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

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A novel anaerobic, thermophilic, carbon monoxide-utilizing bacterium, strain E3-O^T, was isolated from anaerobic sludge from a municipal solid waste digester. Cells were straight rods, 0.6–1 μm in diameter and 2–3 μm in length and grew as single cells or in pairs. Cells formed round terminal endospores. The temperature range for growth was 50–70 °C, with an optimum at 65 °C. The pH range for growth was 5.7–8.0, with an optimum at 7.5. Strain E3-O^T had the ability 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 identity). 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 representative of a novel species, *Moorella stamsii*. The type strain of *Moorella stamsii* is E3-O^T (=DSM 26271^T=CGMCC 1.5181^T).

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 micro-organisms (Kochetkova *et al.*, 2011; Oelgeschläger & 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 carbon dioxide, 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, to our knowledge, described as being able to perform this reaction in the dark, was a mesophilic species of the genus *Rhodopseudomonas* (Uffen, 1976). 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). The first thermophilic carboxydotroph described, to our knowledge, was *Carboxydothermus hydrogenoformans* (Henstra & Stams, 2011; Svetlitchnyi *et al.*, 2001). In this work, we describe a novel anaerobic thermophilic carboxydotrophic hydrogenogenic bacterium, strain E3-O^T.

Strain E3-O^T was isolated from a CO-degrading enrichment culture originating from anaerobic suspended sludge from of a thermophilic municipal solid waste digester in 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 (l^{-1}): Na_2HPO_4, 1.63 g; NaH_2PO_4, 1.02 g; resazurin, 0.5 g; NH_4Cl, 0.3 g; $CaCl_2 . 2H_2O$, 0.11 g; $MgCl_2 . 6H_2O$, 0.10 g; NaCl, 0.3 g; 1 ml each of acid and alkaline trace element stock and 0.2 ml vitamin stock. Medium was reduced with 0.8 mM sodium sulfide before inoculation. Trace elements and

Abbreviations: AQDS, anthraquinone-2,6-disulfonate; DGGE, denaturing gradient gel electrophoresis.

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

Two supplementary figures are available with the online version of this paper.

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 shaken at 120 r.p.m. 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 (2 × 20 min, at 121 °C). Purity of the bacterial culture was checked by microscopic examination (DM 2000; Leica). 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), according to the manufacturer's instructions. The 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) 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 neighbour joining method (Saitou & Nei, 1987) was used for the reconstruction of a 16S rRNA 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 thermocycling 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). Gels contained 8 % (w/v) 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 °C in a 0.5 × Tris/acetate–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 analysis were performed by the identification service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Reference strains *Moorella glycerini* JW/AS-Y6^T (=DSM 11254^T) and *Moorella humiferrea* 64-FGQ^T (=DSM 23265^T) were obtained from the DSMZ. *M. glycerini*, *M. humiferrea* and strain E3-O^T were grown with fructose (20 mM) and yeast extract (0.02 %) for determination of membrane fatty-acids composition. Utilization of CO by *M. humiferrea* was also tested using phosphate-buffered medium containing yeast extract (0.02 %); bottles contained 10 % CO in the headspace (diluted with N_2 to a final pressure of 1.7 bar). Utilization of soluble substrates by strain E3-O^T, *M. glycerini* JW/AS-Y6^T and *M. humiferrea* was performed using a bicarbonate-buffered mineral salt medium (Stams *et al.*, 1993) supplemented with 0.01 % yeast extract. Sucrose (20 mM) or glycerol (20 mM) were used as carbon sources for testing the utilization of different electron acceptors. The optimum growth temperature (from 15 to 80 °C) and pH (from 5 to 8) was tested with sucrose (20 mM). Sensitivity to antibiotics and to oxygen was also tested using sucrose as a 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). All tests were incubated for at least 2 weeks, unless stated otherwise. Growth of strain E3-O^T was monitored by measuring OD_{600} with a spectrophotometer (U-1500; Hitachi). Soluble substrates and intermediates (sugars and volatile fatty acids) were measured using HPLC Thermo Electron equipment with a Shodex SH1821 column. The mobile phase used was sulfuric acid (0.005 M) at a flow rate of 0.6 ml min^{-1}. Column temperature was set at 60 °C. Ionic species were analysed by chromatography using a HPLC DIONEX system, equipped an Ionpac AS22 column and ED40 electrochemical detector. Column temperature and pressure varied between 35 and 40 °C and 130 and 160 bar. Cultures were routinely observed using phase-contrast microscopy (DM 2000; Leica). Gaseous compounds (CO, CO_2 and H_2) were analysed by gas chromatography using a GC-2014 (Shimadzu), fitted with a thermal conductivity detector and equipped with two columns: a CP Poraplot Q column, 25 m × 0.53 mm, stationary phase film thickness 20 μm, using helium as carrier gas at a flow rate of 15 ml min^{-1}; and, a Molsieve 13X column, 2 m × 3 mm, using argon as carrier gas at a flow rate of 50 ml min^{-1}. For the 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 °C 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.* (2009).

The DGGE profile showed the presence of a single band. Microscopic observations showed that vegetative cells of strain E3-O^T were straight rods, 0.6–1 μm × 2–3 μm, occurring singly or in pairs (Fig. S1 available in IJSEM Online). Although species of the genus *Moorella* are known to stain Gram-stain-positive (Collins *et al.*, 1994), the cells

of strain E3-O^T stained Gram-variable. This result was always obtained regardless of whether the cells were 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 at between 50 and 70 °C, with an optimum temperature of 65 °C. The optimum pH for growth was 7.5 with a range of 5.7–7.8. 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 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, v/v, 1.7 bar). The main product detected from sugar (fructose, glucose, raffinose, sucrose and xylose) and pyruvate fermentation was acetate. Most of the described strains of species of the genus *Moorella* 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 mol^{-1} 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-O^T 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. S2). Species of the genus *Moorella* are well known CO-utilizers, but the only hydrogenogenic *Moorella* described thus far is *Moorella 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 maximum CODH activity in cell-free extracts at 55 °C for strain E3-O^T was 15.3 ± 2.6 U mg protein^{-1}. Strain E3-O^T was able to reduce nitrate (20 mM), perchlorate (10 mM) and anthraquinone-2,6-disulfonate (AQDS) (20 mM). The isolate did not reduce sulfate (20 mM), thiosulfate (20 mM) and nitrite (10 mM). Strain E3-O^T can also be distinguished from other species of the genus *Moorella* because all the other described species could use thiosulfate as an electron acceptor and strain E3-O^T could not. Penicillin, ampicillin, chloramphenicol and kanamycin (all at 100 μg ml^{-1}) completely inhibited growth. Streptomycin at 100 μg ml^{-1} did not inhibit growth. Strain E3-O^T is an obligate anaerobic micro-organism, 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-$C_{15:0}$ (26.18 %), iso-$C_{15:0}$ DMA (15.11 %) and $C_{16:0}$ (7.11 %). The differences detected in the lipid composition of strain E3-O^T and the phylogenetically closely related species, *M. glycerini* and *M. humiferrea*, are shown in Table 1. In *M. glycerini*, the predominant fatty acids detected are the same as in strain E3-O^T: iso-$C_{15:0}$ (37.62 %) and iso-$C_{15:0}$ DMA (18.15 %), although the fatty acids composition were less diverse. The fatty acids $C_{16:0}$ and iso-$C_{17:0}$ were substantially more abundant in *M. humiferrea* than in the strain E3-O^T and *M. glycerini*. Besides these differences, iso-$C_{15:0}$ fatty acid is present in substantial amounts in the analysed strains. 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 *M. glycerini* with 97 % 16S rRNA gene identity (Slobodkin *et al.*, 1997), followed by *M. humiferrea* with 16S rRNA gene 96 % identity (Nepomnyashchaya *et al.*, 2012) (Fig. 1). Phenotypically, strain E3-O^T is similar to all other described species of the genus *Moorella* with validly published names, but phylogenetic similarity values between strain E3-O^T and *Moorella mulderi*, *M. thermoacetica*, *Moorella thermoautotrophica* and *Moorella perchloratireducens*, were only around 95–93 %. 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 novel species of the genus *Moorella*, which was in line with the 97 % 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 *M.*

Table 1. Cellular fatty acid composition (%) of strain E3-O^T in comparison with phylogenetically closely related species

Strains: 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). The strains were grown in bicarbonate-buffered medium supplemented with fructose (20 mM) and yeast extract (0.2 g l^{-1}). ND, Not detected; DMA, dimethyl acetal. Products shown in bold type are the predominant fatty acids.

Fatty acid	1	2	3
$C_{14:0}$	0.71	2.07	0.97
iso-$C_{15:0}$	**26.18**	**37.62**	**20.58**
anteiso-$C_{15:0}$	2.23	ND	ND
$C_{15:0}$	1.86	ND	ND
iso-$C_{15:0}$ DMA	**15.11**	**18.15**	1.60
iso-$C_{16:0}$	5.39	ND	ND
$C_{16:0}$	7.11	**10.56**	**21.65**
$C_{16:0}$ DMA	3.35	2.50	3.29
iso-$C_{17:0}$	6.52	**11.30**	**21.85**
anteiso-$C_{17:0}$	2.15	ND	ND
anteiso-$C_{17:0}$ DMA	2.27	ND	ND
$C_{17:0}$ DMA	ND	1.15	2.99
$C_{17:0}$ cyclopropane	ND	ND	2.21
$C_{18:0}$ DMA	ND	ND	1.12
$C_{18:0}$	1.38	1.79	**13.49**
$C_{18:1}\omega 9c$	ND	ND	0.90
$C_{19:0}$ cyclo 11–12 DMA	ND	1.44	ND

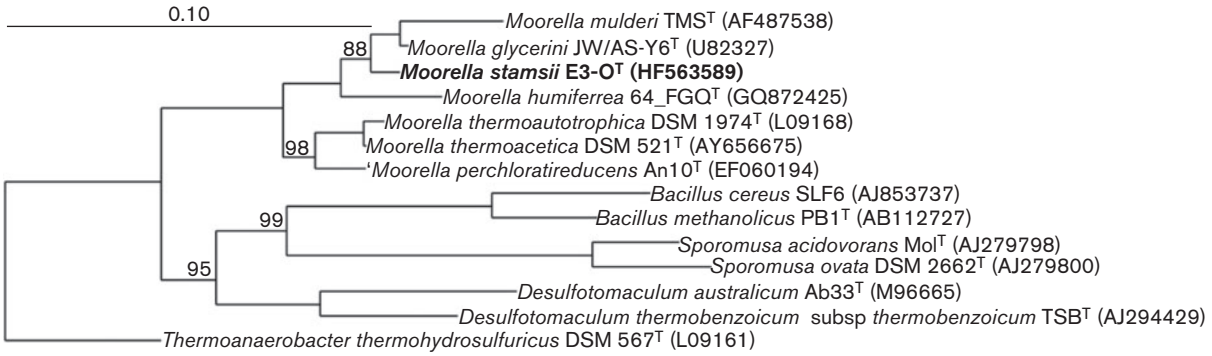

Fig. 1. Phylogenetic tree of 16S rRNA gene sequences showing the position of strain E3-O^T relative to other species of the genus *Moorella* as well as selected reference sequences of bacteria. The phylogenetic tree was calculated using the ARB software package (Saitou & Nei, 1987) and applying the neighbour-joining 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.

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* was 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 acceptors. Based on phylogenetic results and physiological properties, it is proposed that strain E3-O^T represents a novel species of the genus *Moorella*, *Moorella stamsii* sp. nov.

Description of *Moorella stamsii* sp. nov.

Moorella stamsii (stam′si.i. N.L. masc. gen. n. *stamsii* of Stams, named after Alfons J. M. Stams, a Dutch

Table 2. Selected morphological and physiological characteristics that differentiate strain E3-O^T from its phylogenetically closest relatives

Strains: 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). +, Utilized; −, not utilized. Except where noted, all data were obtained in this study.

Characteristic	1	2	3
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†
Substrate utilization			
CO	+	+‡	−§
Glucose	+	+	−
Mannose	+	+	−
Sucrose	+	−	+
Xylose	+	+	−
Lactate	−	+	−
Glycerol	−	+	−
Electron acceptors			
AQDS	+	−	+
Nitrate	+	−	+
Thiosulfate	−	+	+

*Taken from Slobodkin *et al.* (1997).

†Taken from Nepomnyashchaya *et al.* (2012).

‡*M. glycerini* could grow with CO in concentrations up to 50 % in the gas-phase; no hydrogen production was observed.

§*M. humiferrea* was not able to use CO in concentrations of 10 % in the gas-phase (after 2 weeks of incubation).

microbiologist, in recognition of his contribution to the advancement of anaerobic microbial physiology).

Cells are straight rods (approximately 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. An obligate anaerobic bacterium. The most abundant fatty acids are iso-$C_{15:0}$, iso-$C_{15:0}$ DMA and $C_{16:0}$. The optimum temperature for growth is 65 °C and the optimum pH for growth is pH 7.5. Able to grow on (substrates tested in a concentration of 20 mM): fructose, galactose, glucose, mannose, pyruvate, raffinose, ribose, sucrose and xylose. Additionally it can grow utilizing carbon monoxide (100 %, pCO=1.7 bar) and produces hydrogen from CO oxidation. 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.

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 (from Barcelona, Spain). The G+C content of the DNA of strain E3-O^T is 54.6 mol%.

Acknowledgements

This work was possible through the financial support provided by the Portuguese Science Foundation (FCT) and the European Social Fund (POPH-QREN) through a PhD grant SFRH/BD/48965/2008 to J. I. A.

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