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ORIGINAL PAPER

Themoanaerobacterium calidifontis sp. nov., a novel anaerobic, thermophilic, ethanol-producing bacterium from hot springs in China

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Abstract A novel thermophilic Gram staining positive strain Rx1 was isolated from hot springs in Baoshan of Yunnan Province, China. The strain was characterized as a hemicellulose-decomposing obligate anaerobe bacterium that is rod-shaped (diameter: 0.5–0.7 μm; length: 2.0–6.7 μm), spore-forming, and motile. Its growth temperature range is 38–68 °C (optimum 50–55 °C) and pH range is 4.5–8.0 (optimum 7.0). The maximum tolerance concentration of NaCl was 3 %. Rx1 converted thiosulfate to elemental sulfur and reduced sulfite to hydrogen sulfide. The bacterium grew by utilizing xylan and starch, as well as a wide range of monosaccharide and polysaccharides, including glucose and xylose. The main products of fermentation were ethanol, lactate, acetate, CO_2, and H_2. The maximum xylanase activity in the culture supernatant after 30 h of incubation at 55 °C was 16.2 U/ml. Rx1 DNA G + C content was 36 mol %. 16S rRNA gene sequence analysis indicated that strain Rx1 belonged to the genus *Thermoanaerobacterium* of the family '*Thermoanaerobacteriaceae*' (Firmicutes), with *Thermoanaerobacterium aciditolerans* 761–119 (99.2 % 16S rRNA gene sequence similarity) being its closest relative. DNA–DNA hybridization between Rx1 and *T. aciditolerans* 761–119 showed 36 % relatedness. Based on its physiological and biochemical tests and DNA–DNA hybridization analyses, the isolate is considered to represent a novel species in the genus *Thermoanaerobacterium*, for which the name *Thermoanaerobacterium calidifontis* sp. nov. is proposed, with the type strain is Rx1 (=JCM 18270 = CCTCC M 2011109).

Keywords *Themoanaerobacterium calidifontis* sp. nov. · Hot springs · Thermophilic · Phylogeny

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Rx1 is AB544080.

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Introduction

The biotechnological potential and evolutionary significance of thermophiles has led to intensive research focused on anaerobic, saccharolytic, and thermophilic bacteria, which are members of the genera *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Clostridium* (Xue et al. 2001). Within the genus *Thermoanaerobacterium*, eight species have been isolated, and more recently the taxonomic relationships between some of these species have been better defined (Romano et al. 2010). Most studies of eubacterial thermophilic anaerobes have focused on the saccharolytic bacteria, which form ethanol and lactate and are promising tools for creating alternative fuels from plant biomass (Shaw et al. 2008; Patel et al. 2006). Moreover, their ethanol production capacity involving utilization of glucose and the other hexose transformed from cellulose materials has

been the major focus of those studies. Since only a few of the natural anaerobic thermophilic microorganisms identified to date are capable of efficiently fermenting xylose and the other pentose transformed from hemicellulose materials, we aimed to isolate novel thermophilic anaerobic hemicellulose-decomposing bacteria from hot springs sample.

The Baoshan region is situated in the southwest of Yunnan Province and encompasses a large number of natural hot springs. Water and sediment samples were collected from the region's hot springs and measured temperature (50–70 °C) and pH (6.0–7.5). Using oat spelt xylan as substrate, we isolated a Gram staining positive, obligately anaerobic, thermophilic bacterium (strain Rx1).

In this paper, we describe the isolation and characterization of the ethanol-producing strain Rx1. The physiological, biochemical, and phenotypic features of Rx1 were determined, and the results of DNA–DNA relatedness studies indicated that Rx1 is a new member of the genus *Thermoanaerobacterium*. The name *Themoanaerobacterium calidifontis* is proposed.

Materials and methods

Strains and culture conditions

Thermoanaerobacterium aciditolerans DSM 16487^T and *Thermoanaerobacterium saccharolyticum* DSM7060^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and were cultured in modified medium 640 at 55 °C.

All culturing of Rx1 was performed in modified DSMZ medium 640, containing (per liter distilled water) 0.9 g NH_4Cl, 0.9 g NaCl, 0.4 g $MgCl_2 \times 6\ H_2O$, 0.75 g KH_2PO_4, 1.5 g K_2HPO_4, 2.5 mg $FeCl_3 \times 6\ H_2O$, 0.75 g cysteine-HCl $\times\ H_2O$, 2 g tryptone, 1 g yeast extract, 1 ml trace element solution SL-10 (see medium 320, DSMZ), 0.5 mg resazurin, 5 ml vitamin solution (see medium 141, DSMZ, filter-sterilized), 10.0 g substrate. After adjusting pH to 7.0, the medium was prepared anaerobically under 100 % N_2 and dispensed 5 ml portions into 15 ml Hungate tubes. Finally, the medium solutions were autoclaved.

Initial enrichment was carried out at 60 °C by inoculating the samples of mixed sediment and water into 250 ml anaerobic reagent bottles (50 ml modified medium 640). Oat spelt xylan was added as substrate, and the culture incubated until visible growth was observed. The ethanol-producing cultures were isolated through several serial liquid dilutions and applied to the anaerobic phytagel (1.2 %, w/v) shake-roll tube technique (Ljungdahl and Wiegel 1986) with xylose as substrate. Pure strains were stored as liquid cultures under anaerobic conditions at 4 and −80 °C. One of the isolated strains was strain Rx1 for further characterization.

Morphological, physiological, and biochemical analysis

Gram reaction was determined using a Gram staining kit (Guangdong Huankai Microbial Sci. & Tech Co., Ltd. Guangdong, China). Cell morphology was examined by phase-contrast microscopy (Leica, Germany). Bacteria tolerance of NaCl (0–5 %, w/v), temperature (from 35 to 70 °C), pH (from 3.5 to 8.5), and substrates (complete list in Table 1) was determined by growing on the modified 640 medium for four days and measuring the optical density (OD) at 600 nm using a 4802 UV/VIS double-beam spectrophotometer (Unico, Dayton, NJ, USA). Metabolite products were measured by high-performance liquid chromatography (HPLC) and gas chromatography (GC-SC2), as previously described (Shaw et al. 2008; Romano et al. 2010; Ren et al. 2008). Ethanol tolerance was determined by supplementing the modified 640 medium with ethanol (from 0 to 5.0 %, v/v) after autoclaving. The ability of Rx1 to convert thiosulfate, sulfate, sulfite, and elemental sulfur was assessed by adding thiosulfate (7 g/l), sulfate (2 g/l), sulfite (0.76 g/l), or elemental sulfur (2 g/l), respectively, to the modified medium 640 (Kublanov et al. 2007; Liu et al. 1996). All the tests were performed in triplicate. Phase-contrast microscopy was used to visualize the sulfur globules formation (Lee et al. 2007). The gas from the headspace (10 ml) culture of Rx1 was transferred via sterile syringe into a test tube containing 5 ml of 50 g/l copper sulfate solution, and the formation a black precipitate of copper sulfide was observed. At the same time, one control sample was used without gas injected in the test tube, and another control sample was used with gas injected from the headspace of Rx1 cultures, but without added sulfate, sulfite, or elemental sulfur in the modified medium 640.

Enzymatic hydrolysis

A 500 ml anaerobic reagent bottle containing 250 ml of modified 640 medium was inoculated with 2.5 ml of an overnight culture of Rx1 in the exponential growth phase. Fermentation was carried out at 55 °C with 10 g/l of oat spelt xylan as substrate. Aliquots of the fermentation liquid were collected at different time points and were centrifuged (12,000 rpm, 5 min) to remove cells. The supernatant (enzyme solution) was then analyzed as described below.

Xylanase activity was determined in triplicate reactions using the dinitrosalicylic acid (DNS) method (Bailey et al. 1992). The initial reaction mixture consisted of 0.5 ml of 1 mM oat spelt xylan in water and 0.5 ml of enzyme solution. After incubation at 55 °C for 10 min, the DNS reagent was added, and the mixture was boiled at 100 °C for 5 min. After cooling, water was added to bring the volume to 20 ml, and the amount of reduced sugar released from the xylan substrate was measured as OD_{540}. The control

Table 1 Phenotypic characteristics that differentiate strain Rx1 from the other recognized species of the genus *Thermoanaerobacterium*

Characteristic	1	2	3	4
Spore formation	+	+	+	−
Flagellation		Single flagellum	Peritrichous	Peritrichous
Temp range/ optimum temp (°C)	38–68/50–55	37–68/55	35–66/60–63	45–70/60
pH range/optimum pH	4.5–8.0/7.0	3.2–7.1/5.7	3.8–6.8/5.2	5.0–7.5/6.0
NaCl range for growth (%, w/v)	0-3 %	0–3 %	ND	ND
DNA G + C content (mol %)	36	34 ± 0.5	34.5–35	36
Cell size (μm)	0.5–0.7 × 2.0–6.7	0.4 × 3.0–12.0	0.7–1.0 × 2.1–14.3	0.8–1.0 × 3.0–15.0
Utilization of				
Arabinose	+	+	+	ND
D-galactose	+	+	ND	+
D(+)-cellobiose	+	+*	ND	+
Lactose	+	+	+	+
Cellulose	−	−*	ND	−
Xylan from oat spelt	+	+	+	+
Pectin	+	+*	+	ND
Xylitol	−	−*	ND	ND
Pyruvate	+	−	−	ND
Ethanol	−	−	−	ND
Ribose	ND	+	+	−
Sorbitol	−	+	ND	−
Glycerol	−	−	−	ND
Sucrose	−	+	ND	+
Reduction of				
S^0	−	−	−	ND
Thiosulfate	+ (S^0)	+ (S^0)	+ (S^0)	+ (S^0)
Sulfite	+ H_2S	+ H_2S	ND	ND
Sulfate	−	−	−	ND

Strains: *1* Rx1, *2 T. aciditolerans* DSM 16487^T, *3 T. aotearoense* DSM10170^T, *4 T. saccharolyticum* DSM 7060. Data for Rx1 are from this study; strain 2 is from (Kublanov et al. 2007) and this study; strain 3 is from (Liu et al. 1996; Kublanov et al. 2007); strain 4 is from (Lee et al. 1993; Kublanov et al. 2007). All species utilize glucose, D-xylose, D-mannose*, mannitol, D-fructose*, maltose, and starch soluble, and produce ethanol, acetate, lactate, H_2, and CO_2 to form glucose. +, positive; −, negative; *ND* no data available

* Data are from this study

reaction lacked the enzyme solution. Measurements of a series of xylose dilutions were used as standards to calculate the quantity of reduced sugar. One unit of xylanase activity was defined as the activity that released 1 μmol of xylose in 1 min under the assay conditions.

Polyacrylamide gel electrophoresis (PAGE) of soluble proteins

SDS-PAGE of whole cell protein extracts is used widely in bacterial taxonomy and is suitable for grouping strains at the species level (Bandyopadhyay et al. 2013; Dicks et al. 1990). For total soluble cell protein analysis, 10 ml aliquots of late exponential phase cultures grown at 55 °C in the modified medium (pH 6.5) containing 1.0 % glucose as the substrate was harvested by centrifugation. The total cellular protein was extracted as described previously (Liu et al. 1996), and the protein concentration was determined by the Bradford method (Bradford 1976). Samples containing 50 μg of protein from each strain were analyzed by SDS-PAGE, as previously described (Laemmli 1970).

16S rRNA gene sequencing and phylogenetic analysis

Bacterial 16S rRNA was amplified by PCR using the previously described conditions (Rivas et al. 2009) and the following universal primers: reverse 1541R, 5′-TGYG GNTGGATCACCTCCTT-3′ (*Escherichia coli* positions

1,509–1,522) and forward 8F, 5′-AGAGTTTGATCTG GCTCAG-3′ (*E. coli* positions 8–27). Sequence comparison of the 16S rRNA gene was carried out using the BLAST program (http://www.ncbi.nlm.nih.gov/blast) and the global alignment algorithm of the EzTaxon server (http://www.eztaxon.org/; Chun et al. 2007). After multiple sequence alignment by ClustalX (Thompson et al. 1997), a phylogenetic tree was constructed by the neighbor-joining and minimum evolution methods using Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (Tamura et al. 2011) and evaluated by bootstrap resampling with 1,000 replicates.

Determination of G + C and DNA–DNA hybridization

Genomic DNA was extracted, and G + C content was evaluated in triplicate samples by HPLC using the method described by Murray and Thompson (1980). Briefly, purified DNA samples were mixed with 45 % perchloric acid (DNA: 45 % perchloric acid = 10 μg:1 μl) and boiled at 98 °C for 2 h. Supernatants were harvested by centrifugation (5,000*g*, 10 min, 4 °C) and diluted 50-fold using distilled water for HPLC analysis (Mesbah et al. 1989).

The DNA–DNA hybridization technique has been especially successful in resolving taxonomic relationships among closely related organisms (at the species level and below) (Lee et al. 1993), Here, this technique was carried out according to the spectrophotometric method (Ley et al. 1970; Huss et al. 1983; Jahnke 1992), using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a DCW-2008 thermo bath (Yuan et al. 2008). G + C content and DNA–DNA hybridization assays were performed in triplicate.

Results

Isolation and characterization of a novel anaerobic, thermophilic, hemicellulose-decomposing bacteria

An anaerobic, thermophilic, ethanol-producing strain (Rx1) was obtained from samples of mixed sediment and water of hot springs. Colonies of Rx1 grown within the phytagel shake-roll tubes appeared as white, circular, 0.5–1 mm in diameter, and slightly convex (colonies were adhered to the inner surface of the phytagel shake-roll tubes). Analysis of the single cells indicated that the strain was Gram staining positive, motile, rod-shaped, 0.5–0.7 μm in diameter, and 2.0–6.7 μm in length (Table 1). The average (mean of $n = 15$) length of the individual organisms was longer in the stationary phase than in the exponential growth phase. The formation of terminal spores was observed during the late exponential and early stationary phase (Fig. 1).

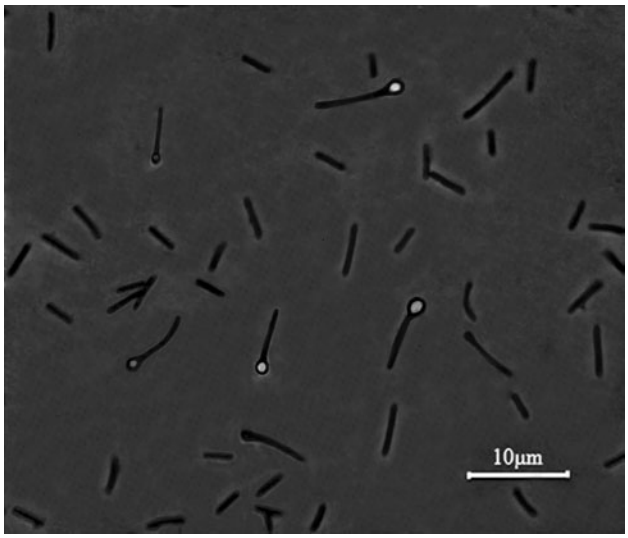

Fig. 1 Cell morphology of strain *T. calidifontis* Rx1 grown in modified 640 medium with 1 % xylose as substrate. Phase-contrast micrograph of late exponential growth cells and spores shows the formation of terminal spores. *Bar* 10 μm

Approximately 6.0 % of the cells sporulated in liquid media, and the spores were oval, 1.5–1.7 μm in length and 1.0–1.5 μm in diameter. The length of the end of the mother cells ranged between 5.9 and 7.6 μm, so that they were longer than normal cells. The strain did not grow in oxidized medium, as evidenced by the pink color of resazurin. The strain had a temperature growth range from 38 °C to 68 °C, with the optimal temperature being between 50 and 55 °C. For up to 96 h, no growth was observed at temperatures ≤37 °C or ≥70 °C. The pH range for growth at 55 °C was between pH 4.5 and 8.0, with the optimum pH being 7.0. For up to 96 h, no growth was observed at pH ≤ 4.3 or ≥8.2. The growth curve of Rx1 under optimal growth condition with 5 g/l xylose substrate is shown in Fig. 3; the doubling time under the optimal growth condition was approximately 50 min. Growth of Rx1 occurred at NaCl concentrations ranging from 0 to 3 %, but no growth was observed in 4 % NaCl.

Rx1 was able to grow in the presence of the following polysaccharide substrates: xylan, starch, and pectin. It also was able to utilize various monosaccharides and disaccharides, including glucose, xylose, fructose, mannose, mannitol, galactose, maltose, lactose, cellobiose, arabinose, and pyruvate (filter-sterilized). However, no growth was observed in the presence of sucrose, xylitol, sorbitol, cellulose, glycerol, or ethanol (Table 1). The fermentation products from xylan, starch, cellobiose, xylose, and glucose substrates were mainly ethanol, acetate, lactate, CO_2, and H_2 (production of H_2 and CO_2 was detected by gas chromatography), respectively. Under optimal conditions, when Rx1 was grown on 5 g/l of glucose for 48 h,

the fermentation products were ethanol (29.3 mM), acetate (13.1 mM), lactate (16.2 mM), H_2 (38.1 mM), and CO_2 (8.6 mM). Ethanol yield reached 58 % of the theoretical yield (we assumed that all of consumed glucose/xylose was converted into ethanol). In optimal conditions, when Rx1 was grown on 5 g/l of xylose for 48 h, the amounts of ethanol, acetate, lactate, H_2 and CO_2 were 45.1, 1.3, 10.5, 29.7, and 8.8 mM, respectively. Ethanol yield reached 81 % of the theoretical yield. When Rx1 was grown on 5 g/l of xylan for 48 h, the amounts of ethanol, acetate, and lactate were 25.2, 0.8, and 7.3 mM, respectively. These results indicated that Rx1 was able to produce ethanol from the majority of hexose and pentose, even polysaccharides; the ethanol yields were 1.0–4.3 and 2.2–34.7 times those of lactate and acetate, respectively. However, ethanol yields of most of the known *Thermoanaerobacterium* species were lower or equal to those for lactate and acetate (Romano et al. 2010; Kublanov et al. 2007; Liu et al. 1996). Therefore, Rx1 is considered as potentially useful strains for converting biomass to produce ethanol.

Rx1 was able to grow in the presence of 3 % (v/v) ethanol. Moreover, addition of thiosulfate (7 g/l) to the modified 640 medium significantly stimulated growth of Rx1. Rx1 was found to convert thiosulfate to sulfur globules (S^0), which were detected both inside the cells and in the medium (Fig. 2), but the ethanol yield of Rx1 decreased under this condition by 22 %. Rx1 also grew well in the presence of sulfate, elemental sulfur and sulfite, and sulfite was reduced to hydrogen sulfide, but neither sulfate nor elemental sulfur was reduced (Fig. S1); however, the bacterial growth and ethanol production capability were not remarkably influenced by these medium conditions (Fig. 3).

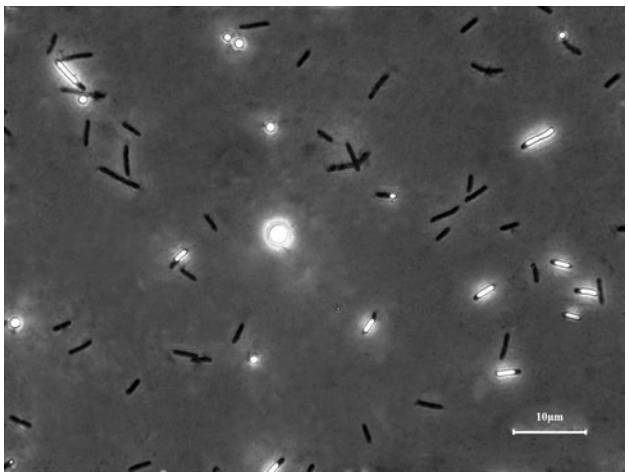

Fig. 2 *Thermoanaerobacterium calidifontis* Rx1 grown in modified 640 medium containing 1 % xylose and 7 g/l $Na_2S_2O_3$ at 55 °C. The cells of strain Rx1 are shown during the late exponential or early stationary phase. Note that the phase-bright sulfur globules accumulated in the medium and in the cells. *Bar* 10 μm

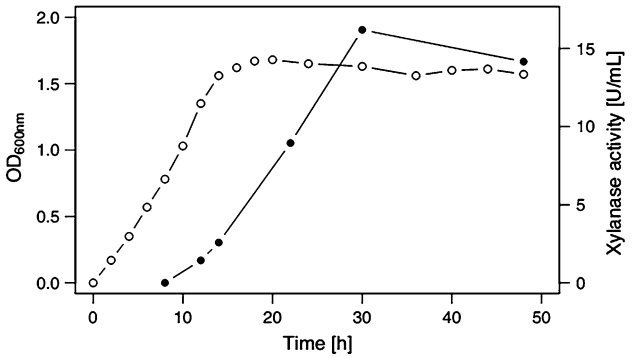

Fig. 3 Growth curve and xylanase activities of supernatants from Rx1 cultures grown at 55 °C. *Filled circles* xylanase activities, Rx1 grown in 1 % xylan. *Open circles* growth curve, Rx1 grown in 1 % xylose

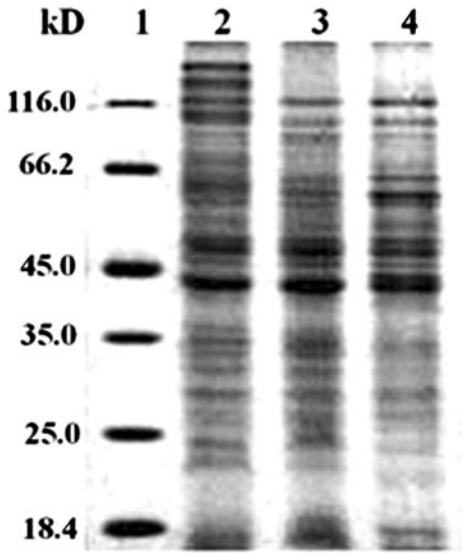

Fig. 4 Electrophoretic comparison of cellular proteins from different strains. *Lane 1* marker; *lane 2* Rx1; *lane 3*, *Thermoanaerobacterium aciditolerans* (DSM 16487^T); *lane 4*, *Thermoanaerobacterium saccharolyticum* (DSM 7060^T)

Rx1 grew on xylan, and xylanase activities were detected in culture supernatants after 8 h. The highest enzyme activity was 16.2 U/ml after culture for 30 h at 55 °C (Fig. 3). Xylanase activity in culture supernatants were compared with those detected from *T. thermosulfurigenes* (0.023 U/ml), *T. xylanolyticum* (0.067 U/ml), and *T. saccharolyticum* (0.108 U/ml) strains of the *Thermoanaerobacterium* genus, and found to be up to 703, 241 and 150 times higher (Lee et al. 1993). Xylanase may be a thermostable enzyme, giving it potential in applications for the ethanol-producing industry. The properties, production, and applications of xylanase need to be further researched.

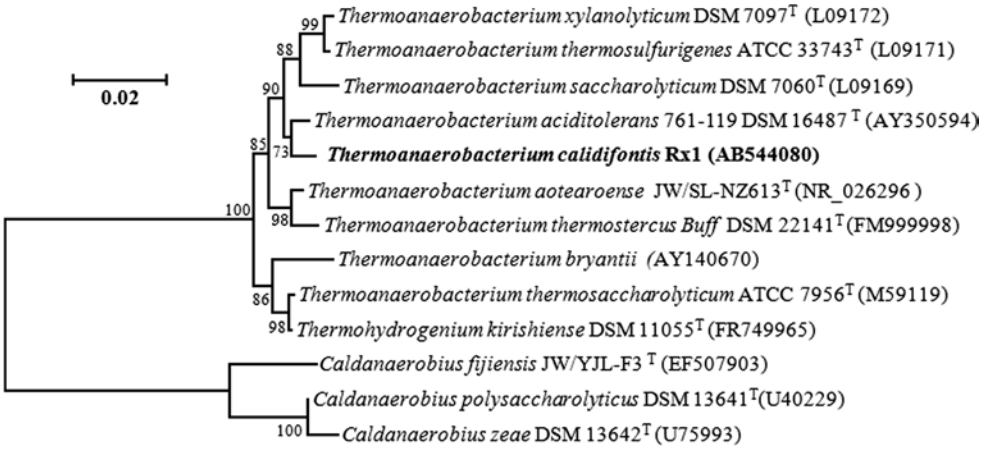

Fig. 5 Phylogenetic tree based on 16S rRNA gene sequences, showing the position of isolate *T. calidifontis* Rx1 in the genus *Thermoanaerobacterium*. The tree was constructed by neighbor-joining method with 1,000 replicates of bootstrapping. GenBank accession numbers are given in *parentheses*. Bootstraps are the confidence values (expressed as percentages) obtained from 1,000 replications. *Bar* 5 nucleotide substitutions per 100 nucleotides

Protein profile

Strain Rx1 showed distinct protein pattern from those of its closest related reference strains (Fig. 4), indicating that strain Rx1 is different from the other two species of the *Thermoanaerobacterium* genera and supporting its identification as a novel species.

Phylogenetic relationships

BLAST search of the 1,473 nucleotide sequence of the Rx1 16S rRNA gene revealed that this novel strain was a member of genus *Thermoanaerobacterium* of the family *Thermoanaerobacteriaceae*, which belongs to the order *Thermoanaerobacteriales* of the class *Clostridia* in the phylum *Firmicutes* (Garrity et al. 2003). Rx1 showed the highest identity with *T. aciditolerans* 761–119^T (99.2 %) (Kublanov et al. 2007). The phylogenetic tree indicated that stain Rx1 was a member of the genus *Thermoanaerobacterium* (Fig. 5). Similar tree topologies were also found by the tree maximum evolution method (data not shown). DNA–DNA hybridization between strain Rx1 and *T. aciditolerans* 761–119^T showed relatedness of only 36 %. The DNA G + C content of strain Rx1 was 36 mol %, which was higher than that of *T. aciditolerans* 761–119^T (34 ± 0.5 mol %).

Discussion

In this paper, we describe a new thermophilic anaerobic bacteria strain, Rx1, which was isolated from hot springs in Baoshan of Yunnan Province, China. Strain Rx1 showed the most sequence similarity (99.2 %) with *T. aciditolerans*. However, despite several phenotypic similarities, strain Rx1 differs from *T. aciditolerans*. As reported in Table 1, the pH range of Rx1 (4.5–8.0) was different from that of both *T. aciditolerans* (Kublanov et al. 2007) and *T. aotearoense* (Liu et al. 1996) (3.2–7.1 and 3.8–6.8, respectively). The optimum pH of Rx1 (7.0) was also higher than *T. aciditolerans* (5.7) and *T. aotearoense* (5.2). Rx1 could utilize pyruvate, but not sorbitol or sucrose, the opposite of *T. aciditolerans*. In addition, Rx1 has a high yield xylanase and was able to ferment a series of carbohydrates, which suggests that it may be applied in the ethanol production process.

On the basis of the above distinctive physiological and biochemical properties, G + C content and DNA–DNA hybridization values, the isolate Rx1 appears to represent a new species of the genus *Thermoanaerobacterium*. As such, the name *Thermoanaerobacterium calidifontis* sp. nov., is proposed.

Description of *Thermoanaerobacterium calidifontis* sp. nov.

Thermoanaerobacterium calidifontis: ca.li.di.fon'tis. L. adj. calidus, hot; L. n. fons fontis, spring, fountain; N.L. gen. n. calidifontis, of a hot spring.

Cells are Gram staining positive, rod-shaped, motile, an average diameter of 0.5–0.7 μm and length of 2.0–6.7 μm, obligate anaerobe, hemicellulose-decomposing bacteria. Colonies are circular, 0.5–1 mm in diameter, white, and slightly convex. Oval terminal spores were formed in the late exponential to early stationary phases of growth, and the spores were oval, 1.5–1.7 μm in length and 1.0–1.5 μm in diameter. The organism is a moderate thermophile that grows between 38 and 68 °C (no growth at ≤37 °C or ≥70 °C), with the optimum temperature between 50 and 55 °C, at pH between 4.5 and 8.0 (no growth at pH ≤ 4.3 or ≥8.2), with the optimum pH of 7.0, and low salinity (NaCl range 0–3 %). The bacteria grow by fermentation of xylan, starch, pectin, mannitol, glucose, xylose, fructose, galactose, maltose, lactose, cellobiose, mannose, arabinose, and pyruvate. Fermentation end products are ethanol, lactate, acetate, H_2, and CO_2.

Under optimal growth conditions, the Rx1 strain fermented xylose and glucose for 48 h and produced ethanol yields that were 81 and 58 % of the theoretical yields, respectively. The bacteria did not utilize sucrose, xylitol, sorbitol, cellulose, glycerol, or ethanol. The bacteria were able to convert thiosulfate to elemental sulfur, which accumulated both in the cells and in the culture medium. Sulfite was reduced to hydrogen sulfide. The genomic DNA G + C content (measured by HPLC) of strain Rx1 was 36 mol %. The type strain Rx1 (= JCM 18270 = CCTCC M 2011109) was isolated from a hot spring in Baoshan, Yunnan Province, China.

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