison in yeast systematics, delimitation of species within the genera *Schwanniomyces*, *Saccharomyces*, *Debaryomyces*, and *Pichia*. Microbiol. Rev. 42:161–193.
11. **Rhodes, M. E.** 1958. The cytology of *Pseudomonas* sp. as revealed by a silver-plating staining method. J. Gen. Microbiol. 18:639–648.
12. **Russell, J. B.** 1983. Fermentation of peptides by *Bacteroides ruminicola* B_14. Appl. Environ. Microbiol. 45:1566–1574.
13. **Sawyer, S. J., J. B. MacDonald, and R. J. Gibbons.** 1962. Biochemical characteristics of *Bacteroides melaninogenicus*: a study of thirty-one strains. Arch. Oral Biol. 6:685–691.
14. **Schildkraut, C. L., J. Marmur, and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430–433.
15. **Wahren, A., and R. J. Gibbons.** 1970. Amino acid fermentation by *Bacteroides melaninogenicus*. Antonie van Leeuwenhoek J. Microbiol. Serol. 36:149–159.
16. **Wiegel, J., and L. G. Ljungdahl.** 1981. *Thermoanaerobacter ethanolicus* gen. nov., spec. nov., a new extremely thermophilic anaerobic bacterium. Arch. Microbiol. 128:41–48.
17. **Zeikus, J. G., P. W. Hegge, and M. A. Anderson.** 1979. *Thermoanaerobium brockii* gen. nov. and sp. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch. Microbiol. 122:41–48.