Characterization of two novel saccharolytic, anaerobic thermophiles, *Thermoanaerobacterium polysaccharolyticum* sp. nov. and *Thermoanaerobacterium zeae* sp. nov., and emendation of the genus *Thermoanaerobacterium*

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Two anaerobic, thermophilic, Gram-positive, non-spore forming bacteria with an array of polysaccharide-degrading enzymes were isolated from the leachate of a waste pile from a canning factory in Hoopeston, East Central Illinois, USA. The results of 16S rDNA sequence homology indicated that their closest relatives belong to the saccharolytic, thermophilic and anaerobic genera of *Thermoanaerobacterium* and *Thermoanaerobacter*. Although, the evolutionary distances between these bacteria and their closest relatives are greater than 11%, there is no defining phenotypic characteristic for the creation of a new genus. It is proposed that these bacteria should be placed in the genus *Thermoanaerobacterium*, which requires emendment of the genus description with regard to the reduction of thiosulfate to sulfur, because neither isolate is capable of this reduction. *Thermoanaerobacterium polysaccharolyticum* reduces thiosulfate to sulfide, whereas *Thermoanaerobacterium zeae* is unable to reduce thiosulfate. The cells of both isolates are rod-shaped and exist as single cells or sometimes in pairs. Cells are motile by means of flagella. Growth occurs between 45 and 72 °C, with optimum temperature of 65–68 °C at pH 6–8. The pH range for growth is from 4 to 8 at a temperature of 65 °C. Both organisms ferment glucose, arabinose, maltose, mannose, rhamnose, sucrose, trehalose, xylose, cellobiose, raffinose, melibiose and melezitose. The major end products of fermentation with glucose are ethanol and CO₂, with lesser amounts of acetate, formate, lactate and hydrogen. The DNA G+C contents of *Thermoanaerobacterium polysaccharolyticum* sp. nov. and *Thermoanaerobacterium zeae* sp. nov. are 46 and 42 mol%, respectively. The type strains are KMTHCJ^T (= ATCC BAA-17^T = DSM 13641^T) and mel2^T (= ATCC BAA-16^T = DSM 13642^T), respectively.

Keywords: thermophilic, bacteria, *Thermoanaerobacterium*, *Thermoanaerobacterium polysaccharolyticum*, *Thermoanaerobacterium zeae*

INTRODUCTION

The importance of thermostable biomolecules in the growing field of biotechnology has spurred research

into organisms capable of growth at high temperatures. During the last two decades, many reports have described the isolation of novel thermophilic organisms from both the domains *Archaea* and *Bacteria*

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The GenBank accession numbers for the 16S rDNA sequence of strains KMTHCJ^T and mel2^T are U40229 and U75993, respectively.

(Cayol *et al.*, 1995; Cook *et al.*, 1996; Engle *et al.*, 1995, 1996; Fiala & Stetter, 1986; Freier *et al.*, 1988; Jones *et al.*, 1983; Liu *et al.*, 1996b; Schink & Zeikus, 1983; Wiegel & Ljungdahl, 1981). Most of the saccharolytic, anaerobic thermophiles belonging to the bacterial domain have received attention for their potential in the bioconversion of substrates of plant origin to end products such as lactate and ethanol, compounds with potential for the production of bulk chemicals and fuels (Lee *et al.*, 1993b).

Members of the genera *Thermoanaerobacterium* and *Thermoanaerobacter* have been isolated from unique areas such as deep surface oil wells (Cayol *et al.*, 1995), geothermally heated water outlets (Cook *et al.*, 1996) and hot springs (Wiegel & Ljungdahl, 1981). Several thermostable enzymes have either been purified or cloned from the members of this group of organisms. For example, polysaccharide-hydrolysing enzymes from this group are thermostable endoxylanases (Lee *et al.*, 1993a; Liu *et al.*, 1996a) which can be used in biomass conversion and the pulp and paper industry.

Several thermophilic bacteria have recently been isolated from the leachate of a waste pile from a canning factory in Illinois, USA. In this paper, two unique, non-spore-forming, Gram-positive *Thermoanaerobacterium* members capable of using a wide range of carbohydrate sources for the heterofermentative production of lactate or ethanol are described. A recent publication describes the cloning and sequencing of a thermostable multidomain mannanase from one of the novel isolates (Cann *et al.*, 1999).

METHODS

Sample site. A canning factory situated in Hoopeston, Illinois, USA, which seasonally processes sweet corn and other vegetables, was selected for study. Organic waste from the canning process is dumped 1 mile west of town. The waste pile and surrounding run-off were characterized by active gas evolution, low pH (5.5), high concentrations of short-chain fatty acids (31.6 mM acetate, 8.2 mM propionate, 26.0 mM butyrate and 6.4 mM valerate) as well as heat generation. Temperature was not recorded, but exhibited a steep gradient away from the centre of the waste pile. A subsurface sample of the leachate from this waste pile was collected in screw-cap flasks and transported under anaerobic conditions and ambient temperature to the laboratory. The sample was kept at 4 °C until used to inoculate enrichment medium.

Enrichments. To isolate strain KMTHCJ^T, aliquots (0.2 ml) of samples were inoculated into a volume of a minimal medium containing raffinose as sole added carbon and energy source [0.25 g Trypticase-peptone l⁻¹ (BBL Microbiology Systems), 5 g raffinose l⁻¹, 50 ml Pfennig's mineral solution (g l⁻¹: KH₂PO₄, 10; MgCl₂ · 6H₂O, 6.6; NaCl, 8.0; NH₄Cl, 8.0; CaCl₂ · 2H₂O, 1.0), 1 ml Pfennig's trace elements solution (g l⁻¹: ZnSO₄ · 7H₂O, 0.1; MnCl₂ · 4H₂O, 0.03; H₃BO₃, 0.3; CoCl₂ · 6H₂O, 0.2; CuCl₂ · 2H₂O, 0.01; NiCl₂ · 6H₂O, 0.02; Na₂MoO₄ · 2H₂O, 0.03; FeCl₂ · 4H₂O, 1.5; Na₂SeO₃, 0.01) and 1 ml 0.1% (w/v) resazurin]. The medium was prepared under anaerobic conditions using the following procedure. The medium was boiled in a microwave

oven and cooled under a stream of oxygen-free carbon dioxide prepared by passage through a column of heated copper filings. This was then dispensed in 9.0 ml amounts into 15 × 180 mm Balch tubes in an anaerobic chamber (Coy Laboratory Products). Dispensed medium was autoclaved for 15 min at 110 °C. After cooling, 0.2 ml 1.25% cysteine · HCl/Na₂S (sterile), 0.05 ml B vitamin solution (0.2 g thiamin · HCl, 0.2 g calcium D-pantothenate, 0.2 g riboflavin, 0.2 g pyridoxine · HCl, 0.01 g *p*-amino benzoic acid, 0.25 g biotin, 0.25 g folic acid and 0.01 g vitamin B₁₂) and 0.7 ml 7% (w/v) NaHCO₃ (filter-sterilized) were injected into each tube. The initial samples were incubated at 60 °C until visible growth was observed and passed through a series of serial dilutions until a uniformly pure culture was obtained, as determined by microscopic examination of wet mounts and Gram-stained preparations.

Strain mel2^T was isolated using the enrichment procedure described for strain KMTHCJ^T, except that melibiose was the carbon and energy source. Aliquots from tubes showing growth were streaked on plates of the same defined medium. A colony was picked and grown in the defined medium based on melibiose and passed through several serial dilutions until a uniformly pure culture was obtained.

Physiological properties. The isolates were grown on representative carbon sources (glucose, arabinose, lactose, maltose, rhamnose, sucrose, trehalose, xylose, cellobiose, mannose, raffinose, sorbose, melibiose, melezitose, pyruvate, mannitol, fumarate, malate or citrate) to determine whether different substrates could be used as sole carbon source. Bacterial growth was monitored by determining the OD₆₀₀ (Spectronic 70; Bausch and Lomb). In addition, ability to grow on cracked corn, ground corn cob and ground corn stalk was evaluated. The carbon sources were suspended separately under anaerobic conditions in distilled water and sterilized by autoclaving. They were then added to sterile defined medium to a final concentration of 0.5% (w/v). Nitrate reduction, catalase and indole production tests were carried out as described by Smibert & Krieg (1994). The defined medium described above with glucose as energy source was used to grow both bacteria above 37 °C to determine their respective optimum temperatures for growth. In the optimum pH studies, the isolates were grown at 68 °C in the defined medium with glucose as the carbon source and the initial pH adjusted over the ranges 5.0–8.0 for strain KMTHCJ^T and 1.0–9.0 for strain mel2^T. The reduction of thiosulfate, sulfate and sulfur at a 20 mM concentration was tested. Excess iron (0.5 mM) in the form of FeCl₂ · 4H₂O was added to the Balch tubes in order to detect sulfide precipitation. At our incubation temperature, it was necessary to use a low cysteine · HCl concentration of 0.05% (w/v), in order to prevent precipitation of the excess iron with the breakdown of the cysteine · HCl. Microscopic examination was used to check for sulfur formation. Reductive acetogenesis was examined by including 80% H₂ and 20% CO₂ in the headspace above the basal medium without glucose and measuring the change in OD₆₀₀ compared to the basal medium.

Morphological characteristics. Routine examination for purity was carried out using an Olympus BH-2 microscope. Gram staining was performed according to the method of Huckner (Murray *et al.*, 1994) and compared to positive controls stained on the same slide. Morphology was examined by phase-contrast microscopy, TEM and negative staining. For TEM, cells were immersed in a modified Karnovsky's fixative overnight and treated with a solution

of 1% (w/v) osmium tetroxide. The sample was dehydrated and a solution of 100% (v/v) ethanol and propylene oxide in the ratio 1:1 was finally used to rinse the sample. Embedding was with Epon 812 epoxy resin. The thin sections that were generated were placed on a grid for EM (JEOL 100C at 80 kV). For negative staining, a carbon-coated Formvar grid was suspended on a drop of specimen. This was then placed on a drop of 2% (w/v) ammonium molybdate (pH 6.2) and dried for 20 min, after which it was observed by EM.

Analytical methods. Gas composition was analysed using a GC (Gow-Mac Series 580) with an Alltech column (30' × 1/8" × 0.085" SS packed with GAS CHROM MP-1 100/120 mesh) and a thermal conductivity detector. The carrier gas was nitrogen at a flow rate of 30 ml min⁻¹. Solvents produced were determined and quantified with a GC (model 5710A Hewlett Packard) on an 80/100 Carbowack C/0.1% SP-1000 column of size 6' × 1/8" SS. The carrier gas was nitrogen at a flow rate of 20 ml min⁻¹. The oven, flame-ionization detector and injection temperatures were 120, 200 and 200 °C, respectively. Volatile fatty acids were determined by GC (model 5890A Hewlett Packard) on a glass column (180 cm × 4 mm i.d.) packed with GP 10% SP-1200/1% H₃PO₄ on 80/100 Chromasorb WAW (Supelco). The carrier gas was nitrogen at a flow rate of 75 ml min⁻¹. The oven, detector and injection temperatures were 125, 180 and 175 °C, respectively. Formate and lactate were determined by HPLC using an ion exclusion Bio-Rad Aminex HPX-87H column. The eluent was 0.0025 M H₂SO₄ at 0.6 ml min⁻¹. The column was operated at 60 °C with a UV detector reading at 210 nm. Fatty acid methyl ester analysis was performed by Microchek. This analysis consisted of a GC with a 5% methyl phenyl silicone capillary column and flame-ionization detector.

PCR amplification of 16S rDNA. Approximately 200 ng genomic DNA was used in a 50 µl PCR mixture. The reaction mixture contained 250 mM of each dNTP, 50 mM KCl, 2 mM MgCl₂ and other ingredients as described by the manufacturer (LA PCR kit; Takara Shuzo). The primers used were 008F and 1517R and were each at a concentration of 1.1 µM. PCR involved 29 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min and extension at 72 °C for 3.5 min. An aliquot of amplified DNA fragment was analysed by electrophoresis on a 0.9% agarose gel, stained with ethidium bromide and examined under UV light.

Cloning and sequencing. The 1.4 kb fragment obtained was cloned by directly ligating the fragment into a pGEM-T vector DNA (Promega) and the product was used to transform *Escherichia coli* DH5α by electroporation. The *E. coli* cells with recombinant plasmids were selected on Luria-Bertani plates incorporating X-Gal and IPTG. Plasmids were extracted according to the method of Birnboim & Doly (1979) and purified by using Qiagen columns. An ABI automated DNA sequencer (Applied Biosystems) was used for sequencing the entire DNA insert in both directions to confirm the integrity of the sequence obtained.

16S rRNA sequence analysis. Species used for the analysis (with gene accession numbers in parentheses) are as follows: *E. coli* (AE000460); *Moorella thermoacetica* (M59121); *Moorella thermoautotrophica* (L09168); *Thermoanaerobacter thermocopriae* (L09167); *Thermoanaerobacter ethanolicus* (L09164); *Thermoanaerobacter brockii* (L09165); *Thermoanaerobacter finnis* (L09166); *Thermoanaerobacter*

kivui (L09160); *Thermoanaerobacter thermohydrosulfuricus* (L09161); *Thermoanaerobacter acetoethylicus* (L09163); *Thermoanaerobacter wiegelsii* (X92513); *Thermoanaerobacterium xylanolyticum* (L09172); *Thermoanaerobacterium aotearoense* (X93359); *Thermoanaerobacterium saccharolyticum* (L09169); '*Thermoanaerobium lactoethylicum*' (L09170); and *Thermoanaerobacterium thermosulfurigenes* (L09171). Using the program CLUSTAL v (Higgins *et al.*, 1991), the 16S rDNA sequences obtained were aligned. Regions showing alignment uncertainties and gaps were excluded from the alignments and 1238 unambiguous nucleotide positions were used in computing evolutionary distances by the method of Jukes & Cantor (1969). The evolutionary distance matrix was used in constructing a dendrogram using the neighbour-joining method (NEIGHBOR). The programs used for the phylogenetic analysis can all be found in the PHYLIP package (Felsenstein, 1995).

DNA base composition. The G + C content of the DNA was determined according to the method described by Marmur & Doty (1962). Genomic DNA from *Butyrivibrio fibrisolvens* strain 49 (University of Illinois, Department of Animal Sciences Culture Collection), *Micrococcus luteus* ATCC 4698^T and *Clostridium perfringens* strain 14115-15 (USDA-ARS), with G + C contents of 41, 70.0–75.5 and 24–27 mol %, respectively, were used as references. Genomic DNA from *Butyrivibrio fibrisolvens* strain 49 was used for melting temperature (*T_m*) calculations. Analysis was performed on a Beckman model DU-640 Spectrophotometer equipped with a *T_m* analysis accessory.

RESULTS AND DISCUSSION

Morphology

The cells of strain KMTHCJ^T and strain mel2^T occurred as straight rods, singly or in pairs (Fig. 1). Chain formation was observed on rare occasions. The cells were motile as observed under light microscopy. Strain KMTHCJ^T exhibited a tumbling motility and flagella could be observed (Fig. 1a). Strain mel2^T was actively motile through flagella which were peritrichously arranged (Fig. 1b). The cells stained Gram-variable. Thin sections of both species showed a Gram-positive ultrastructure under EM (Fig. 1c, d). Both genera *Thermoanaerobacter* and *Thermoanaerobacterium* have members that stain Gram-positive and -negative (Cayol *et al.*, 1995; Cook *et al.*, 1996; Liu *et al.*, 1996b). Differences in Gram staining and other characteristics between the two new strains and the type species of *Thermoanaerobacter* and *Thermoanaerobacterium* are listed in Table 1. These bacteria are likely to exhibit a surface-layer protein (see Fig. 2) as in related organisms (Liu *et al.*, 1996a; Brechtel & Bahl, 1999), which is supported by the presence of an S-layer-like protein region observed at the C-terminal region of a mannanase gene that has been cloned and sequenced from strain KMTHCJ^T (Cann *et al.*, 1999). Strain mel2^T grew readily on agar surfaces, whereas strain KMTHCJ^T did not grow readily on nutrient agar plates, but could be maintained on agar slants. Spore formation has been observed within the genera *Thermoanaerobacterium*

Fig. 1. TEM of negatively stained cells (a, b) and ultrathin sections (c, d) of *Thermoanaerobacterium polysaccharolyticum* (a, c) and *Thermoanaerobacterium zeae* (b, d) grown on minimal medium containing 0.5% glucose at 65 °C. Intracellular granules (Δ) and invagination of the cytoplasmic membrane prior to cell division ($\blacktriangle$) in *Thermoanaerobacterium zeae* are marked (d). Bars: a and b, 1 μ m; c and d, 0.5 μ m.

and *Thermoanaerobacter* (Cayol *et al.*, 1995; Cook *et al.*, 1996; Liu *et al.*, 1996b); however, this characteristic was not observed with either strain under the conditions of growth investigated. According to Cato & Stackebrandt (1989), spore formation is not an effective taxonomic characteristic and the variation in this characteristic between members of the genera *Thermoanaerobacterium* and *Thermoanaerobacter* provides further evidence to support this. Cell division appeared to occur through a septation mechanism as described for *Thermoanaerobacter wiegelii* (Cook *et al.*, 1996). The major fatty acids (Table 2) were determined to be iso 15:0 (70.6 and 61.6% in strains KMTHCJ^T and mel2^T, respectively) and iso 17:0 (19.2 and 26.8% in strains KMTHCJ^T and mel2^T, respectively). The only other fatty acid detected was straight chain 16:0 (10.1 and 9.4% in strains KMTHCJ^T and mel2^T, respectively). These results are remarkably similar to those reported for *Thermoanaerobacter thermohydrosulfuricus*, *Thermoanaerobacter thermocopriae* and *Thermoanaerobacterium thermosulfurigenes* by Yamamoto *et al.* (1998). *Thermoanaerobacterium thermosaccharolyticum* differed from the other five strains reported in Table 2 by having high levels of 14:0 straight chain (32.5%) and an elevated level of 16:0 straight chain (25.5%) fatty acids. Although

reasonably consistent, the cellular fatty acid profiles do not provide a basis for differentiating *Thermoanaerobacter* from *Thermoanaerobacterium* or for reliably differentiating between the five strains reported in the Table.

Growth and physiology

Strain KMTHCJ^T and strain mel2^T did not require yeast extract for growth. This is unlike most members of the genera *Thermoanaerobacter* (Cayol *et al.*, 1995; Cook *et al.*, 1996; Wiegel & Ljungdahl, 1981) and *Thermoanaerobacterium* (Lee *et al.*, 1993b). In the presence of oxygen in the headspace, characterized by the pink colour of resazurin (oxidized medium), growth occurred with subsequent reduction of resazurin (colourless). However, both isolates would only grow in the anaerobic region of a stab culture. Thus, both isolates are obligate anaerobic bacteria that exhibit aerotolerance. This is similar to the members of the genera *Thermoanaerobacter* (Cayol *et al.*, 1995; Cook *et al.*, 1996) and *Thermoanaerobacterium* (Liu *et al.*, 1996b), which require anoxic conditions for growth. Strain KMTHCJ^T grows between 45 and 70 °C, with the optimum temperature for growth on glucose occurring between

Table 1 Characteristics that differentiate strains KMTHCJ^T and mel2^T from other phylogenetically related thermophilic bacteria

+, Positive; —, negative; ND, not determined.

Character	Strain KMTHCJ ^T	Strain mel2 ^T	<i>Thermoanaerobacterium thermosulfurigenes</i> strain 4B ^{T*}	<i>Thermoanaerobacter ethanolicus</i> strain JW200 ^{T†}
Gram stain	Variable	Variable	Negative	Variable
G + C content (mol %)	46	42	32.6	32
Spore formation	—	—	+	—
pH range	5.0–8.0	3.9–7.9	4.0–7.5	4.4–9.8
Optimum pH	6.8–7.0	ND	5.5–6.5	5.8–8.5
Optimum temp. (°C)	65–68	65–70	≥ 60	69
Max. temp. (°C)	70	72	75	78
Formate produced	+	+	—	—
Utilization of:				
Cracked corn	—	+	ND	ND
Starch	—	+	+	+
Xylan	—	+	—	+
Melibiose	+	+	+	—
Melzitose	+	+	—	—
Mannose	+	+	ND	+
Arabinose	+	+	+	ND
Rhamnose	+	+	+	—
Raffinose	+	+	—	—
Lactose	+	+	—	+
Sorbose	—	—	ND	ND
Fructose	+	+	ND	+
Mannitol	—	—	+	—
Malate	—	—	ND	ND
Fumarate	—	—	ND	ND
Citrate	—	—	ND	ND
Trehalose	+	+	+	—
Salicin	+	+	+	ND
Pyruvate	+	+	—	+
Pectin	—	—	+	ND
H ₂ /CO ₂	—	—	—	ND

* Data from Schink & Zeikus (1983).

† Data from Wiegel & Ljungdahl (1981).

65 and 68 °C at pH 6.8. *Thermoanaerobacter wiegelsii* shows similar optimum temperature for growth (Cook *et al.*, 1996). No growth was observed at 45 or 75 °C. Similarly, strain mel2^T grew between 55 and 72 °C, with the optimum temperature for growth on glucose occurring between 65 and 70 °C at pH 6.8. Growth was not observed above 72 °C or below 37 °C. The end products of glucose fermentation for both isolates included ethanol and carbon dioxide, with hydrogen, acetate, formate and lactate being produced in lesser amounts. Strain mel2^T produced more formate than acetate from glucose fermentation, whereas strain KMTHCJ^T produced more acetate than formate. This fermentation is similar to that of other thermoanaerobes found in the domain *Bacteria*, although the high amounts of formate produced are unique (Lee *et*

al., 1993b). The ability of *Thermobrachium celere* to produce formate was used to differentiate it from the genus *Thermoanaerobacter* (Engle *et al.*, 1996). Although strains KMTHCJ^T and mel2^T both produce formate, cell morphology and significant 16S rDNA sequence differences exclude placement of the new strains in the genus *Thermobrachium*. Both organisms fermented a wide variety of complex and simple carbohydrates including melibiose, raffinose, arabinose, galactose, lactose, maltose, mannose, rhamnose, sucrose, trehalose, xylose, cellobiose and melizitose, whereas cellulose was not utilized. A modular enzyme has been cloned and sequenced from strain KMTHCJ^T (Cann *et al.*, 1999) and the presence of an enzyme with carboxymethylcellulase activity has been demonstrated in strain mel2^T. When glucose and xylose were

Fig. 2. Cell wall of *Thermoanaerobacter polysaccharolyticum* (top) and *Thermoanaerobacter zeae* (bottom) under high magnification of ultrathin sections, showing multilayered structure and cytoplasmic membrane characteristics of Gram-positive ultrastructure. The outer cell surface is covered by a regular array of repeating units (Δ) characteristic of bacterial cell surface or S-layer proteins. Bars, 0.2 μ m.

present in the medium in equal proportions, both substrates were used simultaneously by strain KMTHCJ^T (data not shown). This lack of diauxic

growth has been observed in both *Thermoanaerobacter ethanolicus* (Carreira *et al.*, 1983) and *Thermoanaerobacter wiegelii* (Cook *et al.*, 1996). Catalase activity was negative for both isolates, which is consistent with the genera *Thermoanaerobacter* (Cayol *et al.*, 1995; Holt *et al.*, 1994) and *Thermoanaerobacterium* (Holt *et al.*, 1994; Lee *et al.*, 1993b; Liu *et al.*, 1996a). Neither isolate reduced nitrate or produced indole. Similar observations have been made for the genus *Thermoanaerobacter* (Holt *et al.*, 1994). Of the *Thermoanaerobacterium* species, only *Thermoanaerobacterium thermosulfurigenes* has been described as not reducing nitrate, whereas other species have not been tested (Lee *et al.*, 1993b; Liu *et al.*, 1996b). The inability to produce indole has been reported only for *Thermoanaerobacterium aotearoense* (Lee *et al.*, 1993b; Liu *et al.*, 1996b). The doubling times were 2.1, 3.4 and 5.5 h at 68 °C with glucose, raffinose and melibiose as the carbon source in a minimal medium (described previously), respectively, for strain KMTHCJ^T. On the other hand, the doubling time was 1.8 h at 65–70 °C with glucose as the carbon source in a minimal medium for strain mel2^T. Growth on cracked corn, starch and xylan in a minimal medium was only observed for strain mel2^T. Both strains grew in an acidic to neutral pH. The pH range for strain KMTHCJ^T was between 5.0 and 8.0, whereas strain mel2^T grew between pH 3.9 and 7.9. The optimum pH range for strain KMTHCJ^T was 6.8–7.0 at a temperature of 65 °C. The ability to grow at low pH was expected, since the leachate from the waste pile from which these bacteria were isolated was acidic (pH of 5.5) in nature.

Both isolates were tested for their ability to reduce sulfate, sulfur and thiosulfate (20 mM). Neither organism reduced sulfate or sulfur. Strain KMTHCJ^T reduced thiosulfate to sulfide, but strain mel2^T did not reduce thiosulfate. The thiosulfate reduction assay has been proposed as a method for distinguishing between the genera *Thermoanaerobacter* (reduction of thio-

Table 2 Cellular fatty acid composition (%) of species of *Thermoanaerobacter* and *Thermoanaerobacterium* and novel strains KMTHCJ^T and mel2^T

Abbreviations: ND, not detected; TR, trace amount (< 0.5 %).

Type strain	Saturated												Unsaturated		Growth temp. (°C)
	Straight chain						iso-Branched chain						Straight chain		
	14:0	15:0	16:0	17:0	18:0	13:0	14:0	15:0	16:0	17:0	18:0	16:1	18:1		
Genus <i>Thermoanaerobacter</i>															
<i>T. thermohydrosulfuricus</i> DSM 567 ^{T†}	0.5	7.6	11.2	ND	1.2	ND	ND	47.3	3.1	23.3	2.1	ND	3.7	60	
<i>T. thermocopriae</i> IAM 13577 ^{T†}	TR	5.0	1.1	10.3	1.8	TR	ND	54.1	1.6	24.7	0.5	ND	ND	60	
Genus <i>Thermoanaerobacterium</i>															
<i>T. thermosulfurigenes</i> DSM 2229 ^{T†}	TR	4.1	12.7	3.6	1.2	TR	TR	42.4	4.6	23.3	4.0	ND	2.6	60	
<i>T. thermosaccharolyticum</i> DSM 571 ^{T†}	32.5	ND	25.5	TR	1.5	ND	ND	21.6	ND	16.7	1.3	ND	TR	60	
Novel isolates															
Strain KMTHCJ ^T	ND	ND	10.1	ND	ND	ND	ND	70.6	ND	19.2	ND	ND	ND	65	
Strain mel2 ^T	ND	ND	9.4	ND	ND	ND	ND	61.6	ND	26.8	ND	ND	ND	65	

* Number of carbon atoms: number of double bonds.

† Data from Yamamoto *et al.* (1998).

sulfate to sulfide) and *Thermoanaerobacterium* (reduction of thiosulfate to elemental sulfur) (Lee *et al.*, 1993b). Based on these results, neither organism can be described as a member of the genus *Thermoanaerobacterium* and strain KMTHCJ^T should be considered as a member of the genus *Thermoanaerobacter* based on this characteristic.

DNA base composition

The DNA G+C contents of strain KMTHCJ^T and strain mel2^T, as determined by the thermal denaturation method (Marmur & Doty, 1962), were 46 and 42 mol%, respectively. These values are higher than those observed for the type species of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* as shown in Table 1 (Schink & Zeikus, 1983; Wiegel & Ljungdahl, 1981). However, they are close to the G+C content of 40 mol% reported for *Clostridium thermocellum* JW20 (Freier *et al.*, 1988). Similarity of the G+C contents among members of polysaccharide-degrading bacteria belonging to the genera *Clostridium*, *Thermoanaerobacterium* and *Thermoanaerobacter* rendered it unfeasible to separate them into groups based on this criterion (Lee *et al.*, 1993b).

16S rRNA gene sequence analysis

Almost complete 16S rDNA sequences of strain KMTHCJ^T (1438 bases) and strain mel2^T (1449 bases) were PCR-amplified and cloned in *E. coli* DH5 α . Using six primers, the 5' and 3' strands were sequenced. These sequences [positions 8–1517 of *E. coli* consensus numbering (Winker & Woese, 1991)] were aligned to the fifteen most closely related sequences and phylogenetic analysis was performed. The sequence comparison revealed that both strain KMTHCJ^T and strain mel2^T have no known close relatives among previously described bacteria. Their phylogenetic position is, however, within the large subgroup consisting of the low-G+C-content, Gram-positive bacteria (*Bacillus*/*Clostridium* cluster). Collins *et al.* (1994) have previously separated this large group into smaller clusters with three of them corresponding to thermophilic genera (V, *Thermoanaerobacter*; VI, *Moorella*; and VII, *Thermoanaerobacterium*). Of these, cluster VII, *Thermoanaerobacterium*, appears to be the closest match (relative distance > 11.1%) as shown in Fig. 3.

It has been proposed that with bacteria falling within the loosely defined *Clostridium* cluster, a relative distance of more than 8–10% constitutes a separate genus, as long as the placement is consistent with physiological data (Engle *et al.*, 1995). Although the organisms described have unique characteristics, such as formate production, absence of spore formation and variable thiosulfate reduction, this is not sufficient for the formation of a new genus. From these results, it is proposed that both isolates be designated members of the genus *Thermoanaerobacterium*, although their physiological properties warrant an emended genus

description. This emendment will effectively eliminate any distinguishing characteristic between the genera *Thermoanaerobacter* and *Thermoanaerobacterium*. Clearly, analysis of more isolates representing this new branch (based on 16S rDNA sequence) and an extensive DNA–DNA hybridization study are needed to determine the proper taxonomic relationship amongst this extensive group of thermophiles.

Emendation of the description of the genus *Thermoanaerobacterium*

The genus *Thermoanaerobacterium* has been previously described (Lee *et al.*, 1993b; Liu *et al.*, 1996b). According to the description, members of the genus reduce thiosulfate to elemental sulfur. However, both *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* are unable to reduce thiosulfate to elemental sulfur and, therefore, the reduction of thiosulfate to elemental sulfur is not a distinguishing characteristic of the genus *Thermoanaerobacterium*. This emendment to the genus description also rectifies the placement of *Thermoanaerobacterium thermosaccharolyticum*, another organism which reduces thiosulfate to sulfide, not elemental sulfur (Collins *et al.*, 1994; McClung, 1935).

Description of *Thermoanaerobacterium polysaccharolyticum* sp. nov.

Thermoanaerobacterium polysaccharolyticum (poly. sac. cha. ro. ly' ti. cum. Gr. n. polysacchar many sugars; Gr. adj. lyticus dissolving; N.L. neut. adj. polysaccharolyticum many sugars dissolving).

Cells of *Thermoanaerobacterium polysaccharolyticum* are straight rods, occurring singly or sometimes in pairs, motile and Gram-positive. Growth occurs under anaerobic conditions. Formation of spores has not been observed. Flagella are present. Anaerobic cultures are catalase-negative and nitrate is not reduced. Indole is not produced. Thiosulfate is reduced to sulfide. Sulfate and sulfur are not reduced. The bacterium exhibits a surface-layer protein. The optimum temperature for growth is 65–68 °C. The maximum temperature at which growth occurs is 70 °C. Growth does not occur at 45 °C. The optimum pH is 6.8–7.0. Doubling times at 68 °C with glucose, raffinose and melibiose as the carbon source are 2.1, 3.4 and 5.5 h, respectively. The end products of fermentation on a minimal medium with 0.5% (w/v) glucose as the carbon source are ethanol (30 mM), acetate (20 mM), formate (11 mM) and lactate (5 mM). Utilizes glucose, arabinose, galactose, lactose, maltose, mannose, rhamnose, salicin, sucrose, trehalose, xylose, cellobiose, raffinose, melibiose, melizitose and pyruvate. Cellulose, cracked corn, starch, xylan, pectin, sorbose, mannitol, malate, fumarate, citrate, glycerol and H₂/CO₂ are not utilized. The G+C content is 46 mol%, as determined by the thermal denaturation method. The type strain of *Thermo-*

Fig. 3. Phylogenetic dendrogram comparing the 16S rRNA gene sequence of strains KMTHCJT and mel2T and sequences of the genera *Thermoanaerobacter*, *Thermoanaerobacterium* and related genera constructed using the neighbour-joining method. Numbers given at the nodes represent bootstrap values and indicate the percentage probability for the appropriate branches of the tree. Bar, 10% difference in sequence.

anaerobacterium polysaccharolyticum is strain KMTHCJT (= ATCC BAA-17T = DSM 13641T).

Description of *Thermoanaerobacterium zeae* sp. nov.

Thermoanaerobacterium zeae (ze.ae. Gr. n. *zeae* of corn, describing the use of corn as a substrate for growth).

Cells are motile, Gram-positive rods and occur singly or sometimes in pairs. Growth occurs under anaerobic conditions. Formation of spores has not been observed. Peritrichous flagella are present. Grows on solid media. Anaerobic cultures are catalase-negative and nitrate is not reduced. Indole is not produced. Thiosulfate, sulfate and sulfur are not reduced.

The optimum temperature is 65–70 °C. The maximum temperature at which growth occurs is 72 °C and growth does not occur at 37 °C. The pH range is 3.9–7.9. The fastest doubling time observed for *Thermoanaerobacterium zeae* grown on glucose in a minimal media is 1.8 h for the optimum temperature range. Growth is completely inhibited by tetracycline (50 µg ml⁻¹), rifampicin (50 µg ml⁻¹), kanamycin

(50 µg ml⁻¹) and erythromycin (50 µg ml⁻¹). Growth occurs in the presence of concentrations of 300 mM NaCl, 450 mM KCl and 150 mM NH₄Cl. The end products of fermentation on a minimal medium with 0.5% (w/v) glucose as the carbon source are ethanol (33 mM), formate (21 mM), acetate (14 mM) and lactate (7 mM). Utilizes cracked corn, starch, xylan, glucose, arabinose, galactose, lactose, maltose, mannose, rhamnose, salicin, sucrose, trehalose, xylose, cellobiose, raffinose, melibiose, melizitose and pyruvate. Cellulose, pectin, sorbose, mannitol, malate, fumarate, citrate, glycerol and H₂/CO₂ are not utilized. The G + C content is 42 mol% as determined by the thermal denaturation method. The type strain of *Thermoanaerobacterium zeae* is strain mel2T (= ATCC BAA-16T = DSM 13642T).

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