

***Caloramator coolhaasii* sp. nov., a glutamate-degrading, moderately thermophilic anaerobe**

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An obligately anaerobic, moderately thermophilic, glutamate-degrading bacterium (strain Z^T) was isolated from an enrichment culture obtained from anaerobic thermophilic granular sludge. The cells were rod-shaped to filamentous and showed no motility or spore formation. The cell wall had a Gram-positive structure, which was revealed by electron microscopy. Optimum growth of the strain was observed under neutrophilic conditions at 50–55 °C. The doubling time of strain Z^T grown in rich medium was approximately 1 h at optimal pH and temperature. Strain Z^T was able to grow on a variety of organic compounds. Most carbon sources were converted to acetate, CO₂, H₂, and traces of propionate and lactate. Strain Z^T oxidized glutamate to acetate, CO₂, NH₄⁺, traces of propionate and H₂. The doubling time on this substrate was 1.6 d. The strain fermented glutamate syntrophically in co-culture with *Methanobacterium thermoautotrophicum* Z-245^T to the same products, but the co-culture had a fourfold higher growth rate. 16S rDNA sequence analysis revealed a relationship with *Thermobrachium celere*, *Caloramator indicus* and *Caloramator proteoclasticus*. The G+C content was 31.7 mol%. Based on its morphological, phylogenetic and physiological characteristics, it is proposed that strain Z^T should be classified in the genus *Caloramator* as a new species, *Caloramator coolhaasii*.

Keywords: *Caloramator coolhaasii* sp. nov., interspecies hydrogen transfer, glutamate degradation, methanogenic archaea, syntrophic consortia

INTRODUCTION

Proteins are important substrates in anaerobic digestion (Barker, 1981; McInerney, 1989). Amino acids, the hydrolysis products of proteins, can serve as carbon and energy sources for many types of anaerobic bacteria. Utilization of amino acids has been used as a classification characteristic for clostridia (Mead, 1971; Elsdén & Hilton, 1979). Several anaerobic bacteria have been described that can grow on glutamate. Most of them are members of the low-G+C *Clostridium* subphylum, which convert glutamate to acetate, butyrate, CO₂, NH₄⁺ and traces of hydrogen (Buckel & Barker, 1974; Laanbroek *et al.*, 1979). Also, fermentative anaerobes are described that utilize gluta-

mate in co-culture with hydrogen-scavenging methanogens (Cheng *et al.*, 1992; Tarlera, 1997; Baena *et al.*, 1999a; Nanninga & Gottschal, 1985; Stams & Hansen, 1984). Some of these organisms are mesophilic, but *Selenomonas acidaminovorans*, recently renamed *Thermanaerovibrio acidaminovorans* (Baena *et al.*, 1999b), and *Caloramator proteoclasticus* are moderately thermophilic. These bacteria grow slowly in pure culture, but more rapid growth is achieved when grown syntrophically with a partner organism which removes the hydrogen. These bacteria degrade one glutamate to two acetate, one HCO₃⁻, one NH₄⁺ and one H₂, or to one propionate, two HCO₃⁻, one NH₄⁺ and two H₂, where the ratio is dependent on the hydrogen partial pressure.

In this paper, the properties of strain Z^T, a novel organism isolated from anaerobic methanogenic granular sludge, are described. This bacterium degrades glutamate in pure culture, but much faster growth is achieved in co-culture with hydrogenotrophic methanogens.

Abbreviations: MPN, most probable number; TEM, transmission electron microscopy.

The GenBank accession number for the 16S rDNA sequence of strain Z^T is AF104215.

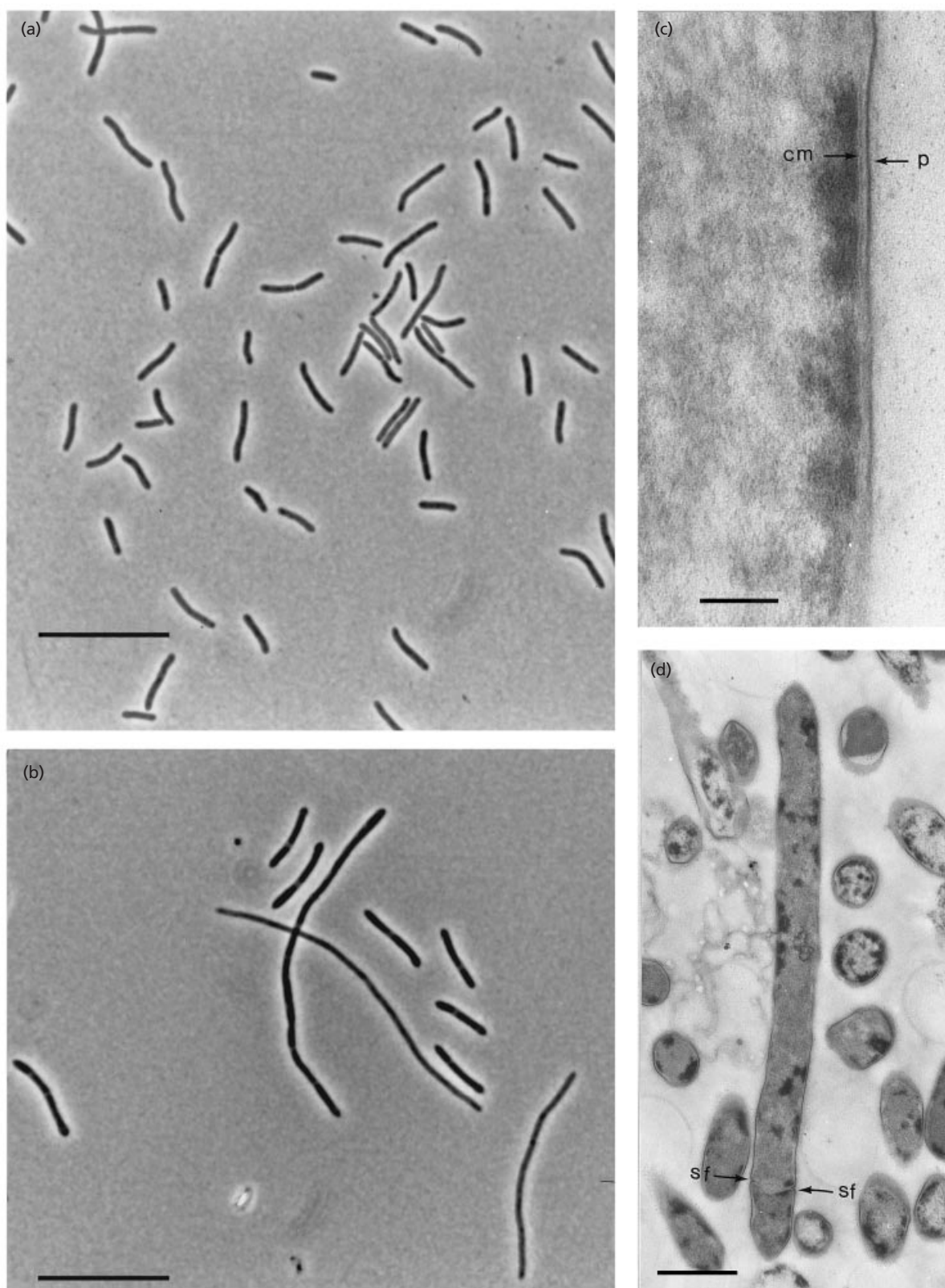


Fig. 1. (a, b) Phase-contrast micrographs of cells of strain Z^T during growth on glutamate (a) and WC broth (b); bar, 10 µm. (c, d) Electron micrographs showing (c) the single-layered structure of the cell wall (bar, 0.1 µm; cm, cytoplasmic membrane; p, peptidoglycan layer) and (d) cell division (bar, 1 µm; sf, septum formation). Electron-dense structures are probably intracellular protein precipitates (d).

METHODS

Organisms, cultivation and isolation procedures. Strain Z^T was isolated from thermophilic methanogenic granular sludge converting glutamate as the sole source of carbon and energy. *Methanobacterium thermoautotrophicum* Z-245^T (= DSM 3720^T) and *Thermobrachium celere* DSM 8682^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Caloramator proteoclasticus* DSM 10124^T was from our own culture collection.

A bicarbonate-buffered anaerobic medium as described previously by Stams *et al.* (1993), supplemented with 0.02 % yeast extract, was used for enrichment and cultivation of strain Z^T. Wilkens–Chalgren broth (WC broth; Oxoid; 16 g l⁻¹) was used for the isolation of strain Z^T. Direct dilution series in liquid WC broth as well as agar plates supplied with WC broth were used. All incubations were done at 55 °C in the dark, unless otherwise stated. For direct comparison of strain Z^T with *Caloramator proteoclasticus* and *T. celere*, all cultures were cultivated on: WC broth (16 g l⁻¹); bicarbonate-buffered anaerobic medium (Stams *et al.*, 1993); bicarbonate-buffered anaerobic medium supplemented with 0.02 % yeast extract; bicarbonate-buffered anaerobic medium supplemented with 0.2 % yeast extract; and YTG medium. YTG medium contained (l⁻¹): Na₂CO₃, 5.3 g; Na₂HPO₄·2H₂O, 0.356 g; KCl, 0.075 g; yeast extract 5 g; tryptone, 10 g; cysteine·HCl, 0.2 g; Na₂S·9H₂O, 0.2 g; resazurin, 1 mg. Before use, the pH of the YTG medium was adjusted to 10 by adding sterile 3 M NaOH. Glucose (10 mM) was used as the carbon source, except in the case of WC broth, where no additional carbon source was added.

Determination of growth parameters. The growth rate of strain Z^T on WC broth was determined by measuring the increase in OD₆₀₀ in triplicate with time. The growth rate on glutamate with and without yeast extract was determined by measurement of product formation with time. Growth on different substrates was tested using a bicarbonate-buffered anaerobic medium as described previously by Stams *et al.* (1993), supplemented with 0.02 % yeast extract. The inoculum size of strain Z^T was 1 %. Substrates were added from sterile stock solutions up to a concentration of 10 mM unless stated otherwise. For syntrophic growth tests, 1 % of strain Z^T was inoculated in hydrogen pregrown cultures of *M. thermoautotrophicum* Z-245^T. In all cases, growth was followed by visual examination of culture turbidity and by product formation after 8 weeks of incubation at 55 °C. The temperature optimum was determined in WC broth at pH 7 in triplicate bottles at a temperature range of 15–80 °C. The pH optimum was tested in WC broth adjusted with NaOH or HCl, and triplicate bottles were incubated at 55 °C at a pH range of 4–10. Both temperature and pH were determined by measuring OD after 2 weeks incubation.

Cellular characterization. The Gram type was determined using Gram staining and electron microscopy. Cells from active cultures were stained for Gram type using 2 % (w/v) crystal violet and 2 % safranin S (w/v) as counterstain. Additionally, a 2 % (w/v) KOH solution was used to test lysis of bacteria. For transmission electron microscopy (TEM), cells were fixed for 2 h in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 0 °C. After rinsing in the same sodium cacodylate buffer, a post-fixation was done in 1 % (w/v) OsO₄ and 2.5 % (w/v) K₂Cr₂O₇ for 1 h at room temperature. Finally, the cells were post-stained in 1 % (w/v) uranyl acetate. Micrographs were taken with a

Philips EM400 TEM. Motility was determined using Gray's method for flagella staining (Gerhardt *et al.*, 1981). For negative staining, cells were fixed for 1 h in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). A Formvar-coated grid (150 mesh) was placed on a drop of a dense cell suspension for 20 min. After rinsing with deionized water, the cells were stained with 1 % (w/v) uranyl acetate and the grid was air-dried. Cells were examined in a JEOL TEM.

G+C content. The G+C content of the DNA was determined at the DSMZ by HPLC (Mesbah *et al.*, 1989).

SDS-PAGE analysis of whole-cell protein profiles. The strains were grown at 55 °C in bicarbonate-buffered anaerobic medium supplemented with 0.2 % yeast extract and 10 mM glucose. Samples from cells in the exponential phase (corresponding to OD₆₀₀ values of approximately 0.25) were centrifuged for 5 min at 16000 g. The pellets were resuspended in 100 mM Tris/HCl buffer (pH 7.2). Protein samples were diluted in denaturing loading buffer and boiled for 15 min. Cell debris was removed by centrifugation (5 min at 16000 g) and samples were stored at 4 °C until electrophoresis. SDS-polyacrylamide running gels with 10 % acrylamide and 4 % acrylamide stacking gels were run in a Bio-Rad Mini Protean II system at 20 mA for 2 h. Approximately 10 µg protein was loaded in each lane. High-molecular-weight markers for SDS-PAGE were from LKB-Pharmacia. The gels were stained with 0.1 % (w/v) Coomassie brilliant blue R250 in methanol/deionized water/acetic acid (4:5:1, by vol.) overnight and destained in methanol/deionized water/acetic acid (4:5:1, by vol.) for 3 h.

16S rDNA sequence analysis. Genomic DNA was isolated from strain Z^T and the 16S rDNA was amplified using bacterial primers 8f and 1510r (Lane, 1991) under previously described conditions (Harmsen *et al.*, 1995). PCR products were purified and concentrated using a QIA Quick kit (Qiagen). Approximately 1 µg purified PCR product was used for sequence analysis using the Sequenase T7 sequencing kit (Amersham) according to the manufacturer's instructions. Infrared Dye 41 (MWG-Biotech) labelled primers 515r, 338f (Lane, 1991) and 968f (Nübel *et al.*, 1996) were used as sequencing primers. The sequences were automatically analysed on a LI-COR DNA sequencer 4000L and corrected manually. The software of the ARB package (Strunk & Ludwig, 1991) was used to check reading errors and for phylogenetic analysis. A neighbour-joining tree was constructed with the closest relatives. FASTA homology searches were done using the GenBank and EMBL databases. The homologies were checked with the ARB programs.

Nucleotide accession numbers. The EMBL database accession numbers of the strains most closely related to strain Z^T are: *Caloramator proteoclasticus* (DSM 10124^T), X90488; *Caloramator indicus* (ACM 3982^T), X75788; *Thermobrachium celere* (DSM 8682^T), X99238; *Clostridium botulinum*, X68187; *Clostridium homopropionicum*, X76744; *Clostridium acetobutylicum*, X78071; and *Clostridium algidicarnis*, X77676.

Other analyses. Substrate and fermentation product concentrations were measured using GC and HPLC methods as described previously (Stams *et al.*, 1993). Amino acids were measured as described by Kengen & Stams (1994), with an extension of the derivatization time to 30 min. Sulfide was

analysed as described by Trüper & Schlegel (1964). Anions were analysed as described by Scholten & Stams (1995).

RESULTS

Enrichment and isolation of strain Z^T

Strain Z^T was enriched from anaerobic methanogenic granular sludge fed with glutamate as the sole carbon and energy source. Strain Z^T was the predominant glutamate-degrading organism in most probable number (MPN) determinations of diluted biomass. Methanogens were present as the only microscopically visible impurity. The enrichment obtained from the MPN determinations was serially diluted in the presence of 5 mM bromoethane sulphonate to inhibit the methanogens. In the absence of methane production, growth of strain Z^T on the mineral salts medium was poor. Strain Z^T was therefore serially diluted and transferred to anaerobic bottles containing WC broth (16 g l⁻¹). The growth yield and growth rate of strain Z^T were considerably higher in this medium. To remove traces of methanogens, strain Z^T was plated on WC broth agar. This resulted in the development of circular, brownish, shiny colonies. Four colonies were picked from the highest-diluted plate with growth and transferred to a dilution series in WC broth. The highest dilution with growth was used to inoculate WC broth agar plates again, resulting in the development of the type of colonies as described above. This procedure was repeated once more and resulted in a pure culture of strain Z^T when checked by light and electron microscopy.

Morphology and cell structure

Cells of strain Z^T growing in WC broth were rod-shaped to filamentous, with a size of 2–40 µm by 0.5–0.7 µm as measured by light microscopy (Fig. 1a, b). During exponential growth, the cells were very long. Rapid lysis was observed as soon as the cells entered the stationary phase. However, in mineral salts medium with glutamate, cells were smaller and did not lyse in the stationary phase. Spores were never observed and no growth occurred after pasteurization of the culture (20 min, 90 °C). Cells stained Gram-negative, but the cell wall ultrastructure resembled that of Gram-positive anaerobic bacteria (Fig. 1c).

Physiological characteristics and metabolism

Strain Z^T was a strictly anaerobic bacterium. No growth occurred when traces of oxygen were present in the medium, as indicated by the pink colour of the medium in which resazurin was present. Its optimal growth temperature was 50–55 °C with lower and upper limits of 37 and 65 °C, respectively. The pH optimum was 7.0–7.5. Below pH 6.0 and above pH 8.5, no measurable growth was observed. Growth of strain Z^T was possible in a mineral salts medium supplemented with vitamins and glutamate as the sole source of carbon and energy. However, growth was enhanced

when 0.02 % yeast extract was added. The doubling times of strain Z^T in glutamate media without and supplemented with 0.02 % yeast extract were approximately 3.5 and 1.6 d, respectively. The doubling time of strain Z^T in WC broth was about 1 h.

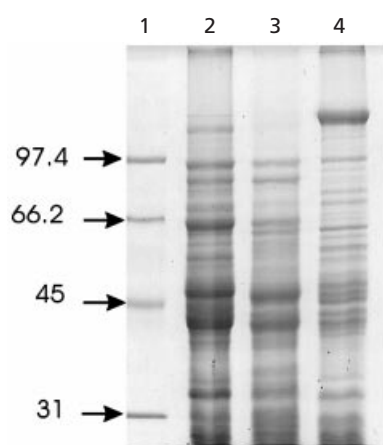
Strain Z^T grew with complex substrates like yeast extract, Casamino acids, gelatin, casitone, peptone (all at 2 %, w/v) and WC broth.

The following single substrates supported growth of strain Z^T: glutamate, aspartate, alanine, arginine, methionine, glucose, galactose, fructose, mannose, maltose, ribose, starch (0.5 %, w/v), xylose, cellobiose (5 mM) and pyruvate. Growth on glutamate, aspartate, alanine, arginine, methionine, mannose, ribose and pyruvate was enhanced when strain Z^T was co-cultivated with *M. thermoautotrophicum* Z-245^T. It produced acetate (13.9 mM), H₂ (10.1 mmol l⁻¹ medium) and traces of propionate (1.3 mM) from glutamate (9.8 mM). The carbon and electron recoveries were 97 % and 84 %, respectively, assuming that glutamate conversion to two acetate yields one CO₂, whereas two CO₂ are formed when glutamate is converted to propionate. From the glutamate originally added, 60–70 % was degraded. Glutamate metabolism was influenced by the presence of *M. thermoautotrophicum* Z-245^T; glutamate (18.6 mM) was converted to acetate (30.7 mM), CH₄ (8.6 mmol l⁻¹ medium; equivalent to 34.4 mmol H₂ l⁻¹ medium) and traces of propionate (1 mM). The carbon and electron recoveries were 102 % and 109 %, respectively, and glutamate was completely converted. The growth rate was about fourfold higher when *M. thermoautotrophicum* Z-245^T was present. When strain Z^T was grown on glutamate under H₂/CO₂ (80/20) only 7–10 % of the glutamate was converted. Under these conditions, it was not possible to obtain a closed electron balance. Glucose was fermented to acetate, lactate, CO₂ and H₂. When the amount of yeast extract in the medium was increased from 0.02 % to 0.2 %, formate and traces of ethanol could be detected as reduced end products as well as the usual products. The following components did not support growth of strain Z^T either in the absence or presence of *M. thermoautotrophicum* Z-245^T: glycine, leucine, cysteine, arabinose, mannitol, melobiose, rhamnose, casein (0.5 %, w/v), cellulose (0.5 %, w/v), citrate, isocitrate, 2-oxoglutarate, fumarate, malate, benzoate, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol or acetoin. A Stickland reaction was not observed with H₂, alanine or leucine as electron donor and glycine, arginine and proline as electron acceptor. The combination of alanine and arginine resulted in a higher production of hydrogen as compared to alanine alone, indicating an oxidative deamination of arginine. Yeast extract stimulated growth on glutamate, but was not required. Sulphide-reduced media were required for growth. Oxygen, nitrate, sulphate, thiosulphate and fumarate could not serve as electron acceptors for growth on glutamate; no measurable decrease in these compounds could be found after incubation.

Table 1. Growth and glucose fermentation products of strain Z^T, *Caloramator proteoclasticus* and *T. celere* on different media

–, No growth; +, growth. acet, Acetate; lact, lactate; form, formate; etoh, ethanol. Products given in parentheses represent minor products.

Medium	Strain Z ^T	<i>Caloramator proteoclasticus</i>	<i>T. celere</i>
WC broth	+	+	+
Bicarbonate-buffered glucose medium	acet, lact, H ₂	–	–
Bicarbonate-buffered glucose medium + 0.02% yeast extract	acet, lact, H ₂	acet, etoh, form, lact, H ₂	–
Bicarbonate-buffered glucose medium + 0.2% yeast extract	acet, (etoh), form, lact, H ₂	acet, etoh, form, lact, H ₂	acet, (etoh), form, H ₂
YTG medium, pH 10	–	–	acet, (etoh), form, H ₂

**Fig. 2.** SDS-PAGE of whole-cell proteins of strain Z^T and closely related strains. Lanes: 1, set of marker proteins with their molecular masses; 2, *Caloramator proteoclasticus*; 3, strain Z^T; and 4, *Thermobrachium celere*.

Direct comparison of strain Z^T with *Caloramator proteoclasticus* and *T. celere*

Strain Z^T, *Caloramator proteoclasticus* and *T. celere* grew well in WC broth and anaerobic bicarbonate-buffered medium supplemented with 0.2% yeast extract and 10 mM glucose. Strain Z^T and *Caloramator proteoclasticus* grew well in a bicarbonate-buffered medium supplemented with 0.02% yeast extract and 10 mM glucose, and strain Z^T was the only strain capable of growing in bicarbonate-buffered medium with glucose without yeast extract. Fermentation products of strain Z^T, *Caloramator proteoclasticus* and *T. celere* grown in glucose/yeast extract medium were different (Table 1).

The total cell proteins of strain Z^T, *Caloramator proteoclasticus* and *T. celere* were separated by SDS-PAGE (Fig. 2). The patterns obtained for the three strains differed indicating that these organisms are different species.

Phylogeny

The nucleotide sequence (1454 bp) of a 16S rRNA gene of strain Z^T was analysed and revealed that this organism belongs to the subphylum of Gram-positive clostridia with low G + C content. Sequence analysis showed that strain Z^T is closely related to *T. celere*, *Caloramator indicus* and *Caloramator proteoclasticus* (Engle *et al.*, 1996; Christostomos *et al.*, 1996; Tarlera *et al.*, 1997) with levels of similarity of 97.8, 97.2 and 96.2%, respectively. A phylogenetic tree showing the relationship of strain Z^T and other related species is depicted in Fig. 3.

The G + C content of strain Z^T was 31.7 ± 0.4 mol %.

DISCUSSION

Phylogenetically, strain Z^T belongs to the low-G + C-containing clostridial group and is closely related to *T. celere*, *Caloramator indicus* and *Caloramator proteoclasticus* (Engle *et al.*, 1996; Christostomos *et al.*, 1996; Tarlera *et al.*, 1997). Within this group, all members are closely related, but have been described and approved as new species. Based on 16S rDNA analysis, *Caloramator indicus* and *T. celere* seem to be almost 100% related. Despite the similarity of the 16S rDNA with these mentioned species, there are several differences between strain Z^T and other members within the genus *Caloramator* (Collins *et al.*, 1994). These include the absence of endospores, immobility, optimum growth temperature and optimum pH for growth (Table 2). Furthermore, the physiological characteristics of strain Z^T also differ considerably from those of the other species (Table 3). All four isolates have a broad saccharolytic activity, but strain Z^T is the only strain capable of fermenting xylose. *Caloramator proteoclasticus* is not able to ferment alanine, but forms alanine as a reduced end product from glutamate fermentation (Tarlera, 1997). Strain Z^T has no proteolytic activity on casein. Direct comparison of strain Z^T, *Caloramator proteoclasticus* and *T. celere* shows that strain Z^T is the only strain able to grow in a bicarbonate-buffered anaerobic medium

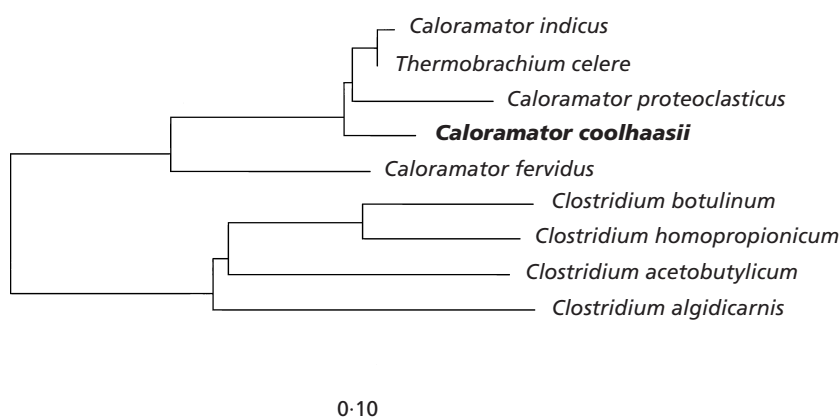


Fig. 3. Dendrogram based on 16S rDNA sequences showing the phylogenetic position of strain Z^T among representatives of the genus *Clostridium*. Bar, 0.1 (evolutionary distance). The type strains of all *Caloramator* species shown and *Thermobrachium celere* were used.

Table 2. General characteristics of strain Z^T compared to *T. celere*, *Caloramator indicus* and *Caloramator proteoclasticus*

Data taken from Engle *et al.* (1996), Christostomos *et al.* (1996) and Tarlera *et al.* (1997).

Characteristic	Strain Z ^T	<i>T. celere</i>	<i>Caloramator indicus</i>	<i>Caloramator proteoclasticus</i>
Gram stain	—	+	—	—
Cell wall type	+	+	+	+
G + C (mol %)	31.7	31	25.6	31
Temperature (°C):				
Optimum	50–55	62–67	60–65	55
Range	37–65	43–75	37–75	30–68
pH optimum	7.0–7.5	8.0–8.5	8.1	7.0–7.5
Motility	—	+	—	+
Spore formation	—	—	—	+
Habitat	Thermophilic granular sludge	Several locations	Thermophilic aquifer	Mesophilic granular sludge
Geographical location	Wageningen, The Netherlands	Several locations	Gujarat, India	Montevideo, Uruguay

with glucose as the sole source of carbon and energy. Strain Z^T and *Caloramator proteoclasticus* show no growth on YTG medium at pH 10, whereas *T. celere* grows very well at this pH.

The glucose fermentation products of the three strains also differ; *T. celere* forms only traces of ethanol and no lactate in a bicarbonate-buffered medium with 0.2% yeast extract, whereas strain Z^T forms traces of ethanol and does produce lactate. *Caloramator proteoclasticus* forms ethanol and lactate as major fermentation products. The whole-cell protein profiles of strain Z^T, *Caloramator proteoclasticus* and *T. celere* on SDS-PAGE also differ. These differences justify the creation of a new species in the genus *Caloramator*. The choice of *Caloramator* as genus was made because this genus was validly published prior to the genus *Thermobrachium* (Collins *et al.*, 1994; Engle *et al.*, 1996).

The glutamate metabolism of strain Z^T is clearly

enhanced by the presence of a hydrogenotrophic methanogen. Complete conversion of glutamate occurred under methanogenic conditions, whereas in pure culture, hydrogen formation inhibited glutamate degradation. *Selenomonas (Thermanaerovibrio) acidaminovorans* (Cheng *et al.*, 1992), another glutamate-degrading, hydrogen-producing moderate thermophile that was tested in co-culture with a methanogen, showed complete glutamate conversion, both in pure as well as in co-culture. However, *S. acidaminovorans* degraded alanine, isoleucine, leucine and valine only in the presence of a methanogen, indicating that its metabolism is also inhibited by hydrogen formation. Similar observations were made with *Clostridium acetireducens*, another moderate thermophile, which is unable to grow on glutamate (Örlygsson *et al.*, 1996). Strain Z^T did not show a shift in product formation when it was co-cultivated with a methanogen, but a more rapid and complete degradation of glutamate occurred.

Table 3. Selected growth substrates that differentiate strain Z^T from *T. celere*, *Caloramator indicus* and *Caloramator proteoclasticus*Data taken from Engle *et al.* (1996), Christostomos *et al.* (1996) and Tarlera *et al.* (1997).

Substrate	Strain Z ^T	<i>T. celere</i>	<i>Caloramator indicus</i>	<i>Caloramator proteoclasticus</i>
Glucose	+	+	+	+
Arabinose	—	—		
Fructose	+	+	+	+
Galactose	+	+		
Mannose	+	—	+	+
Maltose	+	+		
Rhamnose	—	—		
Ribose	—	—		
Xylose	+	—		—
Sucrose	+	+	+	
Lactose	—	—	+	—
Casamino acids	+	—		+
Yeast extract	+	+		+
Casein	—			+
Casitone	+			
Starch	+		+	+
Cellulose	—		—	—
Cellobiose	+	—	+	+
Peptone	+			+
2-Oxoglutarate	—			—
Glutamate	+			+
WC broth	+			
Glycine	—			+
Leucine	—			+
Aspartate	+			+
Alanine	+			—
Pyruvate	+	—		+
Stickland reaction	—			+

Description of *Caloramator coolhaasii* sp. nov.

Caloramator coolhaasii [cool.haas'i.i. M.L. gen. *coolhaasii* of Coolhaas, in honour of Caspar Coolhaas, a Dutch microbiologist, who was the first to describe thermophilic protein degradation under methanogenic conditions (Coolhaas, 1927)].

Rods to filamentous cells that are 2–40 µm long by 0.5–0.7 µm wide. Gram stain negative, with Gram-positive cell wall, non-motile, no spore formation. Strictly anaerobic chemo-organoheterotroph. Utilizes glutamate, aspartate, alanine, arginine, methionine, glucose, galactose, fructose, mannose, maltose, ribose, xylose, Casamino acids, yeast extract, gelatin, casitone, starch, peptone, cellobiose, pyruvate and WC broth for growth. Growth on glutamate, aspartate, alanine, arginine, methionine, mannose, ribose and pyruvate is enhanced in the presence of the methanogen *M. thermoautotrophicum* Z-245^T. Produces acetate, CO₂, H₂ and traces of propionate from glutamate. Produces acetate, lactate, CO₂ and H₂ from glucose. Yeast extract stimulates growth on glutamate, but is not

required. No Stickland reaction could be observed. Sulphide-reduced media were required for growth. No reduction of oxygen, nitrate, sulphate, thiosulphate or fumarate. Moderately thermophilic. Growth range 37–65 °C; optimum 50–55 °C. Growth in pH range 6.0–8.5; optimum pH 7.0–7.5. G+C content is 31.7±0.4 mol%. Type strain is strain Z^T (= DSM 12679^T). Habitat: anaerobic thermophilic methanogenic sludge.

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REFERENCES

- Baena, S., Fardeau, M.-L., Ollivier, B., Labat, M., Thomas, P., Garcia, J.-L. & Patel, B. K. C. (1999a). *Aminomonas paucivorans* gen. nov., sp. nov., a mesophilic, anaerobic, amino-acid-utilizing bacterium. *Int J Syst Bacteriol* **49**, 975–982.
- Baena, S., Fardeau, M.-L., Woo, T. H. S., Ollivier, B., Labat, M. & Patel, B. K. C. (1999b). Phylogenetic relationships of three amino-acid-utilizing anaerobes, *Selenomonas acidaminovorans*, '*Selenomonas acidaminophila*' and *Eubacterium acidaminophilum*, as inferred from partial 16S rDNA nucleotide sequences, and proposal of *Thermanaerovibrio acidaminovorans* gen. nov., comb. nov. and *Anaeromusa acidaminophila* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 969–974.
- Barker, H. A. (1981). Amino acid degradation by anaerobic bacteria. *Annu Rev Biochem* **50**, 23–40.
- Buckel, W. & Barker, H. A. (1974). Two pathways of glutamate fermentation by anaerobic bacteria. *J Bacteriol* **117**, 1248–1260.
- Cheng, G., Plugge, C. M., Roelofsen, W., Houwen, F. P. & Stams, A. J. M. (1992). *Selenomonas acidaminovorans* sp. nov., a versatile thermophilic proton-reducing anaerobe able to grow by the decarboxylation of succinate to propionate. *Arch Microbiol* **157**, 169–175.
- Christostomos, S., Patel, B. K. C., Dwivedi, P. P. & Denman, S. E. (1996). *Caloramator indicus* sp. nov., a new thermophilic anaerobic bacterium isolated from the deep-seated non-volcanically heated waters of an Indian artesian aquifer. *Int J Syst Bacteriol* **46**, 497–501.
- Collins, M., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- Coolhaas, C. (1927). *Bijdrage tot de kennis der dissimilatie van vetzuren zouten en koolhydraten door thermofiele bacteriën*. PhD thesis, Wageningen Agricultural University, The Netherlands.
- Elsden, S. R. & Hilton, M. G. (1979). Amino acid utilization in clostridial taxonomy. *Arch Microbiol* **123**, 137–141.
- Engle, M., Li, Y., Rainey, F., DeBlois, S., Mai, V., Reichert, A., Mayer, F., Messner, P. & Wiegand, J. (1996). *Thermobrachium celere* gen. nov., sp. nov., a rapidly growing thermophilic, alkalitolerant, and proteolytic obligate anaerobe. *Int J Syst Bacteriol* **46**, 1025–1033.
- Gerhardt, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R. & Phillips, G. B. (1981). Gray method for staining flagella. In *Manual of Methods for General Bacteriology*, p. 29. Washington, DC: American Society for Microbiology.
- Harmsen, H. J. M., Stams, A. J. M., Akkermans, A. D. L. & de Vos, W. M. (1995). Phylogenetic analysis of two syntrophic propionate-oxidizing bacteria in enrichment cultures. *Syst Appl Microbiol* **18**, 67–73.
- Kengen, S. W. M. & Stams, A. J. M. (1994). Formation of L-alanine as a reduced end product in carbohydrate fermentation by the hyperthermophilic archaeon *Pyrococcus furiosus*. *Arch Microbiol* **161**, 168–175.
- Laanbroek, H. J., Smit, A. J., Klein Nuland, G. & Veldkamp, H. (1979). Competition for L-glutamate between specialized and versatile *Clostridium* species. *Arch Microbiol* **120**, 61–66.
- Lane, D. J. (1991). 16S/23S rDNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- McInerney, M. J. (1989). Anaerobic hydrolysis and fermentation of fats and proteins. In *Microbiology of Anaerobic Bacteria*, pp. 373–415. Edited by A. J. B. Zehnder. New York: Wiley.
- Mead, G. C. (1971). The amino acid-fermenting clostridia. *J Gen Microbiol* **67**, 47–50.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Nanninga, H. J. & Gottschal, J. C. (1985). Amino acid fermentation and hydrogen transfer in mixed cultures. *FEMS Microbiol Ecol* **31**, 261–269.
- Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. L., Ludwig, W. & Backhaus, H. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *J Bacteriol* **178**, 5636–5643.
- Örlygsson, J., Krooneman, J., Collins, M. D., Pascual, C. & Gottschal, J. C. (1996). *Clostridium acetireducens* sp. nov., a novel amino acid-oxidizing, acetate-reducing anaerobic bacterium. *Int J Syst Bacteriol* **46**, 454–459.
- Scholten, J. C. M. & Stams, A. J. M. (1995). The effect of sulfate and nitrate on methane formation in a freshwater sediment. *Antonie Leeuwenhoek* **68**, 309–315.
- Stams, A. J. M. & Hansen, T. A. (1984). Fermentation of glutamate and other compounds by *Acidaminobacter hydrogeniformans* gen. nov., sp. nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate reducing and methanogenic bacteria. *Arch Microbiol* **137**, 329–337.
- Stams, A. J. M., van Dijk, J. B., Dijkema, C. & Plugge, C. M. (1993). Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. *Appl Environ Microbiol* **59**, 1114–1119.
- Strunk, O. & Ludwig, W. (1991). ARB: a software environment for sequence data. Department of Microbiology, Technical University of Munich, Munich, Germany (e-mail: arb@micro.biologie.tu-muenchen.de).
- Tarlera, S. (1997). *Aspectos microbiológicos y bioquímicos de la degradación termófila anaerobia de proteínas*. PhD thesis, Universidad de la Republica, Uruguay.
- Tarlera, S., Muxi, L., Soubes, M. & Stams, A. J. M. (1997). *Caloramator proteoclasticus* sp. nov., a moderately thermophilic anaerobic proteolytic bacterium. *Int J Syst Bacteriol* **47**, 651–656.
- Trüper, H. G. & Schlegel, H. G. (1964). Sulphur metabolism in *Thiorhodaceae*. I. Quantitative measurements of growing cells of *Chromatium okenii*. *Antonie Leeuwenhoek* **30**, 225–238.