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RESEARCH ARTICLE

Enrichment of anaerobic syngas-converting bacteria from thermophilic bioreactor sludge

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Abstract

Thermophilic (55 °C) anaerobic microbial communities were enriched with a synthetic syngas mixture (composed of CO, H_2, and CO_2) or with CO alone. Cultures T-Syn and T-CO were incubated and successively transferred with syngas (16 transfers) or CO (9 transfers), respectively, with increasing CO partial pressures from 0.09 to 0.88 bar. Culture T-Syn, after 4 successive transfers with syngas, was also incubated with CO and subsequently transferred (9 transfers) with solely this substrate – cultures T-Syn-CO. Incubation with syngas and CO caused a rapid decrease in the microbial diversity of the anaerobic consortium. T-Syn and T-Syn-CO showed identical microbial composition and were dominated by *Desulfotomaculum* and *Caloribacterium* species. Incubation initiated with CO resulted in the enrichment of bacteria from the genera *Thermincola* and *Thermoanaerobacter*. Methane was detected in the first two to three transfers of T-Syn, but production ceased afterward. Acetate was the main product formed by T-Syn and T-Syn-CO. Enriched T-CO cultures showed a two-phase conversion, in which H_2 was formed first and then converted to acetate. This research provides insight into how thermophilic anaerobic communities develop using syngas/CO as sole energy and carbon source can be steered for specific end products and subsequent microbial synthesis of chemicals.

Introduction

Syn(thesis)gas is a gaseous mixture mainly composed by carbon monoxide, hydrogen, and carbon dioxide. Syngas is produced during the gasification of carbon-containing materials, and its further conversion to bulk chemicals and fuels, using chemical or biotechnological processes, can be a way of recycling lignocellulosic biomass or even recalcitrant wastes (Sipma *et al.*, 2006). One of the major problems of using chemical catalytic processes for syngas conversion (to, e.g. methane, organic acids and alcohols, or hydrocarbons) is the requirement of a constant CO/H_2 ratio, worsened by the fact that CO is poisoning to most metal catalysts. Gasification process conditions and feedstocks greatly influence syngas composition – the utilization of microbial catalysts, which are less affected by oscillations in syngas composition and can tolerate the presence of trace contaminants, needs to be explored for the development of novel biotechnological processes for syngas valorization (Heiskanen *et al.*, 2007; Henstra *et al.*, 2007; Tirado-Acevedo *et al.*, 2010).

Carbon monoxide is a direct substrate for a variety of anaerobic microorganisms that can produce different value-added products, such as hydrogen, methane, fatty acids, and alcohols (for complete review see Bruant *et al.*, 2010; Guiot *et al.*, 2011; Henstra *et al.*, 2007; Köpke *et al.*, 2011). Carboxydotrophic hydrogenogenic bacteria, for example, *Rhodospirillum rubrum*, *Carboxydothermus hydrogenoformans*, *Carboxydocella thermoautotrophica*, *Thermincola carboxydiphila*, and *Desulfotomaculum carboxydivorans* can convert CO and water to H_2 and CO_2. *Clostridium* species are able to produce acetate and alcohols from CO (Henstra *et al.*, 2007), while *Moorella* species generally produce acetate or hydrogen (Sokolova *et al.*, 2009; Alves *et al.*, 2013). Methane can be directly produced from CO by methanogenic archaea, such as *Methanosarcina* species and the thermophile *Methanothermobacter thermautotrophicus* (Daniels *et al.*, 1977; Rother

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& Metcalf, 2004). Using a mixed culture approach, the possible routes for CO/syngas conversion increase as well as the robustness of the microbial system. Providing different environmental conditions to open mixed cultures could possibly deviate CO/syngas conversion in different routes. Nevertheless, mixed culture approaches for the conversion of these substrates have received little attention. Sipma *et al.* (2003, 2004) reported CO conversion by six different mesophilic anaerobic bioreactor sludges. Sludges incubated at 30 °C produced methane and/or acetate, while incubation at 55 °C resulted in the formation of mainly methane. Interestingly, CO conversion at thermophilic conditions was significantly faster, despite the fact that the sludges were not acclimated to high temperature. Thermophilic conditions triggered a change in the metabolic route for CO conversion; at 30 °C, conversion of CO to methane was dependent on homoacetogenic CO degraders and acetoclastic methanogens, while at 55 °C, hydrogenogenic CO degraders and hydrogenotrophic methanogens were the main players. Guiot et al. (2011) also observed a higher CO conversion potential during batch incubation of anaerobic granular sludge under thermophilic conditions (fivefold higher than under mesophilic conditions). However, in these studies, the effect of long-term exposure to CO was not studied; nor the microbial composition of the sludges was analyzed. In the present work, we intend to obtain insight into the effect of long-term exposure of a thermophilic anaerobic sludge to syngas and CO, by studying both microbial physiology aspects and community composition after subsequent culture transfer in the presence of syngas/CO.

Material and methods

Source of inoculum

Anaerobic suspended sludge, obtained from an anaerobic reactor treating the organic fraction of municipal solid wastes (Barcelona, Spain), was used as seed sludge for starting the enrichment series under thermophilic conditions (55 °C).

Medium composition

A phosphate-buffered mineral salt medium (20 mM, pH 7.0) was used for cultivation of the enrichment cultures. The medium contained the following (grams per L): Na_2HPO_4, 1.63; NaH_2PO_4, 1.02; NH_4Cl, 0.3; $CaCl_2.2H_2O$, 0.11; $MgCl_2.6H_2O$, 0.10; NaCl, 0.3; resazurin, 0.05. Acid and alkaline trace elements solutions (1 mL per L of each) and vitamins solution (0.2 mL per L) were also supplemented to the medium (Stams *et al.*, 1993). The mineral medium was dispensed into serum bottles, sealed with butyl rubber septa and aluminum crimp caps, and flushed with a mixture of H_2/CO_2 (80:20 vol/vol) or with N_2. Synthetic syngas mixture (60% CO, 30% H_2, 10% CO_2) or pure CO was added to the bottles' headspace to the desired final partial pressure using a syringe. Final total gas pressure in the bottles' headspace was 1.75 bar. The bottles were autoclaved for 20 min at 121 °C. Before inoculation, mineral medium was reduced with sodium sulfide to a final concentration of 2 mM. All the inoculations and transfers, as well as addition of stock solutions, were performed aseptically using sterile syringes and needles.

Enrichment cultures

Thermophilic anaerobic sludge was used for the start-up of two syngas- and CO-converting enrichment series: T-Syn and T-CO ('T' stands for thermophilic (55 °C) enrichments; 'Syn' – syngas and 'CO' – carbon monoxide) (Supporting Information, Fig. S1 for a schematic representation of the experiment setup). Later, a T-Syn-CO enrichment series was started by incubating enrichment culture T-Syn(4) with CO (the number in between parenthesis refers to the number of transfer). Enrichment cultures were developed by successive transfers of active cultures (10% v/v) into fresh medium with the headspace filled with increasing concentrations of CO [from 5% (pCO = 0.09 bar) to 50% (pCO = 0.88 bar)]. Syngas was diluted with H_2/CO_2, and pure CO was diluted with N_2, to obtain the desired final CO partial pressure (total pressure was kept constant at 1.75 bar). The enrichments were carried out in duplicate. Bottles were incubated with agitation (100 r.p.m.) and in the dark.

Analytical methods

Gas samples were analyzed by GC with a GC-Chrompack 9001 with a thermal conductivity detector and equipped with two columns: a Porapack Q (100–180 mesh) 2 m × 1/8″ × 2.0 mm SS column and a MolSieve 5A (80–100 mesh) 1.0 m × 1/8″ × 2.0 mm SS column. Argon was the carrier gas at a flow rate of 16 mL min^{-1}. The oven, injector, and detector temperatures were 35, 110, and 110 °C, respectively. Volatile fatty acids and alcohols were analyzed by high-performance liquid chromatography using an HPLC (Jasco, Tokyo, Japan) with a Chrompack column (6.5 × 30 mm^2) coupled to a UV detector at 210 nm and a RI detector. 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.

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DNA extraction and amplification

20-mL aliquots of well-homogenized microbial cultures were concentrated by centrifugation (13 400 ***g***, 15 min), immediately frozen, and stored at −20 °C. Total genomic DNA was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH) in accordance with the manufacturer's instructions. Bacterial and archaeal 16S rRNA genes were amplified by PCR using a Taq DNA polymerase kit (Invitrogen, Carlsbad, CA); reaction mixtures and PCR programs used were as described elsewhere (Sousa *et al.*, 2007, 2009). Primer sets U968-f/L1401-r and Bact27f/Uni1492r were used for 16S rRNA gene amplification for denaturing gradient gel electrophoresis (DGGE) and sequencing purposes, respectively (Lane, 1991; Nübel *et al.*, 1996). Primer set arch109f/Uni1492r was used for archaeal 16S rRNA gene amplification (Lane, 1991; Grosskopf *et al.*, 1998). A 40-bp GC-clamp was added at the 5′ end sequence of the primer U968-f (Muyzer *et al.*, 1993). Size and yield of PCR products were estimated by electrophoresis in a 1% agarose gel (wt/vol), using a 100-bp BLUE extended DNA ladder (Bioron, Ludwigshafen, Germany) and ethidium bromide staining.

DGGE analysis

DGGE analysis of the PCR products was performed using the DCode system (Bio-Rad, Hercules, CA). Gels containing 8% (wt/vol) polyacrylamide (37.5:1 acrylamide/bisacrylamide) were used with a linear denaturing gradient of 30–60%, with 100% of denaturant corresponding to 7 M urea and 40% (vol/vol) formamide. Electrophoresis was performed for 16 h at 85 V and 60 °C in a 0.5x Tris–Acetate–EDTA buffer. DGGE gels were stained with silver nitrate (Sanguinetti *et al.*, 1994) and scanned in an Epson Perfection V750 PRO (Epson). DGGE gels were scanned at 400 dpi and analyzed using the BioNumericsTM software package (version 5.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium). The normalized banding patterns were used to generate dendrograms by calculating the Pearson's product moment correlation coefficient (Cole *et al.*, 2003). The unweighted pair group method with arithmetic averages (UPGMA) was further applied for clustering.

Cloning and sequencing

PCR products obtained from the enrichment samples genomic DNA were purified using the PCR cleanup kit NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). After purification, PCR amplicons were ligated into the pGEM-T vector using the pGEM Easy Vector Systems (Promega, Madison, WI) and introduced into competent *Escherichia coli* 10G (Lucigen Corporation, Middleton, MI), according to the manufacturer's instructions. Positive transformants were selected (by blue/white screening) and grown in appropriate media supplemented with ampicillin. Insert size was confirmed by PCR amplification with the pGEM-T-specific primers PG1-f and PG2-r to confirm the size of the inserts. To assign the composition of the predominant community visualized in the DGGE patterns, nearly full-length bacterial 16S rRNA gene fragments, retrieved from enrichment cultures, were used to construct clone libraries. Amplified ribosomal DNA restriction analysis (ARDRA) was used to screen clone libraries for redundancy as described elsewhere (Sousa *et al.*, 2009). Clones with the same electrophoretic mobility as that of predominant bands of DGGE patterns were purified using the NucleoSpin Extract II purification kit and subjected to nucleotide sequence analysis. Sequencing reactions were performed at Biopremier (Lisbon, Portugal) using pGEM-T vector-targeted sequencing primers SP6 and T7 and internal specifically tailored primers, when needed. Partial sequences were assembled using the Contig Assembly Program application included in the BioEdit, version 7.0.9 software package (Huang, 1992; Hall, 1999). Consensus sequences obtained were checked for potential chimera artifacts using Bellerophon software (Huber *et al.*, 2004).

Phylogenetic analysis and nucleotide sequence accession numbers

Similarity searches for the 16S rRNA gene sequences derived from the clones were performed using the NCBI BLAST search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990). Nucleotide sequences obtained in this study have been deposited in the European Nucleotide Archive under accession numbers HF562211 to HF562214.

Results

Syngas and CO conversion by thermophilic enrichments

T-Syn cultures were initially incubated with low-CO-content syngas (5%, pCO = 0.09 bar), and by the third transfer, CO concentration was doubled (pCO = 0.18 bar). Carbon monoxide and H_2 were converted to methane by the initial T-Syn enrichments (Table 1). After 3 transfers, T-Syn(3) cultures were able to convert about 82% of the initially supplied CO to methane. However, in T-Syn(4), acetate was the main product detected from CO conversion, and methane was no longer produced. T-CO cultures, which were initially incubated with 20%

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Table 1. Syngas conversion by initial thermophilic (T-Syn(x)) enrichment cultures (P_{total} = 1.75 bar); x represents the number of successive transfers

	T-Syn (initial transfers)		
	T-Syn(1) (5% CO)	T-Syn(3) (10% CO)	T-Syn(4) (10% CO)
Incubation time (days)	14	33	27
Substrate composition			
Carbon monoxide ($mmol\ L^{-1}{}_{medium}$)	2 ± 1	6 ± 0	5
Carbon dioxide* ($mmol\ L^{-1}{}_{medium}$)	28 ± 1	17 ± 1	21
Hydrogen ($mmol\ L^{-1}{}_{medium}$)	93 ± 3	92 ± 0	83
Substrate utilization			
Carbon monoxide (%)	100 ± 0	82 ± 4	100
Hydrogen (%)	100 ± 0	100 ± 0	0
Products formed			
Acetate (mM)	0	0.8	2.1
Methane (mM)	22 ± 2	20 ± 1	0

*Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using the Henry law. Concentration of gases is expressed as milli mol per liter of medium.

CO (pCO = 0.35 bar), and T-Syn-CO cultures [deriving from T-Syn(4)] did not produce methane during the entire experiment. Acetate was the main product formed in T-Syn-CO(1), while T-CO (1) converted CO to mainly hydrogen (data not shown). 16S rRNA gene archaeal-specific PCR indicated the presence of methanogens in the inoculum sample and in the T-Syn samples corresponding to the first 3 initial transfers [T-Syn(1) to T-Syn(3)]; all the other enrichments were negative for the presence of archaeal phylotypes (data not shown).

T-Syn, T-CO, and T-Syn-CO were successfully subcultured for over 1 year (T-Syn, 16 transfers with syngas; T-CO, 9 transfers with CO; and, T-Syn-CO, 4 transfers with syngas followed by 9 transfers with CO), leading to microbial enrichments with stable physiological properties. Highly enriched T-Syn and T-Syn-CO cultures produced mainly acetate from syngas or CO (Fig. 1a and 1b), while in T-CO-enriched cultures, hydrogen was primarily formed and only further converted to acetate (Fig. 1c).

In T-Syn cultures (Fig. 1a), there was a slight increase in H_2 concentration, while CO decreased, suggesting that about 3 $mmol\ L^{-1}{}_{medium}$ of CO was converted to H_2, likely according to Equation 1. The remaining CO (16.1 ± 2.6 $mmol\ L^{-1}{}_{medium}$) was converted to acetate, which was associated with H_2 consumption. This could result from the combination of distinct acetate-producing routes, that is, directly from CO (Eqn. 2) or from the reaction between CO with hydrogen (Eqn. 3). Additional acetate is likely being produced from CO_2/H_2 (Eqn. 4) because, even after complete CO depletion, CO_2 and H_2 decrease steadily. At the end of the incubation, 9.4 ± 1.1 mM acetate had accumulated, which accounts for approximately 97% of product recovery (considering maximum theoretical acetate production from the consumed CO and H_2). Propionate was the only other organic compound produced (maximum 1 ± 0.1 mM); methane or alcohols were not detected in the T-Syn incubation.

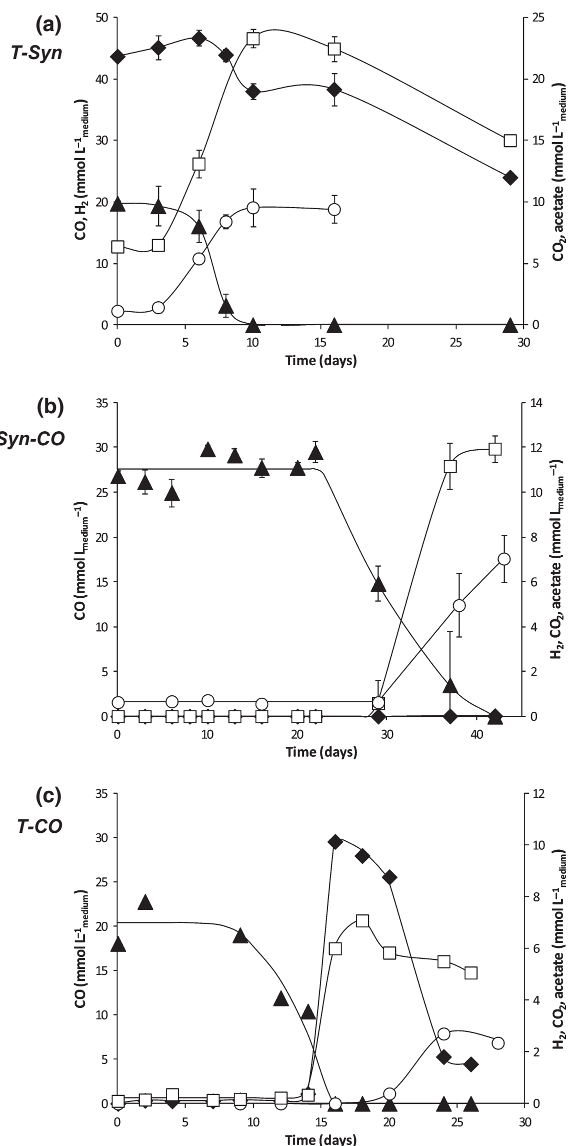

Fig. 1. Substrate consumption and product formation by enriched syngas- and CO-degrading cultures. (a) T-Syn, (b) T-Syn-CO, and (c) T-CO. Symbols: (▲) carbon monoxide, (♦) hydrogen, (□) carbon dioxide, and (○) acetate. Gas products values are expressed in relation to volume of medium, i.e. mmol of gas measured in the headspace divided by the volume of liquid medium. Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using the Henry law.

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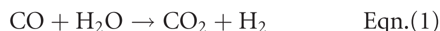
$$CO + H_2O \rightarrow CO_2 + H_2 \quad \text{Eqn.(1)}$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad \text{Eqn.(2)}$$

$$2CO + 2H_2 \rightarrow CH_3COOH \quad \text{Eqn.(3)}$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad \text{Eqn.(4)}$$

Acetogenic activity from CO/H_2 (Eqn.3) seems also to be predominant in cultures T-Syn-CO; in these cultures, 25.6 ± 2.3 mmol $L^{-1}{}_{medium}$ CO was converted to 7 ± 1 mM acetate and 12 ± 0.6 mmol $L^{-1}{}_{medium}$ CO_2 (Fig. 1b). Conversely, in cultures T-CO, an intermediary formation of hydrogen points to a hydrogenogenic route for CO conversion (Eqn.1); by day 16 of incubation, 10 mmol $L^{-1}{}_{medium}$ H_2 has been produced from CO in T-CO cultures (Fig. 1c). However, extension of the incubation time led to the consumption of H_2/CO_2, with the formation of 2.4 mM acetate (Eqn.4).

Molecular characterization of the thermophilic enrichments

Microbial communities present in the thermophilic enrichment series were analyzed by DGGE fingerprinting of PCR-amplified 16S rRNA gene fragments (Fig. 2).

The decrease in the number of predominant bands, from the inoculum sample profile to subsequent transfers, indicates a rapid reduction in bacterial richness. Similarity coefficients of 52%, 33%, and 35% between the inoculum and the samples T-Syn(9), T-Syn-CO(4), and T-CO(5), respectively, were obtained. DGGE profiles from T-Syn(9) and T-Syn-CO(4) transfers onwards showed to be stable, with similarity coefficients between 90% and 97%, for both cultures. Moreover, T-Syn and T-Syn-CO enrichments showed a similar stable DGGE profile, in which only two bands are predominant – bands 1 and 2 (Fig. 2a and 2b). DGGE profile obtained for T-CO was clearly different (Fig. 2c). DGGE profile of T-CO had also two predominant bands – bands 3 and 4 (Figs 2c and 3). During the first period of conversion, corresponding to CO conversion to H_2, band 3 is the predominant band (Fig. 3, sampling point T-CO(9)-1). With the extension of incubation time, and further H_2 depletion, band 4 became also predominant (Fig. 3, sampling point T-CO(9)-2).

Bands 1–4 were identified by 16S rRNA gene sequencing (Table 2). Predominant ribotypes in T-Syn and T-Syn-CO (bands 1 and 2) were most closely affiliated with the genera *Desulfotomaculum* (*D. australicum*, 98% 16S rRNA gene identity) and *Caloribacterium* (*C. cisternae*, 94% 16S rRNA gene identity). Culture T-CO was composed also of two predominant microorganisms (bands 3 and 4), clustering with the genera *Thermincola* (*T. carboxydiphila*, 99% 16S rRNA gene identity) and *Thermoanaerobacter* (*T. thermohydrosulfuricus*, 97% 16S rRNA gene identity).

Discussion

Using thermophilic methanogenic sludge as inoculum, syngas/CO-converting thermophilic communities were obtained after an enrichment period of more than 1 year. The microbial diversity of the cultures declined rapidly upon subculturing. Methanogenic activity of the sludge was rapidly affected by CO. Methane was not produced when the anaerobic sludge was incubated with CO alone (culture T-CO), and in the incubations with syngas,

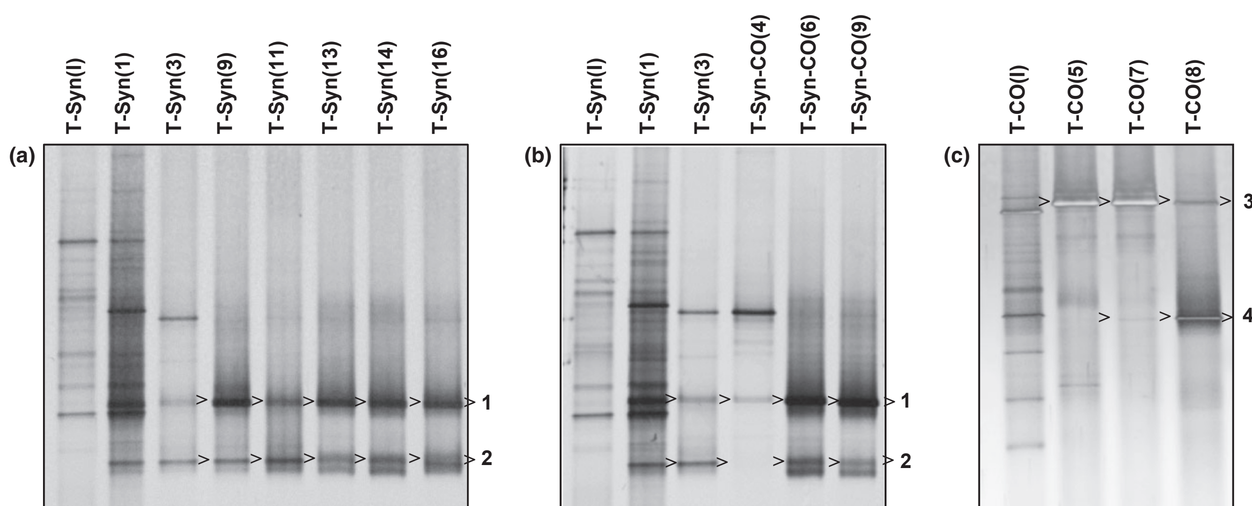

Fig. 2. Bacterial DGGE profile of the thermophilic enrichment series (a) T-Syn, (b) T-Syn-CO and (c) T-CO. I, inoculum; T-Syn(x), T-Syn-CO(x) and T-CO(x), enrichment cultures, where x represents the number of successive transfers at sampling point.

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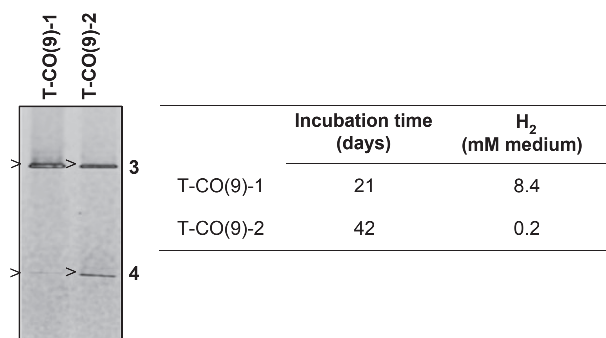

	Incubation time (days)	H_2 (mM medium)
T-CO(9)-1	21	8.4
T-CO(9)-2	42	0.2

Fig. 3. Bacterial DGGE profile of T-CO enrichment in two sampling points during 9th transfer [T-CO(9)]. T-CO(9)-1: early incubation; hydrogen production. T-CO(9)-2: later incubation; hydrogen depletion.

methane production ceased after 4 subsequent transfers. Although there are some methanogenic archaea that are able to convert CO to methane (e.g. *Methanosarcina barkeri, Methanosarcina acetivorans,* and *Methanothermobacter thermautotrophicus*), most methanogens do not tolerate CO (Rother & Metcalf, 2004; Henstra *et al.*, 2007; Guiot *et al.*, 2011). Moreover, acetogenic and hydrogenogenic CO-converting microorganisms grow faster than methanogens (Sipma *et al.*, 2006). Carbon monoxide partial pressure might also influence microbial dynamics; the faster inhibition of the methanogenic activity in T-CO might be related to the higher CO partial pressure applied in the beginning of the enrichment series, when compared to T-Syn (pCO of 0.35 and 0.09 bar for T-CO(1) and T-Syn(1), respectively). Nevertheless, one cannot disregard the presence of H_2 in the syngas mixture that is a direct substrate of hydrogenotrophic methanogens; methanogenic activity in syngas initial enrichments might partially rely on the H_2 present in the substrate. Guiot *et al.* (2011) reported a complete inhibition of methanogenesis in anaerobic sludge subjected to CO partial pressure between 0.30 and 0.83 bar. Also, methane production by *M. thermautotrophicus* from CO is inhibited by a CO partial pressure above 0.3 bar (Daniels *et al.*, 1977). Acetogenic and hydrogenogenic bacteria seem to be more tolerant to CO. For instance, growth of the acetogen *Blautia producta* is stimulated with increasing CO up to a value of CO partial pressure of 0.8 bar, and lag phases appear only at CO partial pressure of 1.5 bar and above (Vega *et al.*, 1989). Also, no lag phase was observed on H_2 production by *Desulfotomaculum carboxydivorans* with 1.8 bar CO (without sulfate) (Parshina *et al.*, 2005).

Enriched cultures T-Syn and T-Syn-CO converted CO to mainly acetate, while cultures T-CO showed a two-phase profile – H_2 was produced from CO and subsequently converted to acetate. DGGE analysis of the predominant bacterial populations present in the enriched cultures revealed phylotypes affiliated with only a few genera: *Desulfotomaculum* and *Caloribacterium* species were predominant in T-Syn and T-Syn-CO, while *Thermincola*- and *Thermoanaerobacter*-related bacteria were predominant in T-CO. Carbon monoxide conversion to acetate and hydrogen by *Desulfotomaculum* species has been reported (Plugge *et al.*, 2002; Parshina *et al.*, 2005; Henstra *et al.*, 2007), and it is likely that the *Desulfotomaculum* sp. in T-Syn and T-Syn-CO cultures is growing on CO. The other predominant bacterium identified in T-Syn and T-Syn-CO enrichments is closely related to *Caloribacterium cisternae*, which is a recently described new species and genus of the *Thermoanaerobacteraceae* family (Slobodkina *et al.*, 2012). The low 16S rRNA gene sequence identity of these microorganisms with *Caloribacterium cisternae* strain SGL43^T (94%) makes it difficult to speculate about their physiological capabilities, especially with respect to the utilization syngas and CO. *C. cisternae* is not known to convert CO.

In T-CO, CO was first converted to H_2 and CO_2, and later, acetate was formed. These results can be directly linked to the microbial community. According to the physiological characteristics of known *Thermincola* species

Table 2. Phylogenetic affiliations of cloned 16S rRNA gene sequences corresponding to the identified bands in DGGE profiles

Band ID	Phylum*	Class*	Relative abundance†	Closest relatives	Identity	Accession no.
1	*Firmicutes*	*Clostridia*	67.5%	*Desulfotomaculum* sp. Hbr7	99%	HF562211
				Desulfotomaculum australicum strain AB33	98%	
2	*Firmicutes*	*Clostridia*	27.9%	Thermophilic anaerobic bacterium K1L1	96%	HF562212
				Caloribacterium cisternae SGL43^T	94%	
3	*Firmicutes*	*Clostridia*	79.2%	*Thermincola* sp. JR	99%	HF562213
				Thermincola carboxydiphila strain 2204	98%	
4	*Firmicutes*	*Clostridia*	7.2%	*Thermoanaerobacter thermohydrosulfuricus* strain JCM 9674	97%	HF562214

*Classified using the RDP Naïve Bayesian Classifier.

†Relative abundance of clones with identical ARDRA profiles (calculated from a total of 74 and 69 clones retrieved from T-Syn/T-Syn-CO and T-CO cultures, respectively).

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[that are able to oxidize CO into H_2 and CO_2 (Sokolova *et al.*, 2005)], it is plausible to infer that *Thermincola*-related bacteria are responsible for hydrogenogenic CO conversion in T-CO cultures. In fact, the band present in T-CO DGGE profile corresponding to a *Thermincola*-like organism is always present and intense when H_2 is produced (Fig. 3). After extended incubation of T-CO, it was observed that the H_2 produced has been used for acetate conversion. DGGE profile of the same culture at this sampling point showed an increase in the band intensity corresponding to the *Thermoanaerobacter*-like bacterium. Results from DGGE profiles together with physiological response of T-CO after extended incubation strongly suggest that *Thermoanaerobacter*-related bacterium converts H_2/CO_2 to acetate. Although *T. thermohydrosulfuricus* strain E100-69^T is not able to convert H_2/CO_2 to acetate (data not shown), *T. kivui* strain LKT-1^T (also a CO-oxidizing bacterium) forms acetate from H_2/CO_2 (Kevbrina *et al.*, 1996).

Bacteria of the genera *Desulfotomaculum* and *Thermincola* and *Caloribacterium* and *Thermoanaerobacter* are associated with syngas and/or CO conversion in thermophilic enrichment cultures. Using the same thermophilic sludge, specialization of the microbial communities depended on the start-up of the experiments. Considering the possibility of using syngas for the production of biofuels and specifically of a microbial process for obtaining H_2-enriched syngas, it could be interesting to previously submit anaerobic biomass to high CO partial pressures in order to select for hydrogenogenic microorganisms. Methane production should also be possible, but more studies on CO toxicity are needed; longer incubation times might be necessary to avoid that methanogens are outcompeted by acetogenic bacteria. Syngas is produced at high temperatures (by gasification processes), and CO occurs naturally at high-temperature environments, such as hot springs or volcanoes – more information about the microbiology of syngas and/or CO conversion at thermophilic conditions can be retrieved from these environments. Moreover, studies on the behavior of anaerobic CO-converting mixed cultures in continuous operation are necessary.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Flowsheet diagram of the experimental procedure used for obtaining syngas/CO thermophilic enrichments.

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