Description of *Caldanaerobius fijiensis* gen. nov., sp. nov., an inulin-degrading, ethanol-producing, thermophilic bacterium from a Fijian hot spring sediment, and reclassification of *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* as *Caldanaerobius polysaccharolyticus* comb. nov. and *Caldanaerobius zeae* comb. nov.

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An obligately anaerobic, spore-forming, Gram-type-positive but Gram-staining-negative thermophilic bacterium, strain JW/YJL-F3^T, was isolated from a Fijian hot spring sediment sample. Cells of strain JW/YJL-F3^T were straight to slightly curved rods, 0.5–1.2 µm in diameter and 1.5–19 µm long. The temperature range for growth was between 40 and 67 °C, with an optimum at 60–63 °C. The pH^{25 °C} range for growth was 4.5–8.4 with an optimum of 6.8. The salinity range for growth was 0–0.5 %. Strain JW/YJL-F3^T utilized a range of substrates including arabinose, cellobiose, galactose, glucose, inulin, lactose, maltose, mannose, raffinose, ribose, trehalose, xylose and yeast extract as carbon and energy sources. The major fermentation end products from glucose were ethanol, acetate and formate. Strain JW/YJL-F3^T converted thiosulfate to elemental sulfur, producing sulfur globules. The DNA G + C content was 37.6 mol% as determined by HPLC. Phylogenetic analysis using the 16S rRNA gene sequence indicated that the isolate is distantly related to the clade of the genus *Thermoanaerobacterium*. However, *Thermoanaerobacterium polysaccharolyticum* (96.7 % similarity to the type strain) and *Thermoanaerobacterium zeae* were the closest relatives, forming a separate, well-supported clade together with the novel isolate. Because *Thermoanaerobacterium polysaccharolyticum*, *Thermoanaerobacterium zeae* and strain JW/YJL-F3^T have different features from other *Thermoanaerobacterium* species, including a higher G + C content and formate production, and are placed distantly from the remaining species of *Thermoanaerobacterium* (greater than 10 % distance) in the 16S rRNA gene sequence analysis, we propose to place the new isolate JW/YJL-F3^T and *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* into the novel genus *Caldanaerobius* gen. nov. as *Caldanaerobius fijiensis* gen. nov., sp. nov. (the type species), *Caldanaerobius polysaccharolyticus* comb. nov. and *Caldanaerobius zeae* comb. nov., respectively. The type strain of *Caldanaerobius fijiensis* is JW/YJL-F3^T (=ATCC BAA-1278^T =DSM 17918^T).

Inulin, a fructose polymer that is poorly digestible for humans, is found as a storage carbohydrate in over 36 000 plant species (Carpita *et al.*, 1989). It consists of 20 to 30

fructose molecules attached to a terminal glucose residue via a β(2→1) linkage (Vandamme & Derycke, 1983). Inulin has potential for various industrial applications and has recently received increased interest as a potential source for fuel ethanol production, food additives, adhesives and prebiotics (Drent *et al.*, 1991; Pandey *et al.*, 1999;

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JW/YJL-F3^T is EF507903.

Roberfroid, 2005; Rowland *et al.*, 1998). Due to inulin's high solubility in water at 58 °C and above, it serves as a suitable substrate for a thermophilic fermentation (Sridhar *et al.*, 2000).

Attempts were made to isolate inulin-degrading bacteria under both anaerobic and thermophilic conditions. Initial enrichment was performed at 60 °C using the modified Hungate technique (Ljungdahl & Wiegel, 1986). A mixed water–sediment sample was inoculated into phosphate-buffered basal medium which contained (per litre distilled water): 0.75 g KH_2PO_4 , 1.5 g K_2HPO_4 , 1 g NaCl, 0.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.5 g NH_4Cl , 0.4 g MgCl_2 , 0.1 g CaCl_2 , 0.05 % cysteine acid, 0.1 % resazurin and 5.0 ml trace element solution and 0.5 ml vitamin solution (Freier *et al.*, 1988). The sample was a clay-containing brown-coloured soil, collected about 5 m downstream from the origin (a concrete basin) of the Wainggele Spring on Vanua Levu island (Fiji), with a temperature of 96.2 °C and a pH at room temperature of 8.1–8.2. Unless otherwise stated, the medium contained 0.5 % (w/v) inulin as a carbon and energy source supplemented with 0.05 % (w/v) yeast extract. The pH^{25 °C} (Wiegel, 1998) was adjusted to 7.0 before degassing, yielding a final medium pH^{25 °C} of 7.01. Pure cultures were obtained by three subsequent rounds of single-colony isolation using the agar–(2.2 % w/v) shake-roll-tube technique (Ljungdahl & Wiegel, 1986). Colonies appeared in 24 h, which were creamy and irregular, 0.5–2 mm in diameter. The cells were straight to slightly curved rods, 0.5–1.2 µm in diameter and 1.5–19 µm in length (Fig. 1). The cells stained Gram-negative, although the species is phylogenetically Gram-type positive (Wiegel, 1981). Spherical terminal spores were observed. Peritrichous flagella were detected using electron microscopy (JEM-1210 transmission electron microscope; JEOL, Inc.). Cell-wall analysis (performed at the DSMZ) revealed *meso*-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan, indicating the peptidoglycan type A1γ (A31 according to <http://www.dsmz.de/species/murein.htm>).

Fig. 1. Phase-contrast micrograph of cells of strain JW/YJL-F3^T showing the formation of spherical, terminal endospores and L-form cells. Bar, 10 µm.

Strain JW/YJL-F3^T grew at pH^{25 °C} 7.0 between 40 and 67 °C, with an optimum at 60–63 °C (no growth at 38 °C or below or at 71 °C or above). The pH^{25 °C} range for growth at 60 °C was 4.5–8.4, with an optimum at pH^{25 °C} 6.8 (no growth at 4.1 or below or at 8.8 or above). The salinity range for growth was 0–0.5 % (NaCl/KCl, 9 : 1); no growth was detected at or above 1.0 %. Doubling times of strain JW/YJL-F3^T were 2.1 h with inulin (0.5 %, w/v) and 2.2 h with glucose (0.5 %, w/v) at optimal temperature (62.5 °C) and pH (pH^{25 °C} 6.8). In addition, the isolate grew slightly better in a medium containing thiosulfate, and formed sulfur globules. The utilization of different substrates was tested in the presence of 0.02 % yeast extract to enhance growth. Strain JW/YJL-F3^T utilized as sole carbon source (0.2 %, w/v) arabinose, cellobiose, fructose, galactose, glucose, inulin, lactose, maltose, mannose, raffinose, ribose, trehalose, xylose and yeast extract. No growth was observed with beef extract, Casamino acids, peptone, inositol, mannitol, sorbitol, xylitol, acetate, lactate, pyruvate, ethanol, glutamate, formate, succinate, cellulose, glycerol, starch or xylan. Yeast extract was not required for growth. The fermentation end products from inulin (0.5 %, w/v) were ethanol, acetate and formate, with traces of pyruvate and lactate. The observed fermentation end products from 20 mM glucose consumed were 28 mM ethanol, 8.7 mM formate and 6.7 mM acetate, without any pyruvate or lactate formation (carbon dioxide and hydrogen were not quantified). At 20 mM, iron(III), manganese, nitrate, nitrite, sulfate and elemental sulfur were not reduced. Strain JW/YJL-F3^T reduced and tolerated sulfite at up to 40 mM. Interestingly, the relative high tolerance of strain JW/YJL-F3 to sulfite and the formation of sulfur globules from thiosulfate are similar to properties reported for the recently described species *Thermoanaerobacter sulfurignens* (Lee *et al.*, 2007). The conversion of thiosulfate to elemental sulfur agreed with the formerly distinguishing characteristic of the genus *Thermoanaerobacterium* (Lee *et al.*, 1993), but differed from its two closely related species, *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* (Table 1), demonstrating again that the end product of thiosulfate reduction can not be used as a genus-specific property (Lee *et al.*, 2007 and literature cited therein). Catalase was negative, and indole was not produced, like other species of the genus *Thermoanaerobacterium* including *Thermoanaerobacterium polysaccharolyticum*, *Thermoanaerobacterium zeae* and *Thermoanaerobacterium aotearoense* (Cann *et al.*, 2001; Liu *et al.*, 1996). Further biochemical tests were performed using the API ZYM system (bioMérieux). Strain JW/YJL-F3^T was positive for alkaline phosphatase, esterase, esterase lipase (C₈), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucosidase, α-mannosidase and α-fucosidase. Morphological and physiological characteristics of strain JW/YJL-F3^T were summarized and compared with those of closely related species and genera, revealing differences (Table 1).

DNA was extracted using a DNeasy Tissue kit (Qiagen) for DNA G + C content and phylogenetic analyses. The G + C

Table 1. Differentiating characteristics between strain JW/SL-YJL-F3^T and type strains of phylogenetically related species

Strains: 1, JW/YJL-F3^T (*Caldanaerobius fijiensis* gen. nov., sp. nov.; data from this study); 2, *Thermoanaerobacterium polysaccharolyticum* KMTHCJ^T (data from Cann *et al.*, 2001); 3, *Thermoanaerobacterium zeae* mel2^T (Cann *et al.*, 2001); 4, *Thermoanaerobacterium thermosulfurigenes* 4B^T (Schink & Zeikus, 1983); 5, *Thermoanaerobacter ethanolicus* JW 200^T (Wiegel & Ljungdahl, 1981). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5
Source	Hot spring	Organic waste pile	Organic waste pile	Hot spring	Alkaline hot spring
Growth conditions					
Temperature range (°C)	40–67	45–70	55–72	35–75	37–78
Optimum temperature (°C)	60–63	65–68	65–70	60	69
pH range	4.5–8.4	5.0–8.0	3.9–7.9	4.0–7.6	4.4–9.9
Optimum pH	6.8	6.8–7.0	ND	5.5–6.5	5.8–8.5
DNA G + C content (mol%)	37.6 (HPLC)	46 (T _m)	42 (T _m)	32.6 (T _m)	32 (T _m)
Gram stain	Negative	Variable	Variable	Negative	Variable
Spore formation	+	–	–	+	–
Formate production	+	+	+	–	–
Product from S ₂ O ₃ ^{2–} reduction	S ⁰	S ^{2–}	None	S ⁰	S ^{2–}
Yeast extract requirement	–	–	–	–	+
Substrate utilization					
Starch	–	–	+	+	+
Xylan	–	–	+	–	+
Pyruvate	–	+	+	–	+

content of the genomic DNA of the isolate was determined by HPLC as described previously (Mesbah *et al.*, 1989) with modifications (Lee *et al.*, 2005) and determined to be 37.6 mol%. The extracted DNA was amplified, purified and sequenced for 16S rRNA gene sequence analysis according to Lee *et al.* (2006). A nearly complete 16S rRNA gene sequence of strain JW/YJL-F3^T was obtained, comprising 1354 bp [positions 53–1451 according to the numbering scheme for *Escherichia coli* ATCC 11775^T (GenBank accession no. X80725)]. The retrieved 16S rRNA gene sequence was analysed using BLASTN and aligned manually with closely related sequences using CLUSTAL_X v1.81 (Thompson *et al.*, 1997), and manual adjustments were performed using GeneDoc v2.6.02 (<http://www.psc.edu/biomed/genedoc>). Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) using the model of Jukes & Cantor (1969) with the phylogenetic analysis package PHYLIP v3.6a2.1 (Felsenstein, 2001). Based on phylogenetic analysis using 16S rRNA gene sequences (Fig. 2), strain JW/YJL-F3^T clustered closely with the type strains of *Thermoanaerobacterium polysaccharolyticum* (96.7%) and *Thermoanaerobacterium zeae* (96.2%); however, it showed only 87.2–87.7% sequence similarity to the type strains of five other species of the genus *Thermoanaerobacterium*. In addition, *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* exhibited only around 88–89% and 87–88% sequence similarity, respectively, to the other members of the genus *Thermoanaerobacterium*. Similarly, strain JW/YJL-F3^T showed less than 86% sequence similarity to the type species of the genera *Caldanaerobacter*, *Thermoanaerobacter* and *Moorella* in the family *Thermoanaerobacteriaceae*. Strain JW/YJL-F3^T, *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium*

zeae have distinct characteristics such as formate production and a slightly higher G + C content of the genomic DNA (37.6–46 mol%) than the other members of genus *Thermoanaerobacterium* (less than 36 mol%).

Based on its physiological and metabolic features and phylogenetic analyses, we propose that strain JW/YJL-F3^T be placed into a novel taxon *Caldanaerobius fijiensis* gen. nov., sp. nov. as the type strain for the type species of the genus *Caldanaerobius*. We also propose to reclassify *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* within the new genus as *Caldanaerobius polysaccharolyticus* comb. nov. and *Caldanaerobius zeae* comb. nov., respectively.

Description of *Caldanaerobius* gen. nov.

Caldanaerobius (Cal.da.nae.ro'bi.us. L. adj. *caldus* warm, hot; Gr. pref. *an* not; Gr. n. *aer* air; Gr. masc. n. *bios* life; N.L. masc. n. *Caldanaerobius* a bacterium which grows in the absence of air at high temperature).

The genus *Caldanaerobius* is placed into the order 'Thermobacteriales' Ludwig *et al.* 2008 in the class *Clostridia* of the phylum *Firmicutes*. Cells are rods and occur singly or in pairs. Anaerobic and thermophilic chemo-organotrophs. Yeast extract is not required for growth. Formate is produced from glucose fermentation. The DNA G + C content is 37–46 mol%. The type species is *Caldanaerobius fijiensis*.

Description of *Caldanaerobius fijiensis* sp. nov.

Caldanaerobius fijiensis (fi.ji.en'sis. N.L. masc. adj. *fijiensis* pertaining to the Fiji islands, reflecting the source of isolation of the type strain).

Fig. 2. Neighbour-joining phylogenetic dendrogram of strain JW/YJL-F3^T based on 16S rRNA gene sequences showing its position amongst members of the family *Thermoanaerobacteriaceae*. Numbers at nodes represent bootstrap values (percentages of 1000 replicates). Bar, 2% substitutions in nucleotide sequence.

Cells are straight or curved rods, 0.5–1.2 µm in diameter and 1.5–19 µm long. Peritrichous flagella are present. Gram-type positive but stains Gram-negative. Spherical, terminal spores, distending the end of the mother cell, are formed at the end of the exponential growth phase, and some L-form cells are observed in the stationary growth phase. The temperature range for growth is 40–67 °C with an optimum at 60–63 °C (no growth at 38 °C or below or at 71 °C or above). The pH^{25 °C} range for growth is 4.5–8.4 with an optimum at 6.8 (no growth at pH^{25 °C} 4.1 or below or at pH^{25 °C} 8.8 or above). The salinity range for growth is 0–0.5%, with no growth at 1.0% w/v NaCl or above. Arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, raffinose, ribose, trehalose, xylose and yeast extract serve as carbon and energy sources. The fermentation products from glucose are ethanol (more than 1 mol ethanol formed per mol glucose consumed), acetate and formate. Up to 40 mM sulfite is reduced. Thiosulfate is converted to elemental sulfur, forming sulfur globules. At 20 mM, iron(III), manganese, nitrate, nitrite, sulfate and sulfur are not reduced. Catalase is negative. Indole is not produced. Tests for alkaline phosphatase, esterase, esterase lipase (C₈), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucosidase, α-mannosidase and α-fucosidase are positive. The DNA G + C content of the type strain is 37.6 mol% (HPLC).

The type strain is JW/YJL-F3^T (=ATCC BAA-1278^T =DSM 17918^T), isolated from Wainggele Spring on Vanua Levu island in Fiji.

Description of *Caldanaerobius polysaccharolyticus* comb. nov.

Caldanaerobius polysaccharolyticus (po.ly.sac.cha.ro.ly'ti.cus. Gr. adj. *polus* many; Gr. n. *saccharon* sugar; N.L. masc. adj. *lyticus* dissolving; N.L. masc. adj. *polysaccharolyticus* dissolving many sugars).

Basonym: *Thermoanaerobacterium polysaccharolyticum* Cann *et al.* 2001.

The description is the same as that given for *Thermoanaerobacterium polysaccharolyticum* by Cann *et al.* (2001). The type strain is KMTHCJ^T (=ATCC BAA-17^T =DSM 13641^T).

Description of *Caldanaerobius zeae* comb. nov.

Caldanaerobius zeae [ze'ae. N.L. gen. n. *zeae* of *Zea mays* (maize or corn), describing the use of corn as a substrate].

Basonym: *Thermoanaerobacterium zeae* Cann *et al.* 2001.

The description is the same as that given for *Thermoanaerobacterium zeae* by Cann *et al.* (2001). The type strain is mel2^T (=ATCC BAA-16^T =DSM 13642^T).

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