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ORIGINAL PAPER

Thermoanaerobacterium thermostercus sp. nov., a new anaerobic thermophilic hydrogen-producing bacterium from buffalo-dung

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Abstract A novel thermophilic, anaerobic, rod-shaped bacterium strain, designated Buff, was isolated from buffalo-dung samples collected from a buffalo-farm located in Caserta (Campania, south of Italy). Strain Buff was Gram-positive, motile and no spore-forming. The growth temperature range was 40–65°C with an optimum at 60°C, while pH growth range at 60°C was 5.5–8.0 with an optimum at about pH 6.5. NaCl growth concentration ranged from 0 to 2.0% with an optimum at 0.5% (w/v); no growth was observed with the presence of NaCl 3.0% (w/v). The strain produced ethanol, acetate, lactate, H_2, H_2S and CO_2 by glucose fermentation. The DNA G + C content was 34.4 mol%. As determined by 16S rRNA sequence analysis, this organism belonged to the genus *Thermoanaerobacterium*. On the basis of the physiological and molecular properties, we propose for strain Buff the new species designation *Thermoanaerobacterium thermostercus* sp. nov. This novel organism represents the first species of the genus *Thermoanaerobacterium* isolated from buffalo-dung. The type strain is Buff (=DSM 22141 = ATCC BAA-1776).

Keywords *Thermoanaerobacterium* · Buffalo-dung · Thermophilic · Hydrogen production · Phylogeny

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16S rRNA gene sequence of *Thermoanaerobacterium thermostercus* is available in the GenBank/EMBL/DDBJ databases under the accession number FM999998.

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Introduction

In the past two decades, due to the biotechnological potential of thermophilic microbes and their thermostable biomolecules, intensive research focused on the anaerobic, thermophilic, carbohydrate-fermenting microorganisms has led to the description of new genera and species in the domains *Archaea* and *Bacteria*. Hyperthermophilic or thermophilic microorganisms were isolated from various environments such as terrestrial hot springs, shallow and deep-sea hydrothermal vents, subsurface petroleum reservoirs, manures and faeces (Cann et al. 2001). In particular, regarding the thermophilic *Bacteria* domain, attention has been paid to the members of the family *Thermoanaerobiaceae*, which includes the genera *Thermoanaerobacter* and *Thermoanaerobacterium*. Concomitantly to the improvement of phylogenetic analyses, the taxonomic relationships between these thermophilic genera have been better defined (Lee et al. 1993; Rainey et al. 1993; Collins et al. 1994). These microorganisms are relevant for their abilities to bioconvert various substrates to end products, such as hydrogen and ethanol, and/or compounds with potential utility in the production of fuels and bulk chemicals. Moreover, several thermostable enzymes have been purified and cloned from the members of this bacterial group, for example the polysaccharide-hydrolysing enzymes

endoxylanases (Lee et al. 1993; Liu et al. 1996) used in biomass conversion and paper industry.

Within the genus *Thermoanaerobacterium*, at the time of writing, 8 species have been isolated (Lee et al. 1993; Collins et al. 1994; Liu et al. 1996; Cann et al. 2001; Kublanov et al. 2007) and more recently the taxonomic relationships between some of these species have been better defined (Lee et al. 2008). In our microbiological investigations carried out under an Italian framework project (F.I.S.R. D.M. 17/02/2002—Metodologie innovative per la produzione di idrogeno da processi biologici) focused on the development of strategies to obtain biohydrogen from thermophilic microorganisms, we isolated from buffalo-dung a new strain, designated as Buff. This bacterium, on the basis of the phenotypic characteristics and of the phylogenetic comparisons, should be classified as a new member of the genus *Thermoanaerobacterium*. In this paper we describe its isolation and characterization as a novel thermophilic, anaerobic, fermentative hydrogen/ethanol-producing bacterium. The name proposed for this new species is *Thermoanaerobacterium thermostercus*.

Materials and methods

Sample site

A buffalo-farm located in Caserta (Campania, south of Italy) was selected as the sample site for our study. Samples of buffalo-dung were collected aseptically in screw-cap flasks and transported to the laboratory. The samples were stored at 4°C until their use as inoculum for the enrichment medium.

Reference strains

Thermoanaerobacterium thermosaccharolyticum DSM 571^T, *Thermoanaerobacterium saccharolyticum* DSM 7060^T, *Thermoanaerobacterium aciditolerans* DSM 16487^T and *Thermoanaerobacterium aotearoense* DSM 10170^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultured routinely using media and conditions recommended by DSMZ.

Enrichment, isolation and growth conditions

Routine growth was performed using a culture medium containing (g/L): NaCl 10.0; KCl 0.1; $MgCl_2 \cdot 6H_2O$ 0.2; NH_4Cl 1.0; K_2HPO_4 0.3; KH_2PO_4 0.3; $CaCl_2 \cdot 2H_2O$ 0.1; cysteine-HCl 1.0; yeast extract 2.0; tryptone 2.0; glucose 5.0; resazurin 0.001; before sterilization, the pH was adjusted at 6.5 with 1 M NaOH at room temperature. After sterilization, the medium was also supplemented with 10 mL/L of both filter-sterilized vitamins and trace element solutions from DSM medium 141. Aliquots (25 mL) of culture medium were dispensed into 120-mL serum bottles. Oxygen was removed by heating the batch-reactors until the solution was colourless and then flushing the medium under a stream of O_2-free N_2 gas. Then bottles were immediately capped with butyl rubber stoppers and sterilized by autoclaving for 5 min at 121°C. Fresh medium was inoculated with 10% (v/v) of buffalo-dung samples and incubated at 50, 60 and 70°C without agitation until visible growth was observed. The hydrogen-producing cultures were isolated through several serial liquid dilutions and streaked on gelrite 1% (w/v) medium plates for obtaining a uniformly pure culture. The incubation was performed in a stainless steel anaerobic jar for 72 h under a 5% H_2, 5% CO_2 and 90% N_2 (v/v/v) gas atmosphere. All transfers and culture sampling were performed using sterile syringes and needles and the bacteria were stored in liquid culture under anaerobic conditions at 4°C. Several anaerobic hydrogen-producing strains were selected and taxonomic properties of one of these, strain Buff, will be presented in this paper.

Morphological, physiological and biochemical tests

For the new isolate the optimum parameters for growth and hydrogen production were determined in the culture medium above-described by ranging the temperature from 40 to 80°C, the pH from 5.0 to 9.0 (determined at room temperature) and the salt from 0 to 5.0% (w/v). Gaseous end products (H_2 and CO_2) were routinely analysed using a gas chromatograph 4200 Carlo Erba equipped with a 3 m Hayesep Q column. The temperatures of the thermal conductivity detector, injector and analytic column were 200, 120 and 60°C, respectively. The carrier gas was nitrogen at a flow rate of 30 mL/min; H_2 and CO_2 were quantified using a calibration curve made by means of pure gaseous products. Bacterial growth was directly monitored in a UV/Vis spectrophotometer DU 730 (Beckman Coulter) by utilizing the change in optical density at 540 nm. Colony morphology was analysed on solid culture medium with a stereomicroscope (Leica Wild M8), while cell morphology was examined during exponential-growth phase by phase contrast microscopy (Nikon Eclipse E400) at a magnification of 40×. Gram reaction was performed assaying aminopeptidase activity by Bactident-Merck and by the KOH lysis method according to Halebian et al. (1981). Motility studies were performed using the aforementioned culture medium solidified with agar 0.4% (w/v) and the tubes were incubated in a stainless steel anaerobic jar for 72 h. For spore formation test, enrichment medium plus $MnCl_2 \cdot 4H_2O$ 0.001% (w/v) was used. The heat-resistance

of cells was determined by heating cultures at 100°C for 30 min and sub-culturing into fresh standard medium (inoculum 20% v/v). These resulting cultures were incubated for 72 h at 60°C and then tested for hydrogen production (Xue et al. 2001). To test the effects on growth and hydrogen production by various electron acceptors, sulphur (0.1% w/v), sodium thiosulphate (20 mM) or sodium hydrosulphite (20 mM) were added to standard culture medium (without cysteine). The utilization by strain Buff of these electron acceptors was monitored by measuring growth rate and hydrogen and H_2S productions. H_2S formation was detected by adding 500 µL of 5 mM $CuSO_4{\cdot}5H_2O$–50 mM HCl solution to 200 µL of the culture; also the gas from the headspace (10 mL) was analyzed using the same method. The sulphide presence was monitored by the formation of a dark-brown precipitate, while microscopic examination was used to check for sulphur formation.

The ability of the strain to utilize different carbon sources for growth and hydrogen production was investigated in the culture medium above described, but deprived of glucose, by adding, at a final concentration of 5 g/L, one of the following compounds: fructose, galactose, mannose, arabinose, rhamnose, xylose, lactose, maltose, trehalose, cellobiose, sucrose, raffinose, xylan, starch (from potato), inulin (from dahlia tubers), molasses, glycerol and pyruvate. Growth and substrate utilization were determined by optical density and hydrogen production. The amounts of ethanol, lactic and acetic acids produced during growth were quantified by ^{13}C- and ^{1}H-Nuclear Magnetic Resonance (N.M.R.) spectra obtained at 300 and 400 MHz, respectively. Ethanol tolerance was determined in the standard culture medium supplemented with ethanol (from 0 to 5.0%, v/v) after autoclaving. To test antibiotic sensitivity, Sensi-discs (6 mm, Oxoid) of erythromycin (5 and 30 µg), fusidic acid (10 µg), chloramphenicol (10 and 50 µg), lincomycin (15 µg), streptomycin (25 µg), ampicillin (25 µg), vancomycin (30 µg), novobiocin (30 µg), neomycin (30 µg), gentamicin (30 µg), kanamycin (30 µg), tetracycline (30 and 50 µg), penicillin G (2 and 10 units), bacitracin (10 units) and nystatin (100 units) were used in 1% (w/v) gelrite plate cultures (Romano et al. 1993). Plates were incubated in a stainless steel anaerobic jar for 48 h. Gelatin hydrolysis was evaluated after 48 h of incubation by examining culture medium supplemented with 12% (w/v) gelatin. For nitrate and nitrite reduction, the culture medium plus 0.1% (w/v) or 0.001% (w/v) of KNO_3 and $NaNO_2$, respectively, was utilized according to Romano et al. (1996). All the tests were performed in triplicate and all the samples were incubated at 60°C. For xylanase and β-xylosidase enzymatic activity determination, the cells were grown in a standard culture medium, collected during stationary growth phase by centrifugation at 9000*g* for 30 min, re-suspended in phosphate buffer 20 mM pH 7.0 and lysed by ultrasonic treatment (Heat System Instruments) for 4 min. Crude extract was assayed as prescribed by Lama et al. (2004).

16S rRNA studies

Total RNA was extracted by the RiboPure-Bacteria kit (Ambion) following the manufacturer's instructions. RNA was dissolved in RNA-storage-solution (Ambion), UV-quantified by a Bio-Photometer® (Eppendorf) and stored at −80°C. To remove contaminating genomic DNA, RNA aliquots (3 µg) were digested by RNAse-free DNAse I (included in the kit) in a 20 µL final volume. After DNAse I digestion, the concentration and purity of rRNA were evaluated by the RNA-6000-Nano® microchip assay, using a 2100 Bioanalyzer® equipped with a 2100-Expert-Software® (Agilent), following the manufacturer's instructions (RNA-Integrity-Number > 7). First-strand cDNA synthesis was performed using an iQ5® (Bio-Rad) thermocycler as follows: 10 µL containing 1 µg of RNA, 2 pmol of *Escherichia coli* 1517-reverse general sequencing primer and 2 mM dNTPs were incubated for 2 min at 70°C and quickly cooled to 4°C. 10 µL containing 2× of a suitable buffer, 20 mM dithiothreitol, 20 units of RNAse inhibitor (Invitrogen) and 200 units of MoMuLV Superscript® III reverse transcriptase (Invitrogen) were added and the resulting mixture was incubated at 55°C for 60 min and then at 75°C for 15 min. Amplification of 16S cDNA was performed by the iQ5® thermocycler in a 50-µL reaction mixture containing: 1× of a suitable buffer, 200 µM dNTPs, 300 nM each of 8-forward and 1517-reverse 16S *E. coli* general sequencing primers and 2.5 units of *High-Fidelity-Platinum*-Taq (Invitrogen). The amplification profile was as follows: initial denaturation of 3 min at 94°C, 25 cycles of 30 s at 94°C, annealing for 30 s at 57°C, elongation for 1 min at 72°C and final elongation of 7 min at 72°C. Amplicon size and purity were evaluated by electrophoresis on 1% (w/v) agarose gels (Agarose-1000, Invitrogen). Sample purification and sequencing were performed at MWG-Eurofin (Ebersberg-Germany) using the primers 8F, 1517R and an internal primer designed on the basis of 8F sequencing data. Sequence alignment and counting were performed by the DNA-Baser v. 2.0 software (Cubic Designer, http://www.dnabaser.com).

The 16S rRNA gene sequence of strain Buff was 1366 nt. in length. This sequence was compared with public sequences in the EMBL gene databases using the BLAST program (National Center for Biotechnology Information; http://ncbi.nlm.nih.gov/) to calculate its phylogenetic position. Multiple sequence alignments were obtained using CLUSTAL_X (Thompson et al. 1997). Phylogenetic analyses were performed using the program

PAUP* v. 4.0b10 (Swofford 2002). Neighbour-Joining (NJ) with Kimura-2-parameters correction (Kimura 1980), Maximum parsimony (MP) with a heuristic search option and Maximum likelihood (ML) analyses were performed. For ML, the optimal model of nucleotide substitution was estimated by the program MODELTEST 3.7 (Posada and Crandall 1998; Posada and Buckley 2004) using the Akaike Information Criterion (AIC). All bootstraps analyses were performed using 1000 replications.

DNA–DNA hybridization experiments, REP and RAPD DNA fingerprint and G + C DNA content

DNA was extracted and purified from bacterial cell cultures (about 250 mg of dry pellet) using the Genomic-DNA-Buffer Set and Genomic-tip-100/G columns (QIAGEN SpA, Italy), according to manufacturer's instructions. DNAs were dissolved in Tris 10 mM pH 8.0, 1 mM EDTA and were serial diluted to a final concentration of 50 μg/mL as evaluated by UV-absorbance and fluorimetric assays (Quant-iT DNA, Invitrogen). DNA size was estimated by electrophoresis through a 0.8% (w/v) DNA-grade agarose (Bio-Rad) gel using λ DNA as a molecular weight marker. DNA–DNA filter hybridization and per cent sequence homology evaluation were performed as previously described (Romano et al. 2007).

PCR-amplification of interspersed-repetitive-sequences (REP-PCR fingerprint) was performed using the GTG_5 primer as described by Versalovic et al. (1994); random-amplified-polymorphic-DNA-PCR (RAPD-PCR) was performed using the OPR2 primer as prescribed by Ronimus et al. (1997). The PCR-mixtures were amplified using an iQ5 thermocycler (Bio-Rad) with 2.5 units of *Platinum*-Taq DNA polymerase (Invitrogen). PCR products were analysed by electrophoresis on microchip using the DNA 7500 kit (Agilent) and a 2100-Bioanalyzer equipped with 2100 EXPERT software (Agilent), following the manufacturer's instructions.

The G + C content was evaluated as previously described (Poli et al. 2009) on the basis of the DNA thermodenaturation profile and the melting point temperature (T_M) evaluation. Briefly, DNA samples, purified as described earlier, were subjected to the melting point iQ5 standard protocol in a reaction mixture containing in a final volume of 25 μL: Tris 10 mM pH 8.0, 1 mM EDTA, 20 mM KCl, 1× of the fluorescent DNA-intercalating dye EVA-green (Biotium) and 200 ng of DNA. Determinations were performed in quadruplicate in a 96-well plate sealed by an optical tape (Bio-Rad). Well factor (FAM channel) was obtained from a replicate plate containing the mixture without DNA, while the experimental plate was inserted in the iQ5 apparatus during a hold step at 37°C. The incubation at 37°C was resumed for further 20 min, followed by a melting protocol from 50 to 96°C in 0.2°C steps, dwell time 15 s and acquisition of fluorescence data for each step. The fluorescence data were analysed by the iQ5 software and exported to the "Melting Profiler" v. 0.7 software (Bio-Rad) for evaluation of T_M. Bacterial DNA samples of known G + C content, ranging from 30 to 60%, were included for a standard curve G + C versus T_M design (linear correlation coefficient 0.999%) and the G + C content of the unknowns was extrapolated from the curve.

Results

Morphological, biochemical and phylogenetic analyses

An anaerobic thermophilic hydrogen-producing culture was obtained by inoculating a standard culture medium with samples of buffalo-dung. Cell growth was observed after incubation at 60°C for 48 h and microscopic examination revealed bacterial populations composed of rods. The strain Buff was the predominant organism in the enrichment culture medium and it was the only colony-forming organism at the highest dilutions.

Comparisons of 16S rRNA gene sequences (1366 nt.) revealed that strain Buff was a member of the genus *Thermoanaerobacterium*. Its closest phylogenetic relatives in terms of sequence similarity were *T. aotearoense* (98.9%), *T. aciditolerans* (97.9%), *T. thermosaccharolyticum* (97.8%), *T. saccharolyticum* (97.6%), *T. xylanolyticum* (97.2%) and *T. thermosulfurigenes* (96.4%) (Table 1). The interspecies distances obtained between the species agree with the interspecies distances previously described by Liu et al. (1996) for this genus, ranging from 99.3 to 97.5% of sequence similarities. The phylogenetic tree obtained with NJ was shown in Fig. 1. The trees obtained using MP and ML tree-building methods displayed similar topology (data not shown). Both the high bootstrap values and the data from comparisons of the local topologies, obtained using several treeing methods, confirm the phylogenetic position of strain Buff.

Optimal temperature for growth and hydrogen production was 60°C, while no growth was observed below 40°C or above 65°C; moreover, growth and hydrogen production occurred in the presence of NaCl concentration from 0 to 2.0% with an optimum at 0.5% (w/v) and an optimum pH of 6.5. No growth was observed with NaCl 3.0% (w/v).

After 3 days of incubation at 60°C, the colony area appeared very small (about 1 mm in diameter), white, uniformly round and with a glossy surface. In liquid medium, in the exponential growth phase, the strain formed straight, motile, rod-shaped cells, both singly and in pairs. Chain formation was rarely observed.

Table 1 Values of 16S rRNA similarity, DNA–DNA hybridization and G + C content for strain Buff and its closest phylogenetic relatives

Species	16S rRNA (%)	DNA–DNA (%)	G + C (mol%)
T. thermostercus	100	100	34.4
T. aotearoense	98.9	18	34.5
T. aciditolerans	97.9	14	34
T. thermosaccharolyticum	97.8	34	ND
T. saccharolyticum	97.6	23	36
T. xylanolyticum	97.2	ND	36.1
T. thermosulfurigenes	96.4	ND	32.6

ND not determined

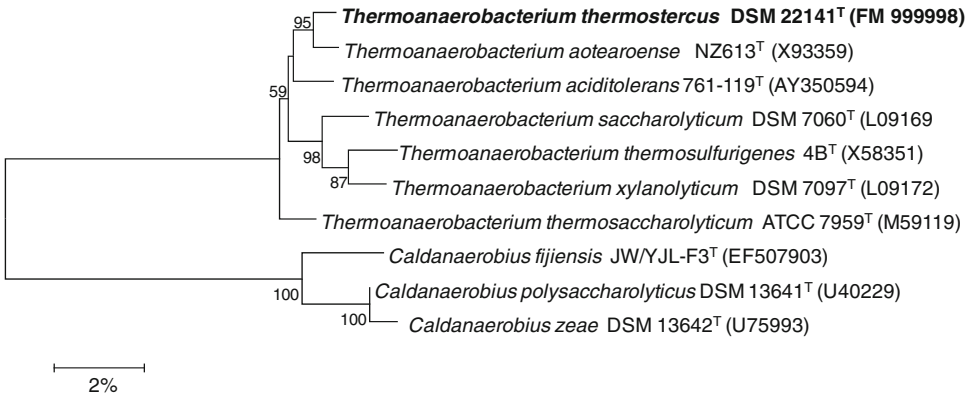

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1366 nt.) showing the phylogenetic position of *Thermoanaerobacterium thermostercus*. *Bar* 2% substitutions in nucleotide sequence. Bootstrap values >50% confidences are shown at branching points (percentage of 1000 resamplings). Sequence accession numbers are given in *parentheses*

In the culture medium plus Mn^{2+}, no spores were observed under light microscopy examination after 24, 48 and 72 h of incubation. When strain Buff cultures were autoclaved and sub-cultured, growth was observed after 3 days of incubation at 60°C, thus suggesting the presence of heat-resistant forms.

Strain Buff was able to reduce thiosulphate, sodium hydrosulphite and elemental sulphur to hydrogen sulphide; moreover, thiosulphate was reduced also to elemental sulphur (S^0) globules. Although S^0 started to form during the late exponential growth phase, the majority of the sulphur globules appeared at the end of the stationary growth phase. When Buff grew on glucose (5 g/L) in the presence of cysteine-HCl (1 g/L), the fermentation products were H_2 (9.1 mM), CO_2 (3.3 mM), H_2S (2.3 mM), ethanol (15.7 mM), acetate (10.5 mM) and lactate (7.4 mM). Instead, in the presence of glucose (5 g/L) and thiosulphate (20 mM), the fermentation products were H_2 (6.1 mM), CO_2 (3.2 mM), H_2S (9.7 mM), ethanol (5.9 mM), acetate (23.5 mM) and lactate (9.8 mM). So in this case, H_2S was the main gaseous product, while, in the presence of cysteine, a reduced growth-rate of H_2S was observed and H_2 was the main gas produced.

The strain Buff was a chemoorganotrophic bacterium able to utilize only a few carbohydrates for growth in comparison with its phylogenetic relatives (Table 2). Little growth was observed both on a yeast extract-tryptone medium (or yeast extract-peptone) without a fermentable carbon source and on a glucose medium without yeast extract and tryptone. These results indicated that for the optimal growth the presence of yeast extract, tryptone and a fermentable carbohydrate was required. During growth on glucose or other fermentable sugars, the pH of the medium decreased from the initial optimum pH to about 4.0. This pH reduction was attributable to the accumulation of organic acids during fermentation. Moreover, Buff was able to grow in the presence of 2.0% (v/v) ethanol. Gelatin was not hydrolyzed, while both nitrate and nitrite reduction was observed.

Lincomycin, vancomycin, tetracycline, neomycin, chloramphenicol, bacitracin and fusidic acid completely inhibited Buff growth. Finally, strain Buff exhibited also xylanase and β-xylosidase enzymatic activities (data not shown).

In consideration of the high 16S rRNA similarity within the *Thermoanaerobacterium* genus, we validated DNA–DNA hybridization data by REP-PCR and RAPD-PCR fingerprints using the GTG_5 (Fig. 2a) and OPR2 primers (Fig. 2b), respectively. Both fingerprint primers were able to discriminate the *Thermoanaerobacterium* species tested and produced relevant, distinct DNA-bands of high molecular weight. Fingerprint profiles of strain Buff were clearly different from that produced by its closest relatives.

The G + C content of Buff-DNA was 34.4 mol% as evaluated on the basis of T_M determination. This value was comparable to G + C content described in literature for the closest phylogenetic relatives (Table 1). In the same table the corresponding DNA–DNA hybridization values were

Table 2 Salient features of strain Buff in comparison to its closest phylogenetic relatives

Characteristic	1	2	3	4
Origin				
Place	NR	North Island (New Zealand)	Kamchatka (Russia)	Caserta (South Italy)
Environment	Canned food	Hot spring	Hot spring	Dung stock-site
Sample	NR	Water and sediment	Water and sediment	Buffalo-dung
Growth conditions				
pH range	6.5–8.5	3.8–6.8	3.2–7.1	5.5–8.0
pH optimum	7.8	5.2	5.7	6.5
NaCl range	0–1%*	0–1.0%*	0–3.0%*	0–2.0%
NaCl optimum	0.2%*	0.1%*	2.5%*	0.5%
Temperature range	37–62°C*	35–66°C	37–68°C	40–65°C
Temperature optimum	60°C	60°C	55°C	60°C
Substrates				
Glucose	+	+	+	+
Fructose	+	+	+	−
Galactose	+	+	+	−
Mannose	+	+	NR	+
Arabinose	+	+	+	−
Rhamnose	−	+	NR	−
Xylose	+	+	+	+
Lactose	+	+	+	−
Trehalose	+	NR	NR	−
Cellobiose	+	+	NR	+
Sucrose	+	NR	+	+
Raffinose	(+)	+	NR	−
Xylan	+	+	+	+
Starch	+	+	+	−
Gelatin hydrolysis	+*	−*	+*	−
Nitrate reduction	−*	+*	−*	+
Nitrite reduction	+*	+*	−*	+
Stimulation of growth by				
Elemental sulphur	NR	+	(+)	+
Thiosulphate	NR	+	ND	+
Reduction of				
S^0	−	−	(+) (H_2S)	+ (H_2S)
Thiosulphate	+	+ (S^0)	+ (S^0)	+ (S^0) (H_2S)

Strains: 1, *Thermoanaerobacterium thermosaccharolyticum* (Collins et al. 1994); 2, *Thermoanaerobacterium aotearoense* (Liu et al. 1996); 3, *Thermoanaerobacterium aciditolerans* (Kublanov et al. 2007); 4, strain Buff (this study)

(+), weakly positive; +, positive; −, negative; *, data from our laboratory; ND, not determined; NR, not reported

also reported. For all the strains tested the hybridization values were plentifully lower than the taxonomic limit.

Discussion

Strain Buff exhibited distinctive phenotipic characteristics with respect to *Thermoanaerobacterium* genus members utilized for the comparative evaluations: indeed, Buff was isolated from a very different place in comparison with its most related strains. As reported in Table 2, the optimum pH of strain Buff differs for about 1.0 pH unit with respect to those of *Thermoanaerobacterium aotearoense* (Liu et al. 1996) and *Thermoanaerobacterium aciditolerans* (Kublanov et al. 2007). Also NaCl optimum concentration was very different from those described for *Thermoanaerobacterium aotearoense* (Liu et al. 1996) and *Thermoanaerobacterium aciditolerans* (Kublanov et al. 2007), fivefold lower and higher, respectively (Table 2). On the contrary both range and optimum temperature resulted

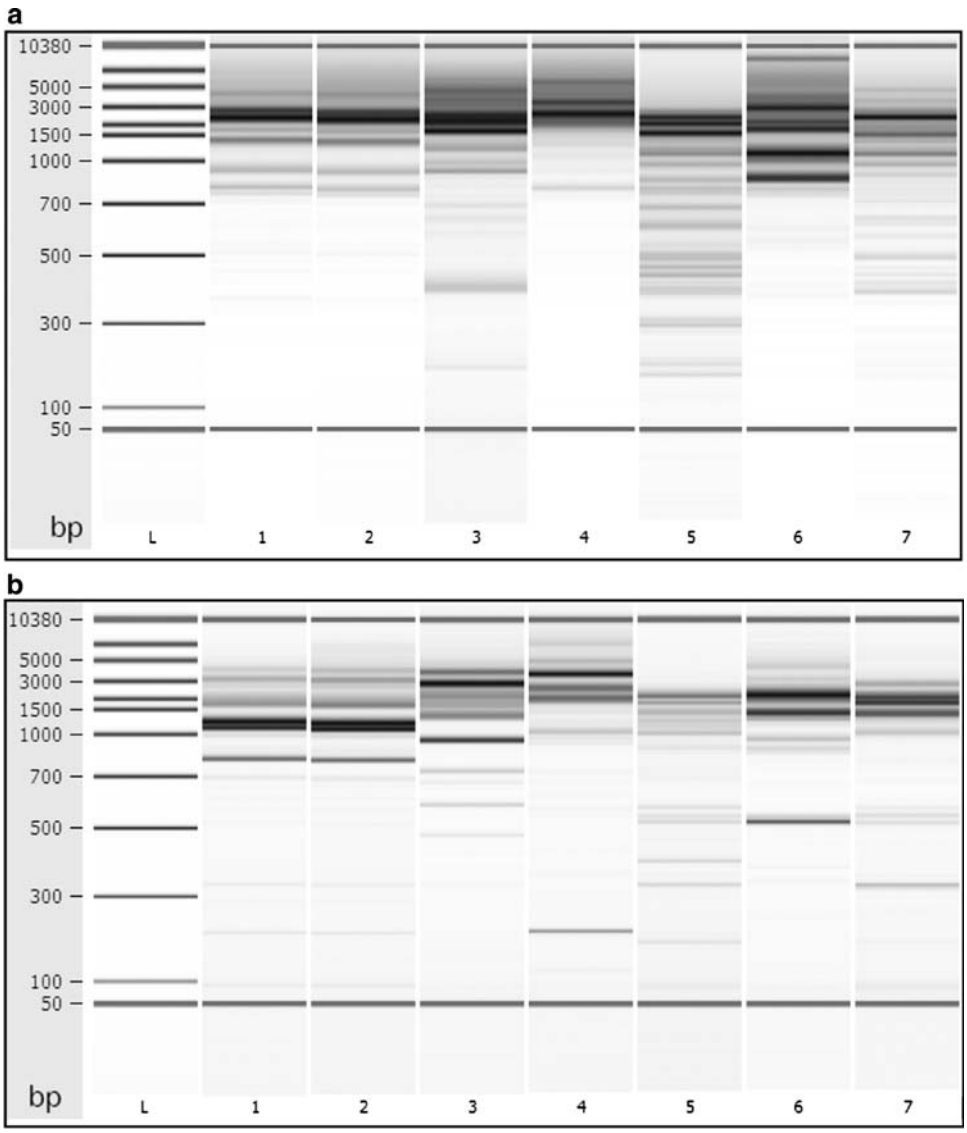

Fig. 2 REP-PCR and RAPD-PCR fingerprints of strain Buff and its closest relatives **a** REP-PCR, GTG_5 primer; **b** RAPD-PCR, OPR2 primer. PCR-amplification and analysis were performed as described in "Materials and methods". Legend: *L* molecular weight DNA ladder; *lanes 1* and *2*: strain Buff (two independent amplification experiments); *lane 3*: *T. aotearoense*; *lane 4*: *T. thermosaccharolyticum*; *lane 5*: *T. acidotolerans*; *lane 6*: *T. saccharolyticum*; *lane 7*: *Clostridium thermoamylolyticum,* outgroup

similar between strain Buff and its closest phylogenetic relatives.

A biochemical difference between the new isolate and its closest relatives was the spectrum of fermentable carbohydrates used for hydrogen production: among several sugars tested, strain Buff was able to grow only in the presence of glucose, mannose, xylose, maltose, cellobiose, sucrose and xylan.

The results obtained by testing various electron acceptors indicated that their presence was required for optimal growth, suggesting a role for these substances in removing the hydrogen produced and conversely a sensitivity of the strain to H_2 partial pressure increase. It is worth noting the greater attitude of Buff to the reduction of sulphur compounds with respect to its closest phylogenetic relatives (Table 2), in particular the ability to reduce elemental sulphur to H_2S.

On the basis of the above-mentioned phenotypic, biochemical and phylogenetic results, we propose to classify strain Buff as a new species of the genus *Thermoanaerobacterium* with the name *Thermoanaerobacterium thermostercus* sp. nov.

Description of *Thermoanaerobacterium thermostercus* sp. nov.

Thermoanaerobacterium thermostercus (ther.mo.ster.cus. Gr. n. *thermos*, hot; L. n. *stercus*, dung; N. L. adj. *thermostercus*, describing the source where this strain was isolated and lived at high temperature).

The bacterium was anaerobic, thermophilic, Gram-positive, non-spore-forming, hydrogen-producer, having the morphology of straight and motile rods, occurring singly and in pairs. Colonies were very small (about 1 mm

of diameter), white and uniformly round with a glossy surface. The optimum growth temperature, pH and NaCl concentration were, respectively, 60°C, 6.5 and 0.5% (w/v). At the optimal conditions, after 24 h of incubation in the standard culture medium, the hydrogen production rate was 25.3 (mL/L/h). Hydrogen sulphide was produced from cysteine, elemental sulphur, thiosulphate, sodium sulphide and sodium hydrosulphite. Thiosulphate was reduced also to elemental sulphur (S^0) globules. Yeast extract, tryptone (or peptone) and a fermentable carbohydrate were required for growth; in the absence of glucose, but in the presence of yeast extract and tryptone, poor growth was obtained. This strain utilized glucose, mannose, xylose, maltose, cellobiose, sucrose and xylan, but not fructose, arabinose, rhamnose, galactose, lactose, trehalose, raffinose, starch, inulin, molasses, glycerol and pyruvate. The end products of glucose fermentation were ethanol, acetate, lactate, H_2, H_2S and CO_2. The strain was incapable of growth with O_2 and was sensitive to lincomycin, vancomycin, tetracycline, neomycin, chloramphenicol, bacitracin and fusidic acid. Nitrate and nitrite were reduced. The DNA G + C content was 34.4 mol%.

The type strain is Buff (=DSM 22141 = ATCC BAA-1776) isolated from buffalo-dung samples collected in a buffalo-farm located in Caserta (Campania, south of Italy).

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