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Moorella stamsii sp. nov., a new anaerobic thermophilic hydrogenogenic carboxydotroph isolated from digester sludge

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Moorella stamsii* strain E3-O is HF563589.

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Summary

A novel anaerobic, thermophilic, carbon monoxide-utilizing bacterium, strain E3-O, was isolated from anaerobic sludge of a municipal solid waste digester. Cells were straight rods, 0.6 to 1μm in diameter and 2 to 3 μm in length, growing as single cells or in pairs. Cells formed round terminal endospores. The temperature range for growth was 50 to 70 °C, with an optimum at 65 °C. The pH range for growth was 5.7 to 8.0, with an optimum at 7.5. Strain E3-O had the capability to ferment various sugars, such as fructose, galactose, glucose, mannose, raffinose, ribose, sucrose and xylose, producing mainly H_2 and acetate. In addition, the isolate was able to grow with CO as the sole carbon and energy source. CO oxidation was coupled to H_2 and CO_2 formation. The G+C content of the genomic DNA was 54.6 mol %. Based on 16S rRNA gene sequence analysis, this bacterium is most closely related to *Moorella glycerini* (97% sequence similarity). Based on the physiological features and phylogenetic analysis, it is proposed that strain E3-O^T should be classified in the genus *Moorella* as a new species, *Moorella stamsii*. The type strain of *Moorella stamsii* is E3-O^T (=DMS 26271^T = CGMCC 1.5181^T).

(**Introduction**)

Carbon monoxide is a gas present in natural and anthropogenic environments that is involved in several important redox reactions. CO metabolism is a significant part of the global carbon cycle. The number of known carboxydotrophic anaerobes is increasing in recent years due to their important role in CO conversion (Sokolova *et al.*, 2009). CO is a potent electron donor (thermodynamic CO_2/CO redox potential = -520 mV) and represents an excellent source of energy for anaerobic microorganisms (Kochetkova *et al.*, 2011; Oelgeschlager & Rother, 2008). Nevertheless, only few anaerobes have been described that exhibit the capacity for hydrogenogenic carboxydotrophy.

Hydrogenogenic CO-oxidizing prokaryotes are able to grow on CO with the production of hydrogen and CO_2, according to the water-gas shift reaction: $CO + H_2O \rightarrow H_2 + CO_2$ (ΔG = -20 kJ mol^{-1}) (Svetlitchnyi *et al.*, 2001). The first organism described to perform this reaction was *Carboxydothermus hydrogenoformans* (Henstra & Stams, 2011; Svetlitchnyi *et al.*, 2001). Since then, other anaerobic CO-oxidizing hydrogenogenic prokaryotes have been described, isolated from a wide range of environments around the world, and spread over different phylogenetic clades (Novikov *et al.*, 2011; Sokolova *et al.*, 2009; Techtmann *et al.*, 2009). In this work, we describe a novel anaerobic thermophilic carboxydotrophic hydrogenogenic bacteria, strain E3-O, isolated from anaerobic sludge of a municipal solid waste digester. This species can utilize CO as sole carbon and energy source, forming mainly H_2 and CO_2. Phylogenetic analysis based on 16S rRNA gene sequences indicated affiliation to the genus *Moorella*. Strain E3-O^T was only moderately related to the recognized species of this genus, with 97% 16S rRNA gene sequence identity, and possesses some unique physiological features. Therefore, the creation of a novel species of *Moorella* is proposed.

(Methods)

Strain E3-O^T was isolated from a CO-degrading enrichment culture originating from anaerobic suspended sludge from of a municipal solid waste digester (Barcelona, Spain).

A phosphate-buffered mineral salt medium (20 mM, pH 7.0) was used for enrichment cultures and isolation of strain E3-O^T.The phosphate-buffered mineral medium contained the following components (per liter): Na_2HPO_4, 1.63 g; NaH_2PO_4, 1.02 g; resazurin, 0.5 g; NH_4Cl, 0.3 g; $CaCl_2 \cdot 2H_20$, 0.11 g; $MgCl_2 \cdot 6H_20$, 0.10 g; NaCl, 0.3 g; 1 mL of acid and alkaline trace element stock each, and 0.2 ml of vitamin stock. Medium was reduced with 0.8 mM sodium sulfide before inoculation. Trace elements and vitamins were prepared as described previously (Stams *et al.*, 1993). Enrichments were performed using CO as sole carbon and energy source. Enrichment cultures were subsequently transferred (10%, v/v) and supplemented with increasing CO partial pressure; total gas pressure was kept constant at 1.7 bar, and pCO varied from 0.34 bar (CO/N_2 mixture) to 1.7 bar (100% CO). Bottles were incubated in the dark, at 55 °C and 120 rpm. Enrichment of strain E3-O^T was possible by culture dilution series and increasing CO partial pressure, but isolation was only effective after culture autoclaving (2x 20 min at 121 °C). Purity of the bacterial culture was checked by microscopic examination (Leica DM 2000, Germany). Direct sequencing of the 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) were also used to check the genetic purity of the bacterial cultures. Total genomic DNA from cultures of strain E3-O^T was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, USA), according to the manufacturer's instructions. 16S rRNA gene was directly amplified from genomic DNA by PCR, using the primer set 027F/1492R (Nübel *et al.*, 1996) and the following PCR program: pre-denaturation, 2 min at 95 °C; 30 cycles of denaturation, 30 s at 95 °C,

annealing, 40 s at 52 °C and elongation, 90 s at 72 °C; and post-elongation, 5 min, at 72 °C. The PCR products were purified using the DNA Clean and Concentrator kit (ZYMO Research, USA) and sequenced directly at BaseClear (Leiden, The Netherlands). Partial sequences were assembled using the alignment editor BioEdit v7.0.9 software package (Hall, 1999). Similarity searches for the 16S rRNA gene sequence derived from strain E3-O^T were performed using the NCBI BLAST search program within the GenBank database (Altschul *et al.*, 1990). Alignment of the 16S rRNA sequences was performed by using the FastAligner V1.03 tool of the ARB program package (Ludwig *et al.*, 2004). The neighbor joining method (Saitou & Nei, 1987) was used for the construction of a 16SrRNA gene based phylogenetic tree. For DGGE analysis, 16S rRNA gene was partially amplified from genomic DNA with primer set U968GC-f/L1401-r (Lane, 1991; Muyzer *et al.*, 1993). The thermo cycling program used for PCR-DGGE amplification was: pre-denaturation, 5 min at 95 °C; 35 cycles of denaturation, 30 s at 95 °C, annealing, 40 s at 56 °C and elongation, 90 s at 72 °C; and post-elongation, 5 min at 72 °C. DGGE was performed using a DCode system (Bio-Rad, Hercules, CA, USA). Gels contained 8% (wt/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) and a linear denaturing gradient of 30–60%, with 100% of denaturant corresponding to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed for 16 h at 85 V and 60 ºC in a 0.5x Trisacetate–EDTA buffer. DGGE gels were stained with silver nitrate (Sanguinetti *et al.*, 1994). G+C content determination, DNA–DNA hybridization and cellular fatty acids composition were performed by the identification service of the DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Reference strain *Moorella glycerini* JW/AS-Y6 (=DSM11254^T), used in physiological tests and DNA-DNA hybridization studies, was obtained from DSMZ (Braunschweig, Germany). Utilization of soluble substrates by

strain E3-O^T was performed using a bicarbonate-buffered mineral salt medium (Stams *et al.*, 1993). Sucrose (20mM) was used as carbon source for testing the utilization of different electron acceptors and the optimum growth temperature (from 15 to 80°C) and pH (from 5.7 to 8.0). Sensitivity to antibiotics and to oxygen was also tested using sucrose as carbon source. Antibiotics were added from freshly prepared anoxic filter-sterilized solutions to a final concentration of 100 µg mL^{-1}. The effect of oxygen on the growth of strain E3-O^T was studied by incubating the culture with different concentrations of oxygen in the headspace, from 2% to 21% O_2 (pO_2=0.03 to 0.32 bar). Growth of strain E3-O^T was monitored by measuring optical absorbance at 600 nm with a spectrophotometer (U-1500 Hitachi, Tokyo, Japan). Soluble substrates and intermediates (sugars, volatile fatty acids) were measured using a HPLC Thermo Electron equipment with a Shodex SH1821 column. The mobile phase used was sulfuric acid (0.01 N) at a flow rate of 0.6 mL min^{-1}. Column temperature was set at 60 °C. Ionic species were analyzed by chromatography using a HPLC DIONEX system, equipped an Ionpac AS22 column and ED40 electrochemical detector. Column temperature and pressure varied between 35-40 °C and 130-160 bar. Cultures were routinely observed using phase contrast microscopy (Leica DM 2000, Germany). Gaseous compounds (CO, CO_2, H_2) were analyzed by gas chromatography using a GC-2014 Shimadzu equipment, appended with a thermal conductivity detector and equipped with two columns: a CP Poraplot Q column, 25 m x 0.53 mm, df 20 µm, using helium as carrier gas at a flow rate of 15 mL min^{-1}; and, a Molsieve 13X column, 2 m x 3 mm, using argon as carrier gas at a flow rate of 50 mL min^{-1}. For CP Poraplot Q column the temperatures of injector, column and detector were 60, 33 and 130°C; and, for the Molsieve column the temperatures were 80, 100 and 130°C, respectively. Cells from active cultures of strain E3-O^T were stained using Gram staining techniques. Carbon

monoxide dehydrogenase (CODH) activity was determined at 55 ºC by following spectrophotometrically the CO dependent reduction of oxidized methyl viologen (Svetlitchnyi *et al.*, 2001). For this measurement, cell-free extract was obtained from cultures grown with CO as the only electron donor (pCO = 0.425 bar), and using the procedure previously described by Balk *et al.* (Balk *et al.*, 2009).

(Results and Discussion)

A CO-oxidizing bacterium was obtained from a CO-degrading culture enriched from anaerobic sludge of a municipal solid waste digester. Isolation of this bacterium was possible after autoclaving the enriched culture two times, for 20 min at 121 °C and sub-culturing it with CO in the gas phase (pCO = 0.43 bar). A DGGE profile of this culture showed the presence of a single band. Microscopic observations showed that vegetative cells of strain E3-O^T were straight rods, 0.6-1µm by 2-3 µm, occurring singly or in pairs (Fig. 1). Although species from the *Moorella* genus are known to be Gram positive bacteria (Collins *et al.*, 1994), the cells of strain E3-O^T stained Gram variable. This result was always obtained indiscriminately of the cells being grown with CO (gas phase) or with sucrose, and did not change with growth phase of strain E3-O^T. Spores were terminal, round and heat-resistant endospores. Strain E3-O^T could grow between 50 and 70°C, with an optimum temperature of 65°C. The optimum pH for growth was 7.5. The doubling time of strain E3-O^T when growing on glucose under optimal conditions was 2.2 ± 0.9 days. Strain E3-O^T could ferment the following substrates (at a concentration of 20 mM): fructose, galactose, glucose, mannose, pyruvate, raffinose, ribose, sucrose and xylose. Slow growth was also observed on arabinose, cellobiose and maltose. Other substrates (at a concentration of 20 mM unless indicated otherwise) were

also tested as the sole substrate, but not utilized for growth by strain E3-O^T: acetate, benzoate, butyrate, ethanol, formate, fumarate, glycerol, lactate, lactose, methanol, propionate, sorbitol, succinate and trehalose, peptone and yeast extract (5 g L^{-1}, each), and H_2/CO_2 (80:20 vol/vol, 1.7 bar). The main product detected from sugar (fructose, glucose, raffinose, sucrose and xylose) and pyruvate fermentation was acetate. Most of the described *Moorella* strains are capable of performing homoacetogenic fermentation of glucose, converting 1 mol of glucose into 3 mol of acetate. Strain E3-O^T converted 1 mol of glucose into 2.16 ± 0.74 mol of acetate. A ratio of 2.31 ± 0.09 mol acetate per mol of glucose has been described for *M. glycerini*, which is the closest relative of strain E3-O^T (Slobodkin *et al.*, 1997). In addition, strain E3-OT was able to grow on CO as sole carbon and energy source with the production of equimolar amounts of H_2 and CO_2 (Fig. 2). *Moorella* species are well known CO-utilizers, but the only hydrogenogenic *Moorella* described thus far is *M. thermoacetica* strain AMP (Balk *et al.*, 2008; Jiang *et al.*, 2009). In agreement with the observed CO-utilization, cell-free extracts from strain E3-O^T were shown to exhibit CODH activity. The specific CODH activity in cell-free extracts at 55 °C for strain E3-O^T was 15.3 ± 2.6 U mg of $protein^{-1}$. Strain E3-O^T was able to reduce nitrate (20mM), perchlorate (10mM) and anthraquinone-2,6-disulfonate (AQDS) (20mM). The isolate did not reduce sulfate (20mM), thiosulfate (20mM) and nitrite (10mM). Strain E3-O^T can also be distinguished from other *Moorella* species, because all the other described species could use thiosulfate as electron acceptor, and strain E3-O^T could not. Penicillin, ampicillin, chloramphenicol and kanamycin completely inhibited growth (100 µg mL^{-1}). Streptomycin at 100 µg mL^{-1} did not inhibit growth. Strain E3-O^T is an obligate anaerobic microorganism, since there was no growth detected in the presence of oxygen. The cellular fatty acid composition revealed that the most abundant fatty acids of strain E3-O^T were iso-C15:0 (45.37%) and C16:0

(16.77%). Table 1 shows a detailed lipid composition of strain E3-O^T. The G+C content of the genomic DNA of strain E3-O^T was 54.6 mol%. Phylogenetic analysis of the almost full-length 16S rRNA sequence showed that strain E3-O^T was most closely related to *Moorella glycerini* with 97% 16S rRNA gene identity (Slobodkin *et al.*, 1997), followed by *Moorella humiferrea* with 16S rRNA gene 96% identity (Nepomnyashchaya *et al.*, 2012) (Fig.3). Phenotypically, strain E3-O^T is similar to all other described *Moorella* species, but phylogenetic similarity values between strain E3-O^T and *M. mulderi*, *M. thermoacetica*, *M. thermoautotrophica* and *M. perchloratireducens*, were only among 95 to 93% identity. Quantitative DNA-DNA hybridization between strain E3-O^T and its closest relative (*M. glycerini*) was performed and the values obtained (in duplicate) were 51.1% - 53.3%, indicating that strain E3-O^T is a new species of the genus *Moorella*, which was in line with the 97% of 16S rRNA gene identity between *M. glycerini* and strain E3-O^T. Phenotypic characteristics of strain E3-O^T in comparison with the phylogenetically closely related species are presented in Table 2. The main differences between strain E3-O^T and the closest related *M. glycerini* were the optimum temperature and pH for growth, but also the conversion of some substrates: strain E3-O^T was able to grow on sucrose but *M. glycerini* not. On the other hand, *M. glycerini* can use glycerol and lactate, but strain E3-O^T did not grow on these two substrates. Furthermore, in contrast to *M. glycerini*, strain E3-O^T can use nitrate and AQDS as electron acceptor. Based on phylogenetic results and physiological properties, it is proposed that strain E3-O^T represents a novel species of genus *Moorella*, *Moorella stamsii* sp. nov.

Description of *Moorella stamsii* sp. nov.

Moorella stamsii (stams'i.i. N.L. masc. gen. n. stamsii, of Stams, named after Alfons J. M. Stams, a Dutch microbiologist, in recognition for his contribution to the advancement in anaerobic microbial physiology).

Cells are straight rods (approx. 0.6-1 µm in diameter and 2-3 µm in length) and show variable response to Gram staining. Usually, cells occur singly or in pairs. Cells produce terminal and round endospores. The most abundant fatty acids are iso-C15:0 (45.4%) and C16:0 (16.8%). The G+C content of the DNA of strain E3-O^T is 54.6 mol% (G+C) (determined by HPLC). The optimum temperature for growth is 65°C and the optimum pH for growth occurs at pH 7.5. Strain E3-O^T is able to grow on (substrates tested in a concentration of 20mM): fructose, galactose, glucose, mannose, pyruvate, raffinose, ribose, sucrose and xylose. Additionally it can grow on carbon monoxide (100%, pCO=1.7 bar). Strain E3-O^T does not require any growth factors. With acetate, benzoate, butyrate, ethanol, formate, fumarate, glycerol, H_2/CO_2, lactate, lactose, methanol, peptone, propionate, sorbitol, succinate, trehalose and yeast extract no growth was detected. Nitrite, sulfate and thiosulfate could not, but AQDS, nitrate, and perchlorate could act as electron acceptors. E3-O^T is an obligate anaerobic bacterium. The type strain is E3-O^T (= DSM 26217^T = CGMCC 1.5181^T), and was isolated in Wageningen, The Netherlands, from a CO-degrading culture enriched from a thermophilic anaerobic suspended sludge of a municipal solid waste digester (Barcelona, Spain).

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TABLE 1. Cellular fatty acid composition (%) of strain E3-O^T.

Fatty acids	Strain E3-O^T
C14:0	4.90
iso-C15:0	45.37
C15:0	0.43
iso-C14:0 3OH	6.52
C16:0	16.77
iso-C17:1	1.20
iso-C17:0	6.88
cyclo-C17:0	4.38
C18:3	0.86
C18:0	1.20
C20:4	0.79
C20:1	1.95

TABLE 2. Comparison of morphological and physiological characteristics of strain E3-O^T and its phylogenetic closest relatives. Species: 1, strain E3-O^T; 2, *Moorella glycerini* DSM 11254^T (Slobodkin *et al.*, 1997); 3, *Moorella humiferrea* DSM 23265^T (Nepomnyashchaya *et al.*, 2012).

	1	2	3
Characteristics:	strain E3-O^T	*Moorella glycerini* DSM 11254^T	*Moorella humiferrea* DSM 23265^T
Origin	thermophilic anaerobic digester treating organic solid wastes	sediment-water from a hot spring	terrestrial hydrothermal spring
Optimum temperature (°C)	65	58	65
Optimum pH	7.5	6.3-6.5	7.0
Gram reaction	variable	positive	positive
DNA G+C content (mol%)	54.6	54.5	51.0
Spore formation	+	+	+
Substrate utilization:			
CO	+	+ (up to 50% gas phase)*	nd
H_2/CO_2	-	-	-
arabinose	+-	-	-
fructose	+	+	+
galactose	+	+	+
glucose	+	+	-
mannose	+	+	-
sucrose	+	-	+
xylose	+	+	-
lactate	-	+	+
succinate	-	-	+
pyruvate	+	+	+
glycerol	-	+	+
Electron acceptors:			
AQDS	+	-	+
nitrate	+	-	+
thiosulfate	-	+	+
perchlorate	+	+	+

* this study.

nd: not determined; +: utilized; +-: poorly utilized; -: not utilized.

Caption to the figures:

Fig.1. Phase-contrast microscopic picture showing the cell morphology of strain E3-O^T. Size bar indicates 10 μm.

Fig. 2. Growth of strain E3-O in mineral medium under a CO atmosphere (pCO = 1.36bar). (▲), carbon monoxide; (♦), hydrogen; (□) carbon dioxide. Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using Henry's law. Gas products values are expressed as amounts in the gas phase per litre liquid culture.

Fig. 3. Phylogenetic tree of 16S rRNA gene sequences showing the position of strain E3-O^T relative to other species of the genus *Moorella* as well as selected reference sequences of bacteria. The phylogenetic tree was calculated using the ARB software package (Saitou & Nei, 1987) and applying the neighbor-joining method with Felsenstein correction. The significance of each branch is indicated at the nodes by bootstrap values (%) based on 1000 replications; only values above 80% are given. GenBank accession numbers of 16S rRNA gene sequences are indicated in parentheses. Bar, 10% sequence divergence.

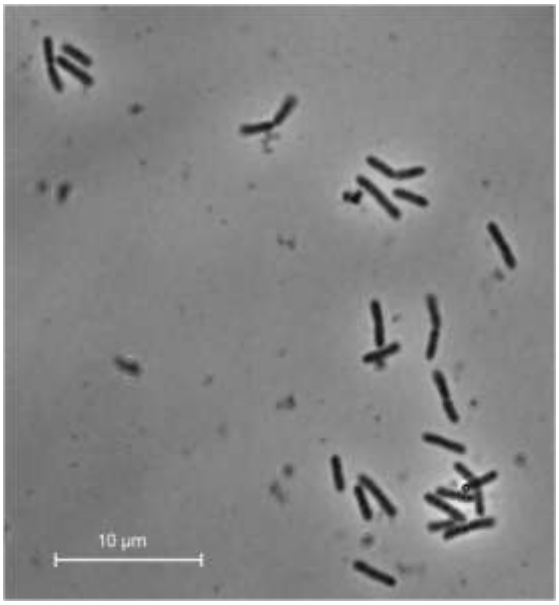


Fig. 1

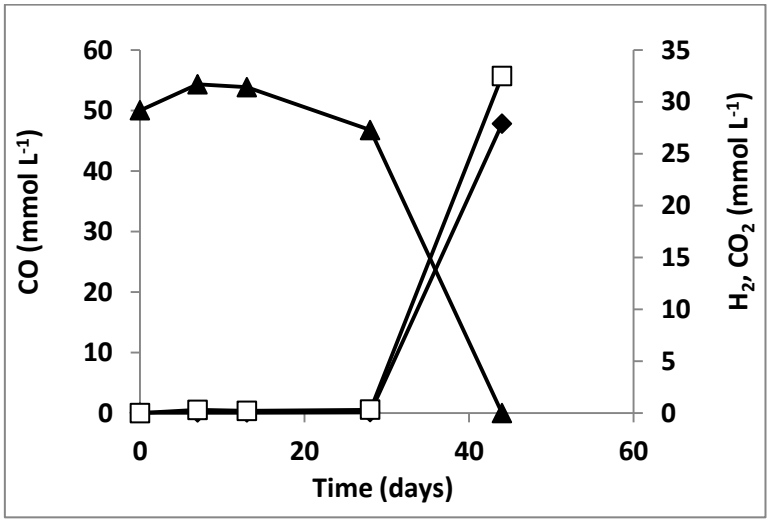
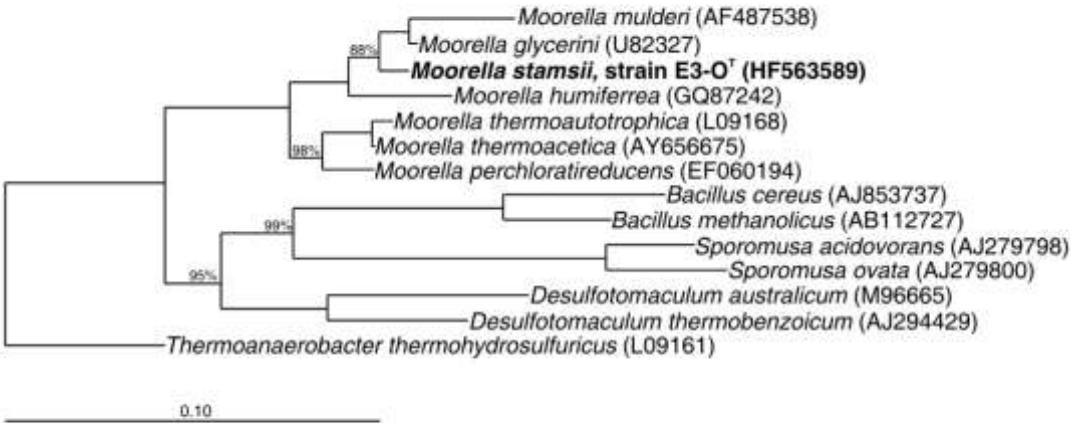

60
50
40
30
20
10
0
CO (mmol L-1)
35
30
25
20
15
10
5
0
H2, CO2 (mmol L-1)
0
20
40
60
Time (days)

Fig. 2

1


Moorella mulderi (AF487538)
Moorella glycerini (U82327)
Moorella stamsii, strain E3-O^T (HF563589)
Moorella humiferrea (GQ87242)
Moorella thermoautotrophica (L09168)
Moorella thermoacetica (AY656675)
Moorella perchloratireducens (EF060194)
Bacillus cereus (AJ853737)
Bacillus methanolicus (AB112727)
Sporomusa acidovorans (AJ279798)
Sporomusa ovata (AJ279800)
Desulfotomaculum australicum (M96665)
Desulfotomaculum thermobenzoicum (AJ294429)
Thermoanaerobacter thermohydrosulfuricus (L09161)
88%
98%
99%
95%
0.10

2

3 Fig. 3

4

5

6