

***Gelria glutamica* gen. nov., sp. nov., a thermophilic, obligately syntrophic, glutamate-degrading anaerobe**

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A novel anaerobic, Gram-positive, thermophilic, spore-forming, obligately syntrophic, glutamate-degrading bacterium, strain TGO^T, was isolated from a propionate-oxidizing methanogenic enrichment culture. The axenic culture was obtained by growing the bacterium on pyruvate. Cells were rod-shaped and non-motile. The optimal temperature for growth was 50–55 °C and growth occurred between 37 and 60 °C. The pH range for growth was 5.5–8 with optimum growth at pH 7. In pure culture, strain TGO^T could grow on pyruvate, lactate, glycerol and several sugars. In co-culture with the hydrogenotrophic methanogen *Methanobacterium thermautotrophicum* strain Z-245, strain TGO^T could grow on glutamate, proline and Casamino acids. Glutamate was converted to H₂, CO₂, propionate and traces of succinate. Strain TGO^T was not able to utilize sulphate, sulphite, thiosulphate, nitrate or fumarate as electron acceptors. The G+C content was 33.8 mol %. Sequence analysis of the 16S rDNA revealed that strain TGO^T belongs to the thermophilic, endospore-forming anaerobes, though no close relations were found. Its closest relations were *Moorella glycerini* (92 %) and *Moorella thermoacetica* (90 %). Strain TGO^T had an unusually long 16S rDNA of more than 1700 bp. The additional base pairs were found as long loops in the V1, V7 and V9 regions of the 16S rDNA. However, the loops were not found in the 16S rRNA. The name *Gelria glutamica* gen. nov., sp. nov. is proposed for strain TGO^T.

Keywords: thermophilic bacteria, interspecies hydrogen transfer, obligate syntrophic glutamate oxidation, proline oxidation, propionate formation

INTRODUCTION

Because proteins are encountered in almost every ecosystem, the biodegradation of amino acids is a very important microbial process. The conversion of amino acids in methanogenic environments has been studied over the last 20 years, especially in temperate environments (Barker, 1981; Nagase & Matsuo, 1982; McInerney, 1989). However, in moderately thermophilic methanogenic environments, the degradation of amino acids has not been studied in detail. Only a few bacterial species have been described to degrade amino acids under thermophilic conditions (Cheng *et al.*,

1992; Örlýgsson, 1994; Tarlera *et al.*, 1997; Plugge *et al.*, 2000).

Glutamate conversion under methanogenic conditions can occur in different ways (Table 1). The formation of acetate and butyrate as the organic end-products has been described for many anaerobes that belong mainly to the genus *Clostridium*. This type of conversion of glutamate is hydrogen independent. Other examples of hydrogen-independent glutamate conversions are the homoacetogenic fermentation (Dehning *et al.*, 1989) and the reductive formation of propionate (Nanninga *et al.*, 1987). The formation of hydrogen is more likely to occur in methanogenic environments, where hydrogen-scavenging methanogens convert the hydrogen to methane with the concomitant reduction of CO₂. In the presence of methanogens, the free energy available from the overall reactions is higher (Table 1). Under

The GenBank accession number for the 16S rDNA sequence of strain TGO^T is AF321086.

Table 1. Change of free energy for the conversion of glutamate to various products at 55 °C under anaerobic conditions

Energy changes were calculated using the van't Hoff equation, standard enthalpy values of compounds (Chang, 1977) and Gibbs' free energy changes at 298 K (Thauer *et al.*, 1977). Values of $\Delta G'$ were calculated under standard conditions: pH 7, 1 M solutes, 1 atmosphere gases and 55 °C (328 K).

Reaction	$\Delta G'$ (kJ)	
	10 ⁵ Pa H ₂	1 Pa H ₂
Glutamate [−] + 2H ₂ O → acetate [−] + HCO ₃ [−] + $\frac{1}{2}$ H ⁺ + NH ₄ ⁺ + $\frac{1}{2}$ butyrate [−]	−61.1	−61.1
Glutamate [−] + 3H ₂ O → 2acetate [−] + HCO ₃ [−] + H ⁺ + NH ₄ ⁺ + H ₂	−41.6	−73.1
Glutamate [−] + 2H ₂ O → $\frac{2}{3}$ acetate [−] + $\frac{1}{2}$ HCO ₃ [−] + $\frac{3}{4}$ H ⁺ + NH ₄ ⁺	−64.0	−64.0
Glutamate [−] + 2H ₂ O → $\frac{1}{3}$ acetate [−] + $\frac{1}{3}$ propionate [−] + $\frac{2}{3}$ HCO ₃ [−] + $\frac{3}{2}$ H ⁺ + NH ₄ ⁺	−69.0	−69.0
Glutamate [−] + 4H ₂ O → propionate [−] + 2HCO ₃ [−] + NH ₄ ⁺ + 2H ₂	−16.0	−79.0

standard conditions, these reactions yield small amounts of energy. In particular, the exclusive formation of propionate from glutamate is very difficult, since the free energy at 55 °C is only -16.0 kJ (mol glutamate)^{−1} (Table 1). It is highly unlikely that a single organism can perform this reaction. However, in a methanogenic consortium, the hydrogen formed is consumed via interspecies hydrogen transfer and the energy that becomes available from this reaction increases to -79.0 kJ (mol glutamate)^{−1}.

Earlier research indicated the presence of a propionate-forming, obligately syntrophic bacterium in a thermophilic, syntrophic, glutamate-degrading, propionate-oxidizing enrichment (Stams *et al.*, 1992). From this mixed culture, we isolated a bacterium that produced propionate as the major product from glutamate, in addition to traces of succinate. In this paper, we present detailed information about the organism and we propose to name the organism *Gelria glutamica* gen. nov., sp. nov.

METHODS

Strains and source of organisms. The glutamate-degrading strain TGO^T was isolated from a thermophilic, syntrophic, propionate-oxidizing enrichment culture as described by Stams *et al.* (1992). Strain TPO, a syntrophic, propionate-oxidizing bacterium, was isolated from the same enrichment culture. *Moorella thermoacetica* DSM 521^T and *Moorella glycerini* DSM 11254^T were obtained from the DSMZ. *Methanobacterium thermautotrophicum* Z-245 (= DSM 3720) has been used before; this methanogen was recently renamed *Methanothermobacter thermautotrophicus* Z-245 (Wasserfallen *et al.*, 2000).

Media and cultivation. A bicarbonate-buffered medium with the following composition was used (l^{−1}): 0.4 g KH₂PO₄, 0.53 g Na₂HPO₄, 0.3 g NH₄Cl, 0.3 g NaCl, 0.1 g MgCl₂ · 6H₂O, 0.11 g CaCl₂ · 2H₂O, 1 ml alkaline trace element solution, 1 ml acid trace element solution, 1 ml vitamin solution, 0.5 mg resazurin, 4 g NaHCO₃, 0.25 g Na₂S · 7–9H₂O and 0.5 g yeast extract. The trace elements and vitamins were as described in Stams *et al.* (1993). All compounds were heat-sterilized except for the vitamins and the solution of Na₂S · 7–9H₂O, which were filter-sterilized. Incubations were done in serum bottles sealed with butyl

rubber stoppers (Rubber bv) and a gas phase of 182 kPa N₂/CO₂ (80:20, v/v). For the cultivation of methanogens, a gas phase of 182 kPa H₂/CO₂ (80:20, v/v) was used and, after growth, the gas phase was changed to N₂/CO₂. Organic substrates were added from anaerobic sterile stock solutions to final concentrations of 20 mM (unless otherwise stated). To obtain an axenic culture, soft agar (0.7–0.8 % agar noble; Difco) was added to the medium described above, supplemented with 20 mM pyruvate as the carbon source. Light microscopy confirmed purity.

For the reconstitution experiments with axenic cultures of strain TGO^T (0.5 %, v/v), strain TPO (2 %, v/v) and *Methanobacterium thermautotrophicum* Z-245 (2 %, v/v) were inoculated in medium with 20 mM glutamate.

Temperature and pH. The temperature optimum was determined in bicarbonate-buffered medium containing 20 mM pyruvate at pH 7 and duplicate bottles were incubated at temperatures ranging from 30 to 75 °C. The pH optimum was tested in medium by adding 0.15 g KH₂PO₄ l^{−1} instead of sodium bicarbonate. The pH value of the medium containing 20 mM pyruvate was adjusted with NaOH or HCl under the N₂ atmosphere. Duplicate bottles were incubated at 55 °C at pH values ranging from 4.5 to 9.5. For determinations of the temperature and pH optimum, OD₆₀₀ and acetate production were measured as indicators for growth.

Growth and substrate utilization. Utilization of substrates by strain TGO^T in pure culture and in co-culture with *Methanobacterium thermautotrophicum* Z-245 was determined by monitoring growth and substrate depletion as well as product formation. All incubations were performed at 55 °C, pH 7. The effect of electron acceptors on the growth of strain TGO^T was tested in medium with 20 mM glutamate.

G + C content. Isolation and purification of genomic DNA was carried out according to Marmur (1961). The G + C content of the DNA was analysed using thermal denaturation as described by Owen *et al.* (1969).

16S rDNA sequence analysis. Total DNA was extracted from strain TGO^T as described previously (Zoetendal *et al.*, 1998). PCR was performed with the bacterial primers 7f and 1510r (Lane, 1991) by using the *Taq* DNA polymerase kit (Life Technologies) to amplify the bacterial 16S rDNA. PCR products were purified with the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Primers 538r, 1100r (Lane, 1991) and 968f (Nübel *et al.*, 1996) labelled with Infrared Dye 41 (MWG-Biotech) were

used as sequencing primers. The sequences were analysed automatically on a LI-COR DNA sequencer 4000L and corrected manually. Phylogenetic analysis and tree construction were performed with the programs of the ARB software package (Strunk & Ludwig, 1991). FASTA homology searches with sequences of the EMBL and GenBank DNA databases were performed and the results were compared with those obtained with the ARB programs. Because strain TGO^T had additional loops in several regions of the 16S rDNA, we did the database comparison with the complete sequence of 1725 bp and also with the sequence without the additional loops.

RNA isolation, RT-PCR and dot-blot hybridizations. RNA was extracted from strain TGO^T as described by Zoetendal *et al.* (1998). Specific probes targeting the V1, V7 and V9 regions of the 16S rDNA of strain TGO^T were applied in order to investigate whether such additional loops were also present in the 16S rRNA. The sequences of these oligonucleotides were 5'-GCTCTTGGGCCTTTTGAA-3' (V1 region), 5'-GTTAACCTCTGGCTTTG-3' (V7 region) and 5'-CTCAATCCGCAAGTTTAA-3' (V9 region). Primer 538r (Lane, 1991) was used as a positive control for Eubacteria. Dot-blot hybridizations were performed with strain TGO^T, *Moorella thermoacetica*, *Moorella glycerini* and *Escherichia coli* as described by Oude Elferink *et al.* (1997). All membranes were hybridized overnight at 40 °C.

RT-PCR of the 16S rRNA genes of strain TGO^T, *Moorella thermoacetica*, *Moorella glycerini* and *Escherichia coli* was performed by means of bacterial primers 7f and 1510r using the Access RT-PCR system (Promega). Prior to the RT-PCR amplification, the samples were incubated with RNase-free DNase (Promega) to remove all traces of DNA. The integrity and size of the nucleic acids were determined visually after electrophoresis on a 1.2% agarose gel containing ethidium bromide in the presence of markers and compared with the 16S rDNA of strain TGO^T.

Other methods. Gases and organic acids were analysed by GC and HPLC as described by Plugge *et al.* (2000). Amino acids were analysed by HPLC as described by Kengen & Stams (1994). Occasionally, glutamate was determined enzymically with glutamate dehydrogenase as described by Bernt & Bergmeyer (1974). Ammonium was analysed by the indophenol-blue method (Hanson & Philips, 1981). Inorganic compounds tested as electron acceptors were analysed by HPLC as described by Scholten & Stams (1995). Gram and flagella staining were done by standard procedures as described previously (Plugge *et al.*, 2000).

RESULTS AND DISCUSSION

Isolation of the glutamate-oxidizing strain TGO^T

A thermophilic, syntrophic, propionate-oxidizing enrichment, as described by Stams *et al.* (1992), was also able to convert glutamate to acetate, NH₄⁺, HCO₃⁻ and CH₄. When the enrichment culture was growing on glutamate, a small, rod-shaped bacterium became predominant. It was not possible to obtain the bacterium in pure culture by adding an inhibitor of methanogenesis (bromoethanesulphonic acid, BES) to the enrichment culture. No degradation of glutamate was observed when BES was added. The bacterium could be purified by serial dilution in media containing agar (0.7–0.8%) with pyruvate as the organic substrate. The colonies that appeared in the agar and on

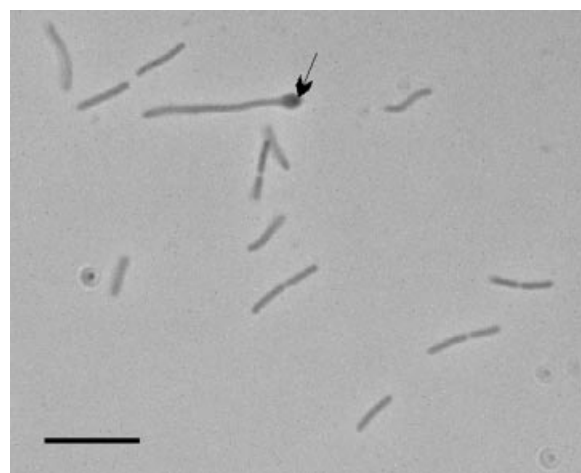


Fig. 1. Phase-contrast micrograph of strain TGO^T grown on pyruvate. The arrow indicates the start of spore formation. Bar, 5 µm.

the surface of the agar were 0.7–1.0 mm in diameter. The colonies were white and round at the surface and lens-shaped in the agar. A single colony picked from the agar grew in medium containing pyruvate and with 0.05% yeast extract. Repeated transfer from liquid medium to soft-agar medium resulted in an axenic culture of a strain designated TGO^T. This strain was characterized further.

Morphology and cellular characterization

The isolated strain TGO^T is a rod-shaped, spore-forming organism. The Gram stain was positive. Cells were 0.5 µm in diameter and 1–1.5 µm in length when grown on pyruvate (Fig. 1). If the bacterium was grown on glucose, the cells were 0.5 µm in diameter and 3–20 µm in length. Spores were located terminally and were 0.5 × 0.5 µm in size and developed in the late-exponential phase. Motility was never observed, nor were flagella found.

Physiological characterization

Strain TGO^T was able to grow on glutamate only in the presence of the methanogenic archaeon *Methanobacterium thermoautotrophicum* Z-245. Glutamate (15 mM) was converted to propionate (12.9 mM), succinate (1.0 mM), NH₄⁺ (14.9 mM) and CH₄ (8.9 mM). The carbon and electron recovery were respectively 93 and 98% (excluding the biomass formed). The doubling time of strain TGO^T in co-culture with *Methanobacterium thermoautotrophicum* Z-245 on glutamate was 0.23 day⁻¹. Several thermophilic bacteria are known to utilize glutamate with the concomitant production of H₂, but these organisms also grow in pure culture on glutamate. *Caloramator proteoclasticus*, *Caloramator coolhaasii* and *Thermanaerovibrio acidaminovorans* (Cheng *et al.*, 1992; Tarlera & Stams, 1999; Plugge *et al.*, 2000) are examples of such thermophilic glutamate-degrading organisms. In pure

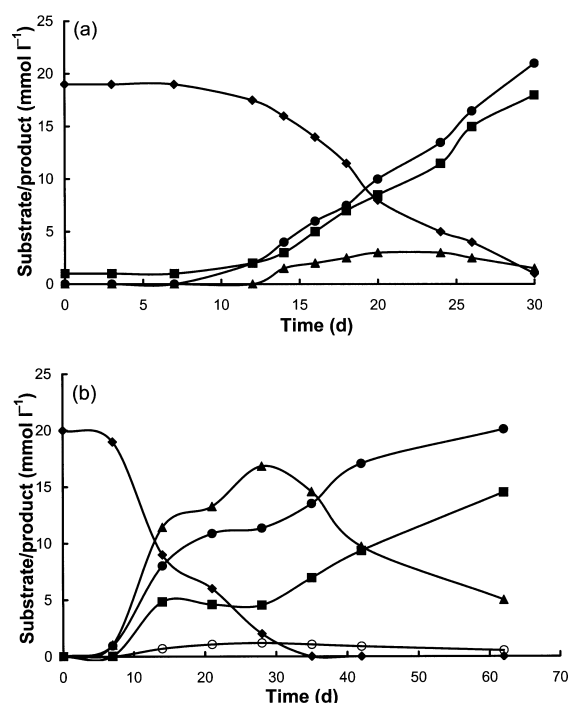


Fig. 2. Utilization of glutamate (◆) and production of propionate (▲), acetate (■), succinate (○) and CH₄ (●) by a consortium of strain TGO^T, strain TPO and *Methanobacterium thermautotrophicum* Z-245. (a) Original enrichment culture as described by Stams *et al.* (1992). (b) Co-culture inoculated with 0.5, 2 and 0.5% (v/v) of pure cultures of strain TGO^T, strain TPO and *Methanobacterium thermautotrophicum* strain Z-245.

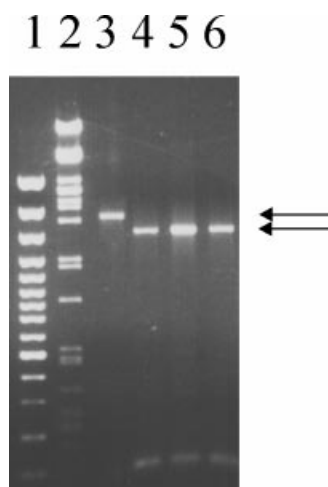


Fig. 3. Agarose gel (1.2%) showing size and integrity of reamplified 16S rDNA (lane 3) and rRNA (lane 4) of strain TGO^T and 16S rRNA of *Moorella glycerini* (lane 5) and *Moorella thermoacetica* (lane 6). Markers: Gene Ruler 100 bp DNA ladder (lane 1) and phage λ DNA digested with *Pst*I (lane 2).

culture, they form acetate, NH₄⁺, CO₂ and H₂. *Thermanaerovibrio acidaminovorans* also forms propionate in pure culture. When *Thermanaerovibrio acidamino-*

vorans is grown in co-culture with a methanogen, the products formed from glutamate conversion shift in favour of propionate formation. However, considerable amounts of acetate are still formed. Examples of mesophilic glutamate-degrading organisms that show the same degradation products as *Thermanaerovibrio acidaminovorans* are *Acidaminobacter hydrogenofor-* *mans* and *Aminobacterium mobile* (Stams & Hansen, 1984; Baena *et al.*, 2000). Like strain TGO^T, *Aminobacterium mobile* only grows on glutamate in the presence of a hydrogen scavenger.

Strain TGO^T is the first example of an organism that is unable to form acetate from glutamate and forms mainly propionate. As a consequence, the organism has to grow in a syntrophic co-culture with a methanogen, since the free energy under standard conditions is only slightly negative (Table 1). It is unclear why strain TGO^T does not form acetate from glutamate, even though acetate production could be detected after growth on several sugars, lactate and pyruvate. The formation of traces of succinate during glutamate utilization suggests that direct oxidation takes place via α -ketoglutarate and succinyl-CoA. This pathway was also suggested to be used in propionate formation in *Thermanaerovibrio acidaminovorans* and *Acidaminobacter hydrogenofor-* *mans* (Cheng *et al.*, 1992; Stams & Hansen, 1984).

Yeast extract (minimum 0.02%) was required for growth. Pyruvate was converted by strain TGO^T to acetate, propionate, succinate (traces), H₂ and CO₂. Glycerol was utilized slowly by the pure culture but, in co-culture, glycerol was converted rapidly to acetate, traces of propionate and CH₄.

Other substrates that could be used by the pure culture of TGO^T were lactate, arabinose, fructose, galactose, glucose, maltose, mannitol, rhamnose and sucrose. Sugars were converted mainly to acetate and propionate with the formation of traces of hydrogen and formate.

In co-culture with the methanogen *Methanobacterium thermautotrophicum* Z-245, strain TGO^T could also grow on Casamino acids, α -ketoglutarate and proline. Proline (18.6 mM) was degraded to propionate (17.3 mM), NH₄⁺ (18.2 mM) and H₂ (27.2 mM) and the amount of hydrogen formed was calculated from the amount of methane measured. Anaerobic proline oxidation has been reported for two other bacteria. *Desulfobacterium vacuolatum* (Rees *et al.*, 1998), a versatile amino acid-utilizing sulphate reducer, and *Geovibrio ferrireducens* (Caccavo *et al.*, 1996), an iron-reducing bacterium, can couple the oxidation of proline to sulphate reduction and dissimilatory Fe(III) reduction, respectively. The study of fermentative proline utilization by anaerobes has focussed on its use as an electron acceptor (McInerney, 1989). Strain TGO^T might be able to convert proline to glutamate with the reverse reactions of proline synthesis via glutamate semialdehyde.

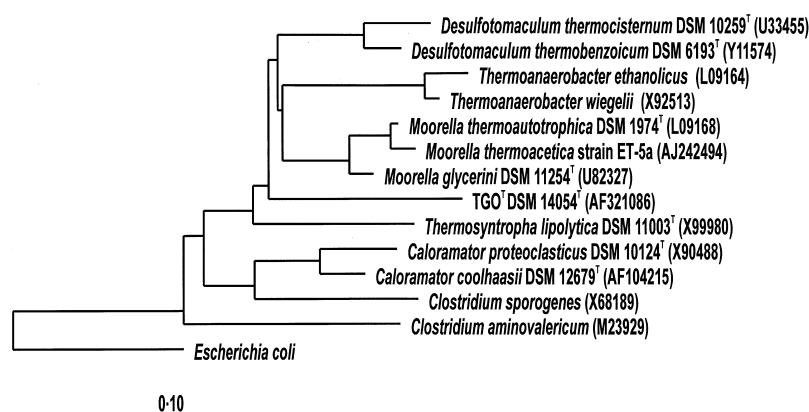


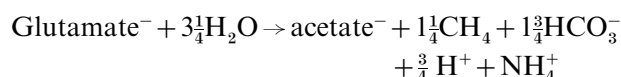
Fig. 4. Phylogenetic tree showing the position of strain TGO^T among representatives of thermophilic, anaerobic spore-forming genera. The tree is based on a distance matrix of 16S rRNA sequences and was constructed using the neighbour-joining method corrected by the method of Felsenstein (1982). Bar, 0.1 (evolutionary distance).

No growth of strain TGO^T was observed in pure culture or in co-culture with *Methanobacterium thermautotrophicum* Z-245 on aspartate, alanine, lysine, threonine, leucine, tyrosine, glycine, fumarate, malate, succinate, propionate, acetate, methanol, ethanol, propanol, butanol, acetone, benzoate, starch or H₂/CO₂. The following mixtures of amino acids were tested but were not utilized by the pure culture of strain TGO^T: alanine + glycine, alanine + arginine, alanine + proline, leucine + glycine, leucine + arginine, leucine + proline, H₂ + glycine, H₂ + arginine and H₂ + proline. The strain could not grow in the presence of traces of oxygen, nor could sulphate, sulphite, thiosulphate or nitrate serve as electron acceptors.

Strain TGO^T could grow on glucose between 37 and 60 °C with an optimum at 50–55 °C. The pH range for growth was 5.5–8, with optimum growth at pH 7.

Reconstitution of the original consortium from axenic cultures of strain TGO^T, strain TPO and *Methanobacterium thermautotrophicum* Z-245

In order to investigate glutamate utilization as originally observed in the propionate-oxidizing enrichment culture, we performed reconstitution experiments with three axenic cultures: strain TGO^T, strain TPO (the syntrophic propionate-oxidizing organism) and *Methanobacterium thermautotrophicum* Z-245. In the original enrichment culture, glutamate was degraded to acetate, CH₄, NH₄⁺ and CO₂, with the intermediate production of propionate (Fig. 2a). The stoichiometry of glutamate conversion was:



In the reconstitution experiments, glutamate was consumed according to the stoichiometry described above (Fig. 2b). This indicated that the axenic cultures of strain TGO^T and strain TPO had the same physiological capabilities as in the original enrichment cultures.

Phylogeny

The nucleotide sequence (1725 bp) of the 16S rDNA of strain TGO^T was analysed and it revealed that this organism belongs to the subphylum of Gram-positive, endospore-forming, thermophilic, anaerobic bacteria. Sequence alignment revealed that strain TGO^T had additional loops in the V1, V7 and V9 helices of the 16S rDNA. There are examples of other organisms with 16S rDNA that exceeds the mean length of 1500 bp. In thermophiles, it is not unusual that the 16S rDNA is longer (Rainey *et al.*, 1996). Dot-blot hybridizations with specific oligonucleotides against these regions showed no hybridization with 16S rRNA from strain TGO^T, *Moorella glycerini*, *Moorella thermoacetica* and *Escherichia coli*. The positive control with primer 538r reacted with all 16S rRNA molecules. This indicated that the loops were not transcribed from the 16S rDNA to the 16S rRNA of strain TGO^T. Comparison of the size of the 16S rDNA and 16S rRNA showed that the sizes were respectively 1700 and 1500 bp. This confirms the absence of the loops in the 16S rRNA observed with the dot-blot hybridizations (Fig. 3). It is unclear how the transcription of the rRNA is regulated.

Sequence analysis showed that strain TGO^T is only distantly related to *Moorella glycerini* and *Moorella thermoacetica* (Slobodkin *et al.*, 1997; Collins *et al.*, 1994), with respective levels of similarity of 92 and 90 %. The similarities were calculated with the use of the 16S rDNA sequence of strain TGO^T without the additional loops. A phylogenetic tree showing the relationship of strain TGO^T and other related species is depicted in Fig. 4.

The G + C content of strain TGO^T was 33.8 mol %.

Taxonomy

Our findings indicate that strain TGO^T differs physiologically and phylogenetically from previously described species. Strain TGO^T is phylogenetically most similar to the genus *Moorella* (90–92 % similarity), but

the phylogenetic relationship is not sufficiently close to classify strain TGO^T in this genus. Also, the ability of strain TGO^T to form solely propionate from glutamate separates it from members of this genus. Therefore, we propose a novel genus and species, *Gelria glutamica* gen. nov., sp. nov.

Description of *Gelria* gen. nov.

Gelria (Gel.ri'a. N.L. fem. n. *Gelria* Gelre or Gelderland, one of the 12 provinces of The Netherlands, in which Wageningen is located).

Non-motile, Gram-positive rods. Formation of terminal spores. Strictly anaerobic. Moderately thermophilic. Saccharolytic growth in pure culture. Hydrogen formed can be transferred to methanogenic partner. Habitat: methanogenic granular sludge. Type species: *Gelria glutamica*.

Description of *Gelria glutamica* sp. nov.

Gelria glutamica (glu.ta'mi.ca. N.L. n. *acidum glutamicum* glutamic acid; N.L. fem. adj. *glutamica* referring to glutamic acid, on which the bacterium grows).

Cells are 0.5 × 0.5–6 µm, varying depending on the growth substrate. In pure culture, the cells can grow on pyruvate, lactate, glycerol, glucose, rhamnose and galactose. In syntrophic association with a hydrogenotrophic methanogen, the organism can utilize glutamate, α-ketoglutarate, proline, Casamino acids and a variety of sugars. Glutamate and proline are oxidized to propionate, H₂, NH₄⁺ and CO₂. Sugars are converted to acetate, propionate, CO₂ and H₂ as main products. Growth occurs between 37 and 60 °C with optimum growth at 50–55 °C and at pH 5.5–8 (optimum pH 7). The DNA G + C content is 33.8 mol %.

The type strain is TGO^T (= DSM 14054^T = ATCC BAA-262^T).

ACKNOWLEDGEMENTS

The determination of the G + C content by Dr A. Lysenko at the Institute of Microbiology of the Russian Academy of Sciences in Moscow is greatly appreciated.

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