Caloramator boliviensis sp. nov., a thermophilic, ethanol-producing bacterium isolated from a hot spring

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A novel moderately thermophilic, anaerobic, ethanol-producing bacterial strain, 45B^T, was isolated from a mixed sediment water sample collected from a hot spring at Potosi, Bolivia. The cells were straight to slightly curved rods approximately 2.5 µm long and 0.5 µm wide. The strain was Gram-stain-variable, spore-forming and monotrichously flagellated. Growth of the strain was observed at 45–65 °C and pH 5.5–8.0, with optima of 60 °C and pH 6.5. The substrates utilized by strain 45B^T were xylose, cellobiose, glucose, arabinose, sucrose, lactose, maltose, fructose, galactose, mannose, glycerol, xylan, carboxymethylcellulose and yeast extract. The main fermentation product from xylose and cellobiose was ethanol (0.70 and 0.45 g ethanol per gram of consumed sugar, respectively). Acetate, lactate, propionate, carbon dioxide and hydrogen were also produced in minor quantities. 1,3-Propanediol was produced when glycerol-containing medium was supplemented with yeast extract. The major cellular fatty acids were anteiso-C_{15:0}, C_{16:0}, iso-C_{16:0}, C_{15:1}, iso-C_{14:0}, C_{13:0} and C_{14:0}. The polar lipids diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an aminoglycolipid and 15 other unidentified lipids were predominant. The DNA G + C content of strain 45B^T was 32.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequence similarity revealed that strain 45B^T is located within the Gram-type positive *Bacillus*–*Clostridium* branch of the phylogenetic tree. On the basis of morphological and physiological properties and phylogenetic analysis, strain 45B^T represents a novel species, for which the name *Caloramator boliviensis* sp. nov. is proposed; the type strain is 45B^T (=DSM 22065^T=CCUG 57396^T).

Growing concerns on environmental issues and finite fossil fuel supplies have stimulated an increasing interest in microbial fuel ethanol production using renewable raw materials (Olsson & Hahn-Hagerdal, 1996; Wheals *et al.*, 1999; Wyman, 1999; Cook & Beyea, 2000; Zaldivar *et al.*, 2001; Galbe & Zacchi, 2002; Dien *et al.*, 2003; Doi, 2003; Demain *et al.*, 2005).

During the last two decades, saccharolytic, thermophilic, anaerobic bacteria have been intensively studied because of their potential for producing ethanol by metabolizing a very broad range of carbohydrates, including pentose sugars and their polymers. In addition, the thermophilic fermentation process has important advantages such as

easier product recovery, minimized contamination risk, reduced costs of pumping and stirring, and no aeration and cooling problems (Cook & Morgan, 1994; Wiegel *et al.*, 1985).

Some thermophilic anaerobic clostridia from clusters III, V and VII (according to the phylogenetic interrelationship established by Collins *et al.*, 1994) have been reported to produce ethanol at high temperatures. Moreover, some other clostridial species that are phylogenetically distinct but conserve some physiological similarities have been reclassified in separate genera, including the genus *Caloramator*. Members of the *Caloramator*/*Thermobrachium* genera also produce ethanol, short chain fatty acids, carbon dioxide and hydrogen as end products of glucose fermentation (Collins *et al.*, 1994; Patel *et al.*, 1987; Plugge *et al.*, 2000; Seyfried *et al.*, 2002; Chrisostomos *et al.*, 1996; Tarlera *et al.*, 1997; Ogg & Patel, 2009; Engle *et al.*, 1996).

In this article, we report the isolation and characterization of a novel anaerobic, moderately thermophilic, fermentative,

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 45B^T is FM244718.

A supplementary figure and a supplementary table are available with the online version of this paper.

spore-forming and ethanol-producing bacterium (strain 45B^T) belonging to the genus *Caloramator*.

Strain 45B^T was isolated from a mixed sediment water sample containing decayed leaves of eucalyptus (*Eucalyptus* sp.). The sample was collected from the hot spring Chaqui (19° 37.469' S 65° 34.302' W) located in Potosi, Bolivia, at 3721 m above sea-level. The temperature and pH at the sampling point were 65 °C and 6.5, respectively. The sample was collected in a sterile plastic flask by completely filling it. Subsequently, it was stored at room temperature prior to study.

For enrichment, isolation and cultivation, anaerobic mineral medium (AMM) was used as described by Angelidaki *et al.* (1990) with the following modifications: cysteine was omitted, the concentration of sodium sulphide was increased to 0.5 g l⁻¹ and the vitamin solution was replaced by vitamin solution DSM-141 (German Collection of Microorganisms and Cell Cultures; 10 ml l⁻¹). Analytical grade chemicals and deionized water were used.

The medium was prepared by flushing with oxygen-free nitrogen according to the modified Hungate technique (Wiegel *et al.*, 1985). Filter-sterilized solutions of vitamins, D-xylose and sodium sulphide were added after autoclaving (121 °C, 20 min) and cooling the medium.

For initial sample enrichment, AMM supplemented with wheat straw (12 g l⁻¹) was used. The presence of ethanologenic micro-organisms was determined by quantifying ethanol production using the method described in this study.

For strain isolation, a biphasic AMM was used. The solid phase contained agar base (3 % agar). D-Xylose (5 g l⁻¹) was supplemented to both phases as a carbon and energy source. Cultures were incubated at 60 °C (pH 6.5) in darkness without shaking, unless otherwise stated. Single colonies were picked from the solid phase, transferred to fresh liquid AMM and incubated at 60 °C for 2 days. The biphasic culture procedure was repeated several times until a pure culture of strain 45B^T was obtained. The axenic culture was examined under a light microscope.

Routine examinations were performed using light microscopy (equipped with phase-contrast optics; Olympus). Gram staining was performed using the method of Hucker (Doetsch, 1981). The presence of spores in a liquid culture of strain 45B^T was examined by microscopy using Malachite green stain. Cells used for staining were harvested from exponential and/or early stationary growth phase.

The ability of the micro-organism to utilize different substrates was tested by using AMM supplemented with 5 g filter-sterilized substrates l⁻¹. The cultures were incubated for 2 weeks and monitored for growth by measuring the optical density and pH.

The effect of temperature and pH on growth was studied by incubating the strain 45B^T in AMM supplemented with cellobiose at temperatures ranging from 45 to 75 °C and

from pH 5 to 9. The pH was adjusted by using 1 M H₂SO₄. All pH measurements were done at room temperature. Bacterial growth was monitored by measuring the increase in optical density at 620 nm (Ultrospec 3000; Pharmacia Biotech). Acidification was studied with an API 20A test system (bioMérieux) after 2 days of incubation at the optimum growth temperature. All tests were repeated in triplicate.

Transmission electron microscopy was performed with a JEOL 100CX electron microscope. Samples were prepared by using the negative stain with uranyl acetate as described by Spurr (1969). Scanning electron microscopy was performed with a JSM-5600 LV microscope. Cells were harvested from a fresh liquid culture. The sample was prepared as described by Reynolds (1963).

Substrates and fermentation products were analysed in acidified and filtered samples by HPLC (JASCO) equipped with an HPX-87H ion-exchange column (Bio-Rad) at 55 °C and a refractive index detector. Sulphuric acid (5 mM) was used as a mobile phase (0.6 ml min⁻¹). Determination of carbon dioxide was performed by GC as described by Parawira *et al.* (2004). Hydrogen was analysed offline by GC (6890 Network GC system, Agilent Technologies). The GC was fitted with an 80/100 Hays Sep-N, 9 ft, 1/8 stainless steel column, a molecular sieve (60/80 Molesieve 5A, 6 ft, 1/8) and a thermal conductivity detector. Argon was used as the carrier gas at a flow rate of 46.6 ml min⁻¹. The column temperature was 60 °C and the injector and detector temperatures were 105 and 150 °C, respectively.

Chemical characterization of strain 45B^T, including the analysis of respiratory quinones, polar lipids and fatty acids, was carried out. Cells subjected to chemotaxonomic analysis were grown under the conditions as described for temperature and pH studies. Respiratory quinones and polar lipids were analysed by the Identification Service of the DSMZ and Dr B. J. Tindall, DSMZ, Braunschweig, Germany. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol/hexane (Tindall, 1990a, b), followed by phase separation into hexane. Polar lipids were extracted by adjusting the remaining methanol/0.3 % aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0.3 % aqueous NaCl mixture 1:2:0.8 (by vol.). The extraction solvent was stirred overnight and the cell debris was pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3 % aqueous NaCl mixture to a ratio of 1:1:0.9 (by vol.).

Respiratory lipoquinones were separated into their different classes (menaquinones, ubiquinones, etc.) by TLC on silica gel (Macherey-Nagel), using hexane/*tert*-butylmethylether (9:1, v/v) as solvent. UV absorbing bands corresponding to the different quinone classes (e.g. menaquinones or ubiquinones)

were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse phase column (Macherey-Nagel, 2 mm × 125 mm, 3 µm, RP18) using methanol/heptane (9:1, v/v) as the eluent. Respiratory lipoquinones were detected at 269 nm.

Polar lipids were separated by two-dimensional silica gel TLC (Macherey-Nagel). The first direction was developed in chloroform/methanol/water (65:25:4, by vol.), and the second was in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected using dodecamolybdo-phosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α -glycols), Dragendorff reagent (quaternary nitrogen) and α -naphthol-sulphuric acid (glycolipids) according to the procedure described by Tindall *et al.* (2007).

For determining the fatty acid profile, fatty acid methyl esters were prepared directly from cells of strain 45B^T, as described by Miller & Berger (1985) with minor modifications. NaOH (100 ml, 1.2 M) in 50 % methanol was added to 4 g freeze-dried cells; the mix was then vortexed and placed in boiling water for 30 min. Subsequently, and after cooling down to room temperature, 200 ml 6 M HCl in methanol (325:275, v/v) was added. The mixture was placed at 80 °C for 10 min. After cooling, 125 ml hexane/*tert*-butylmethylether (1:1, v/v) was added. The mixture was then agitated in a shaker (60 r.p.m.) for 10 min. The lower aqueous phase was discarded and 300 ml 0.3 M NaOH was added. The mixture was agitated for an additional 5 min. The lower aqueous phase was discarded and the same procedure was repeated. Subsequently, the upper phase was centrifuged at 15 300 g for 20 min to remove remaining colloidal particles. The supernatant was then concentrated to 2 ml under a nitrogen gas stream. Prior to the analysis, the sample was diluted 100-fold and filtered through a 0.2 µm polystyrene membrane. Fatty acid methyl ester analysis was carried out using a Varian 3400 GC system equipped with a flame-ionization detector and an automatic sampler. Factor four capillary column (15 m × 0.25 mm; Varian) was used to separate fatty acid methyl esters. The carrier gas was helium at 1.38×10^{-1} MPa. The temperature programme for separation was: initial temperature of 50 °C held for 2 min, then increased to 250 °C at 20 °C min⁻¹ and held for 3 min. Injector temperature was maintained at 270 °C and detector temperature was kept constant at 275 °C.

Genomic DNA was extracted as described by Marmur (1961). Purity and quantification of the DNA was measured by NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies). Amplification of the 16S rRNA gene was performed using Phusion polymerase (Finnzymes) and bacterial universal primers 8-27F and 1422R (Weisburg *et al.*, 1991). PCR products were purified using a QIAquick PCR Purification kit (Qiagen). The 16S rRNA gene was sequenced at GATC Biotech AG (Konstanz, Germany) and

analysed using Vector NTI 10 software (Invitrogen). The 16S rRNA gene sequence of strain 45B^T was submitted to GenBank/EMBL and similar sequences were found using the BLAST algorithm. To construct the phylogenetic tree, only sequences from members of species of the genera *Caloramator*/*Thermobrachium* with validly published names were taken into consideration. *Clostridium butyricum* DSM 2478^T (X68177.1), the type strain of *Clostridium sensu stricto*, was used as an outgroup.

Assays of genomic DNA G + C content (mol%) and DNA–DNA hybridization were carried out by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). DNA–DNA hybridization was performed between strain 45B^T and *Caloramator viterbiensis* DSM 13723^T. Strain 45B^T was cultured as described previously and the reference strain was cultured as recommended in the culture collection catalogue of the DSMZ.

For sample enrichment and primary isolation, AMM containing wheat straw was inoculated with 1 % (v/v) of the sample and incubated at 60 °C until ethanol was detected as a product. In addition, dilution series of this enrichment culture were inoculated in AMM supplemented with D-xylose (5 g l⁻¹). After subsequent transfers, 1 % (v/v) of the final culture was inoculated in the liquid phase of a biphasic medium containing D-xylose and incubated until turbidity was observed. Subsequently, the serum bottle was turned so that the surface of the solid phase was covered by the liquid for approximately 1 h. Then, the serum bottle was turned to its original position and incubated for 48 h. White, uniformly round, convex and mucous colonies of strain 45B^T (1.0–1.5 mm in diameter) were formed.

Single colonies were picked and suspended in about 30 ml AMM containing D-xylose (5 g l⁻¹). Cells (1 % v/v) were subcultured in liquid medium of the same composition to confirm the production of ethanol.

Strain 45B^T was Gram-variable; it stained Gram-negative during exponential phase and Gram-positive when the stationary phase was reached. Cells of strain 45B^T were terminal spore-forming rods of approximately 2.5 µm long and 0.5 µm wide (Fig. 1a). Cells occurred singly or in chains and were monotrichously flagellated (Fig. 1b).

The substrates utilized by strain 45B^T included xylose, cellobiose, glucose, arabinose, sucrose, lactose, maltose, fructose, galactose, mannose, glycerol, xylan, carboxymethylcellulose and yeast extract.

The primary end products formed during D-xylose and cellobiose fermentation included ethanol, acetate, lactate, propionate, carbon dioxide and hydrogen. Fermentation of D-xylose and cellobiose yielded ethanol as the main product (0.70 and 0.45 g ethanol per gram of sugar consumed, respectively). The pattern obtained suggests a conversion according to the following equations.

The fermentation balance when 0.73 mM D-xylose (equation 1) and 11.7 mM cellobiose (equation 2) were

Fig. 1. Electron micrographs of strain 45B^T. (a) Scanning electron micrograph of cells of strain 45B^T showing swollen ends (reminiscent of prespores) and bulging sections in the middle of elongated cells; bar, 2 μm. (b) Transmission electron micrograph of strain 45B^T, negatively stained, showing monotrichous flagellum; bar, 0.5 μm.

consumed by strain 45B^T grown to the stationary phase is described as follows (in mol quantities):

Strain 45B^T grew strictly anaerobically at 45–65 °C, with optimum growth at 60 °C. No growth was observed at temperatures above 65 °C after 3 weeks of incubation (Fig. 2a). The pH range for growth was 5.5–8.0, with an optimum pH between 6.5 and 7.0. No growth was detected at pH 5.0 or 9.0 (Fig. 2b). The shortest doubling time under optimal growth conditions was 1.4 h.

Acid was produced by strain 45B^T from D-glucose, D-mannitol, lactose (bovine origin), sucrose, maltose, salicin, D-xylose, L-arabinose, gelatin (bovine origin), aesculin, ferric citrate, glycerol, cellobiose, D-mannose, melezitose, raffinose, D-sorbitol, L-rhamnose and trehalose, but it is not produced from L-tryptophan.

Chemotaxonomic analysis revealed the absence of respiratory quinones in strain 45B^T. Strain 45B^T possessed a rather complex polar lipid profile as shown by TLC (Fig. S1, available in IJSEM Online). Nineteen resolved lipid spots included diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an aminoglycolipid, 11 other unidentified phospholipids, three unidentified aminolipids and one unidentified lipid. Fatty acid analysis of strain 45B^T showed a complex profile in which saturated and branched acids predominated; anteiso-C_{15:0} was found to be the major compound (35 %) and other branched acids such as iso-C_{14:0} and iso-C_{16:0} were found in minor amounts (7 and 10 %, respectively). The saturated acids detected were C_{13:0} (6 %), C_{14:0} (4 %) and C_{16:0} (16 %). The only unsaturated acid found in strain 45B^T was C_{15:1} (10 %).

The almost complete 16S rRNA gene sequence of strain 45B^T, comprising 1437 nt, was determined. Phylogenetic analysis showed that strain 45B^T clustered together with the type strain of *Caloramator viterbiensis* (Seyfried *et al.*, 2002) as a distinct lineage within the radiation of the previously described genera *Caloramator*/*Thermobrachium* (Fig. 3).

The 16S rRNA gene sequence of strain 45B^T had 91.8–99.4 % similarities to those of other members of the same genus (Table S1), and had 86.6 % similarity with *Clostridium butyricum* DSM 2478^T 16S rRNA gene sequence. Multiple alignment was performed by using the programs CLUSTAL W and MUSCLE and the default settings for the 16S rRNA gene alignment excluded smaller final blocks, gap positions within the final blocks and less strict flanking positions and included contiguous non-conserved positions, if any. The phylogenetic tree was constructed from evolutionary distances using the neighbour-joining method. Tree topology was examined by bootstrap analyses on the branches (Fig. 3) and it clearly supports the close relationship between strain 45B^T and the type strain of *Caloramator viterbiensis* and their distinct lineage within the *Caloramator*/*Thermobrachium* cluster. 16S rRNA gene sequence analysis indicated that strain 45B^T was most closely related to *Caloramator viterbiensis* (99.4 % similarity to the type strain) (Table S1).

In spite of the high similarity of the 16S rRNA gene sequences of strain 45B^T and *Caloramator viterbiensis* DSM 13723^T (>99 %), the DNA–DNA relatedness between them both was only 19.5 %. This value is lower than the recommended value (70 %) required to recognize them as members of the same species (Wayne *et al.*, 1987). The genomic DNA G + C content of strain 45B^T was 32.6 mol %.

The described strain, 45B^T, was isolated based on its ability to ferment D-xylose and cellobiose to ethanol as main

Fig. 2. Effect of temperature (at pH 6.5; a) and pH (at 60 °C; b) on growth of strain 45B^T. t_d, Doubling time.

fermentation product, which is the most remarkable difference between this strain and other members of the genera *Caloramator*/*Thermobrachium*. However, it has been reported that some members of this group produce minor quantities of ethanol from glucose (Collins *et al.*, 1994; Patel *et al.*, 1987; Plugge *et al.*, 2000; Seyfried *et al.*, 2002; Chrisostomos *et al.*, 1996; Tarlera *et al.*, 1997; Ogg & Patel, 2009; Engle *et al.*, 1996).

Most strains of the genera *Caloramator*/*Thermobrachium* produce acetate and minor quantities of ethanol, lactate, propionate, butyrate, formate, valerate, carbon dioxide and hydrogen as end products of the fermentative metabolism of carbohydrates. Moreover, strain 45B^T produces significant amounts of ethanol and acetate, lactate, propionate, carbon dioxide and hydrogen as minor products. Since 45B^T was primarily isolated using wheat straw and, based on the fact that it utilizes various substrates, it may have a wide range of saccharolytic activities, which could be used for direct microbial conversion of lignocellulosic material into ethanol.

In addition, the conversion of glycerol to 1,3-propanediol has been demonstrated for strain 45B^T in liquid medium (AMM); this activity was enhanced by supplementation with yeast extract. This ability has also been reported for its closest relative, *Caloramator viterbiensis* (Seyfried *et al.*, 2002). The ability to utilize glycerol has not yet been studied for other strains from the genera *Caloramator*/*Thermobrachium*.

The temperature range for growth of strain 45B^T is more similar to those of *Caloramator coolhaasii* and *Caloramator viterbiensis* than those of other *Caloramator* species. However, since strain 45B^T has an optimum growth temperature around 60 °C, it is considered to be a moderately thermophilic bacterium.

The shortest doubling time for growth of strain 45B^T under optimum conditions with cellobiose as a substrate was found to be around 1.4 h, which is half of the doubling time reported for *Caloramator viterbiensis*, its closest relative (Seyfried *et al.*, 2002). Moreover, this value is

Fig. 3. Phylogenetic dendrogram based on 16S rRNA gene sequence comparisons. The neighbour-joining tree was reconstructed from distance matrices. Bootstrap values (bootstrap trials, 1000; seed 111; values given as percentages) are shown at branch points. Bar, number of nucleotide substitutions per 100 residues.

several times higher than the doubling times reported for other members of the *Caloramator*/*Thermobrachium* cluster and other taxa of the genus *Clostridium* cluster I.

The formation of spores and/or thermoresistant cells has been demonstrated for two species of *Caloramator* (*Caloramator fervidus* and *Caloramator proteoclasticus*) and for strain 45B^T. In contrast, *Caloramator viterbiensis*, *Caloramator australicus*, *Caloramator coolhaasii* and *Caloramator indicus* lack this ability.

The presence of peritrichous flagella has only been reported for *Thermobrachium celere*, *Caloramator proteoclasticus* and *Caloramator australicus*. In contrast, strain 45B^T possessed a monotrichous flagellum. Other members of the genera *Caloramator*/*Thermobrachium*, including *Caloramator viterbiensis*, lack flagella.

Lipid composition data for strain 45B^T revealed the absence of respiratory lipoquinones. In a previous study carried out by Yamamoto *et al.* (1998), menaquinone MK-7 was found in *Caloramator fervidus*; however, no other reports of quinone composition in *Caloramator*/*Thermobrachium* species are available so far. Although the results obtained in this study revealed the absence of quinones, as in most clostridia and/or related genera, further systematic studies are necessary before considering quinone analyses as a taxonomic tool for this particular genus. Examination of the polar lipid profile of strain 45B^T revealed some unusual features, including the absence of glycolipids and the presence of several unidentified phospholipids. The presence of glycolipids is very significant for saccharolytic bacteria, e.g. *Clostridium thermocellum*, since glycolipids on the surface serve for recognition and/or binding to substrates (e.g. cellulose, xylan) (Herrero *et al.*, 1982). An interesting physiological feature of strain 45B^T is the ability to utilize a wide spectrum of substrates in spite of the absence of glycolipids. In common with other Gram-positive bacteria, strain 45B^T contains PE, DPG and PG. Aminophospholipids such as PE and their respective plasmalogens are major phospholipids of the saccharolytic, butyric-acid-producing clostridia, including *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum* and related species (Johnston & Goldfine, 1983).

Chemotaxonomic analyses revealed the predominance of iso-branched fatty acids, in particular iso-C_{15:0}, in thermophilic clostridia, which have been reclassified into new genera (Yamamoto *et al.*, 1998). Moreover, it was found that iso-C_{15:0} is the major fatty acid present in *Caloramator fervidus*. In contrast, the major cellular fatty acid found in strain 45B^T was anteiso-C_{15:0}.

Phylogenetic analysis (Fig. 3) showed that strain 45B^T is most closely related to the type strains of *Caloramator viterbiensis* and other members of the *Caloramator* cluster including *Thermobrachium celere*. The *Caloramator* cluster forms part of the Gram-type-positive *Bacillus*–*Clostridium* branch and is most closely related to cluster I of the *Clostridium* branch. All species of the *Caloramator* cluster

are thermophilic, chemo-organoheterotrophic, anaerobic bacteria exhibiting low DNA G+C content, which are also characteristics of strain 45B^T. Table 1 shows a comparison of the morphological and physiological features of all *Caloramator*/*Thermobrachium* species.

The DNA G+C content determined for strain 45B^T (32.6 mol%) is close to that of *Thermobrachium celere*, *Caloramator viterbiensis*, *Caloramator proteoclasticus* and *Caloramator coolhaasii* (31–32 mol%), whereas the values reported for *Caloramator indicus*, *Caloramator australicus* and *Caloramator fervidus* differ significantly (25, 34 and 39 mol%, respectively); this may be due to the different methods used for determination.

Although strain 45B^T is related to other *Caloramator*/*Thermobrachium* species and has similar physiological properties, it shows certain characteristics that differ significantly from those of the type strains of species of the genera *Caloramator*/*Thermobrachium* (Table 1).

The differences in morphological and physiological properties and the low level of 16S rRNA gene sequence similarity of strain 45B^T suggest that this strain can be classified as a representative of a novel species of the genus *Caloramator*.

Description of *Caloramator boliviensis* sp. nov.

Caloramator boliviensis (bo.li.vi.en'sis. N.L. masc. adj. *boliviensis* of or belonging to Bolivia, the country from which the organism was first isolated).

Cells are straight to slightly curved spore-forming rods, approximately 2.5 µm long and 0.5 µm wide. Cells are Gram-stain-variable and occur singly or in chains. Cells are strictly anaerobic and motile by means of a monotrichous flagellum. White, uniformly round, convex and mucous colonies (1.0–1.5 mm in diameter) form after 48 h on biphasic AMM. Growth is observed on xylose, cellobiose, glucose, arabinose, sucrose, lactose, maltose, fructose, galactose, mannose, glycerol, xylan, carboxymethylcellulose and yeast extract. Fermentation of xylose and cellobiose yields ethanol as the main end product (0.70 or 0.45 g ethanol per gram of xylose or cellobiose consumed, respectively). Acetate, lactate, propionate, carbon dioxide and hydrogen are also produced in minor quantities. Glycerol is converted to 1,3-propanediol when AMM is supplemented with yeast extract. Acid is produced from D-glucose, D-mannitol, lactose (bovine origin), sucrose, maltose, salicin, D-xylose, L-arabinose, gelatin (bovine origin), aesculin ferric citrate, glycerol, cellobiose, D-mannose, melezitose, raffinose, D-sorbitol, L-rhamnose and trehalose. The shortest doubling time (1.4 h) occurs at 60 °C and pH 6.5, using cellobiose as a sole carbon and energy source. Polar lipids such as DPG, PG, PE, an aminoglycolipid plus 15 other unidentified lipids, and fatty acids including anteiso-C_{15:0}, C_{16:0}, iso-C_{16:0}, C_{15:1}, iso-C_{14:0}, C_{13:0} and C_{14:0} are predominant.

Table 1. Characteristics differentiating strain 45B^T from its phylogenetic relatives

Taxa: 1, strain 45B^T; 2, *Caloramator viterbiensis* JW/MS-VS5^T (Seyfried *et al.*, 2002); 3, *Caloramator fervidus* RT4-B1^T (Patel *et al.*, 1987); 4, *Caloramator indicus* IndiB4^T (Chrisostomos *et al.*, 1996); 5, *Caloramator proteoclasticus* U^T (Tarlera *et al.*, 1997); 6, *Caloramator coolhaasii* Z^T (Plugge *et al.*, 2000); 7, *Caloramator australicus* KCTC 5601^T (Ogg & Patel, 2009); 8, *Thermobrachium celere* JW/YL-NZ35^T (Engle *et al.*, 1996). NR, Not reported.

Characteristic	1	2	3	4	5	6	7	8
Shortest doubling time (h)	1.40	2.80	0.75	0.33	0.50	1.00	NR	0.17
Growth temperature (°C)								
Range	45–65	33–64	37–80	37–75	30–68	37–65	45–70	43–75
Optimum	60	58	68	60–65	55	50–55	60	66
Growth pH								
Range	5.5–8.0	5.0–7.8	5.5–9.0	6.2–9.2	6.0–9.5	6.0–8.5	5.0–9.0	5.4–9.5
Optimum	6.5–7.0	6.0–6.5	7.0–7.5	7.5–8.1	7.0–7.5	7.0–7.5	7.0	8.2
DNA G + C content (mol%)	33	32	39	26	31	32	34	31
Spores or heat-resistant cells	+	–	+	–	+	–	+	–
Flagella*	M	–	–	–	P	–	P	P
Utilization of:								
Xylose	+	–	+	NR	–	+	+	–
Cellobiose	+	+	NR	+	+	+	+	–
Glycerol	+	+	NR	NR	NR	NR	–	–
Ethanol production	+	+	+	+	+	+	+	+

*M, Monotrichous; P, peritrichous.

The type strain, 45B^T (=DSM 22065^T=CCUG 57396^T), was isolated from Chaqui hot spring located in Potosi, Bolivia. The DNA G+C content of the type strain is 32.6 mol%.

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