Enrichment and microbial characterization of syngas converting anaerobic cultures

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Abstract
Bioconversion of recalcitrant biomass/waste into bulk chemicals or biofuels is often not feasible. By gasification of these materials, syngas (mainly composed of CO_2, CO and H_2) is generated and can be used for the production of high value compounds by thermochemical or biotechnological processes. Here, three thermophilic cultures enriched with syngas mixtures or pure CO (T-Syn, T-Syn-CO and T-CO) were studied. Stable enriched cultures obtained by subsequent transfers for over a year, convert syngas/CO to mainly acetate and hydrogen (CO partial pressure up to 0.88 bar). 16S rRNA based techniques (PCR-DGGE) showed that predominant microorganisms in the cultures belonged to *Desulfotomaculum*, *Caloribacterium*, *Thermincola* and *Thermoanaerobacter* genera. Moreover, from the syngas- and CO-degrading cultures, a novel *Thermoanaerobacter* sp. (strain PCO) and a novel *Moorella* sp. (strain E3-O) were isolated.

Keywords
Syngas; carbon monoxide; enrichments; PCR-DGGE; microorganisms; isolation.

INTRODUCTION
Syngas (gaseous mixture composed of mainly H_2, CO and CO_2) can be produced from a vast array of feed stocks, such as petrol fuels, lignocellulosic biomass and carbon-based wastes (Sipma et al., 2006). Production of biofuels and other products from syngas by thermochemical or microbial processes is a promising technological development. Microbial conversion of syngas was thus far mainly directed to the production of ethanol (Datar et al., 2004; Wilkins & Atiyeh, 2011). However, other products such as butanol, acetic acid, butyric acid, hydrogen and methane can be obtained from syngas as well (Guiot et al., 2011; Henstra et al., 2007). The microbiology of syngas conversion to biofuels has been the subject of several reviews (Henstra et al., 2007; Oelgeschlager & Rother, 2008; Sokolova et al., 2009). Several mesophilic anaerobic microorganisms, e.g. *Clostridium carboxidivorans, C. autoethanogenum* and *Butyribacterium methylotrophicum*, produce short-chain fatty-acids and alcohols from CO and H_2. Mesophilic and thermophilic carboxydotrophic hydrogenogenic bacteria, e.g. *Carboxydothermus hydrogenoformans* and *Desulfotomaculum carboxydivorans* convert CO and H_2O to H_2 and CO_2. Direct conversion of CO to CH_4 can be achieved by *Methanosarcina* species. Thus far, syngas and CO conversion was mainly studied using pure cultures or defined co-cultures. Mixed culture approaches for the conversion of these substrates has received little attention and microbial community diversity linked to CO conversion was never assessed. In this study microorganisms involved in syngas and CO batch conversion by anaerobic sludge were investigated. In addition, two new bacterial strains were isolated and characterized.

MATERIAL AND METHODS
Three thermophilic enrichments were started at 55ºC, with anaerobic suspended sludge that was not previously exposed to syngas or CO as inoculum (from a methanogenic digester treating the organic fraction of municipal solid wastes) and using syngas (60% CO, 30% H_2, 10% CO_2) or CO as sole carbon and energy source: T-Syn, T-Syn-CO and T-CO ("T" represents thermophilic; "Syn" and "CO" corresponds to syngas or carbon monoxide used as substrate, respectively). Different amounts of syngas or CO were added to bottles' headspace based on the intended final CO partial pressure

(pCO ranged from 0.09 to 0.88 bar; all the assays were conducted at a total pressure of 1.75 bar, by pressurization with H_2/CO_2 or N_2). A phosphate-buffered mineral salt medium was used for cultivation of the enrichment cultures and for subsequent bacteria isolation. Cultures were transferred to fresh medium once CO was completely used. During incubation, headspace composition was analyzed by GC and fatty-acids and alcohols in the liquid by HPLC. Bacterial strains were isolated from the enriched cultures using serial dilutions, pasteurization, autoclaving and incubation with different substrates. Microbial community changes during enrichment were monitored by 16S rRNA-based techniques (PCR-DGGE). Predominant microorganisms present in stable enrichments were identified by cloning and sequencing. Similarity searches for the 16S rRNA gene sequences were performed using the NCBI BLAST search program within the GenBank database. Alignment of the 16S rRNA sequences was performed by using the FastAligner V1.03 tool of the ARB program package. The neighbor joining method was used for the construction of a 16S rRNA gene based phylogenetic tree.

RESULTS AND DISCUSSION
Syngas and CO conversion by thermophilic enrichments
By repeated transfer in fresh medium, stable enrichments converting 50% of CO in the headspace (pCO = 0.88bar) were obtained. The T-Syn enrichment formed acetate and CO_2 as main products from CO. From an initial substrate composed of 20 mmol L^{-1} CO, 44 mmol L^{-1} H_2 and 6 mmol L^{-1} CO_2, 100% of the CO and 45% of the H_2 were converted, resulting in the production of 15 mmol L^{-1} CO_2 and 10 mmol L^{-1} acetate (Figure 1a). This could result from the combination of two distinct acetate-producing routes, i.e. directly from CO ($4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$) and/or indirectly via hydrogen ($4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$). CO conversion by culture T-Syn-CO, resulted in the production of acetate (7 mmol L^{-1}) and CO_2 (12 mmol L^{-1}) (Figure 1b). Such product profile is in accordance with following equation: $4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$. The main products detected from the conversion of CO by T-CO were H_2 and CO_2, and acetate (Figure 1c). Most of the substrate (18 mmol L^{-1} CO) was converted to H_2 in a first step of conversion, following the equation $CO + H_2O \rightarrow H_2 + CO_2$. After total conversion of CO, there was a decrease of the H_2 concentration (from 10 mmol L^{-1} to 1 mmol L^{-1}) indicating that the H_2 formed was used for acetate production (Figure 1c).

(a)   **(b)**   **(c)**
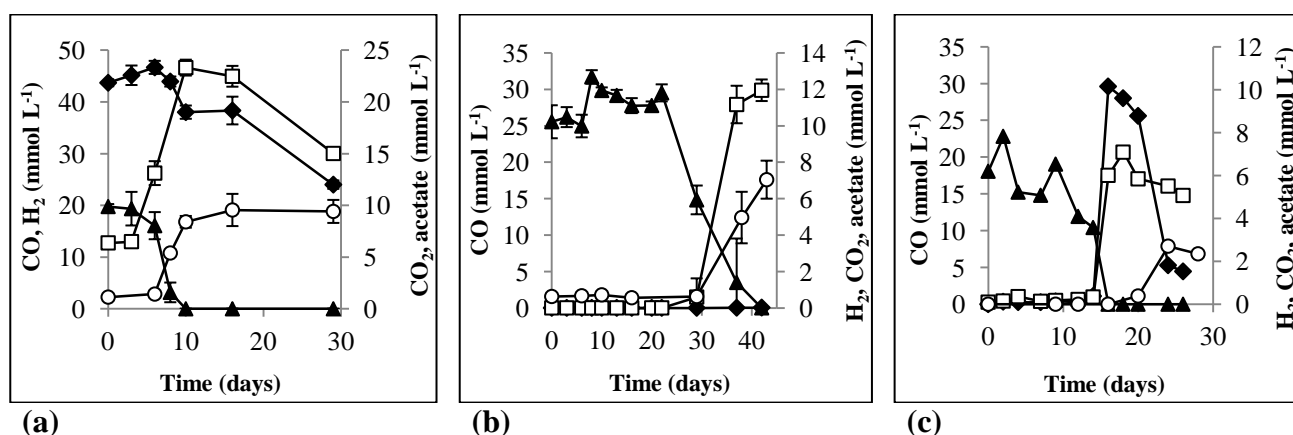
Figure 1. Syngas conversion by T-Syn **(a)**, and CO conversion by T-Syn-CO **(b)** and by T-CO **(c)**. Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using the Henry law. Symbols: (▲) carbon monoxide, (♦) hydrogen, (□) carbon dioxide, and (○) acetate.

Microbiology of thermophilic syngas conversion and bacteria isolation
Based on the DGGE profiles, each enriched culture was mainly composed of two predominant microorganisms (Figure 2). Enrichments T-Syn and T-Syn-CO were composed mostly of two bacteria related to *Desulfotomaculum* (clone SYN-1, band 1) and *Caloribacterium* (clone SYN-2,

band 2) (Figures 2 and 3). Bacteria of the genera *Thermincola* and *Thermoanaerobacter* were present in the T-CO enrichments (clone CO-3, band 3 and clone CO-4, band 4, respectively) (Figures 2 and 3). The presence of bacteria belonging to *Desulfotomaculum*, 98% identity to *Desulfotomaculum australicum*, in T-Syn and T-Syn-CO cultures suggests that these bacteria play an important role in CO conversion. Some *Desulfotomaculum* species with the ability to use CO, producing H_2 or acetate, were described. The other dominant bacterium in both enrichments is closest related to *Caloribacterium cisternae*, but 16S rRNA genes identity is only 94%. CO utilization by this bacterium was not reported. In the T-CO enrichment, the predominant microorganisms were closely related to *Thermincola carboxydiphila* (99% 16S rRNA gene identity) and to *Thermoanaerobacter thermohydrosulfuricus* (97% 16S rRNA gene identity) (Figure 3). All the described *Thermincola* species have the ability to convert CO and produce H_2 and CO_2, which suggests that the *Thermincola*-like bacteria in T-CO culture were responsible for CO conversion to H_2. The role of the *Thermoanaerobacter*-like bacterium is not yet clear. *Thermoanaerobacter thermohydrosulfuricus* subsp. *carboxydovorans* is able to convert CO into H_2, but a *Thermoanaerobacter* strain that we have isolated was unable to use CO (see below). Our results showed a different specialization of the microbial communities in the enriched cultures, suggesting that start-up of the experiments had influence on the further evolution of microbial communities. Two novel bacterial strains were isolated from the obtained enriched cultures. A *Thermoanaerobacter* sp. (strain PCO) was isolated from the T-Syn enriched culture. The bacterium is closest related to *Thermoanaerobacter thermohydrosulfuricus* (97% identity based on 16S rRNA) (Figure 3). Strain PCO does not utilize CO, but is able to grow in the presence of high CO concentration (1.75 bar). A *Moorella* sp. (strain E3-O), phylogenetically related to *Moorella glycerini* (97% idendity based on 16S rRNA) (Figure 3), was isolated from the T-CO enrichment. Strain E3-O is a hydrogenogenic carboxydotrophic bacterium that converts CO to hydrogen, but not to acetate.
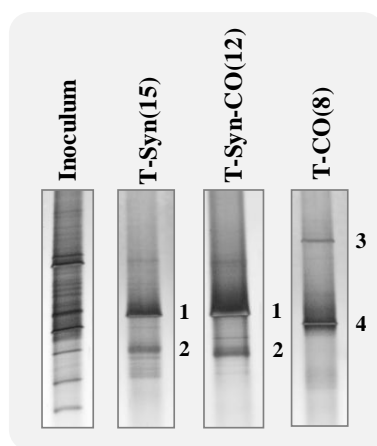

Figure 2. Bacterial DGGE profile of stable thermophilic enrichment series T-Syn, T-Syn-CO and T-CO (after 15, 12 and 8 successive transfers, respectively).

Our results point to unknown physiological properties of known anaerobic bacteria. Despite the fact that methanogenic sludges were used as starting material, in none of the cultures methanogens were enriched, which may point to the sensitivity of thermophilic methanogens for high levels of CO. Futhermore, high CO partial pressure seems to inhibit methane formation and stimulate acetate production. Besides, acetogenic and hydrogenogenic CO-converting microorganisms have much lower doubling times than methanogens (Sipma et al., 2006), which give them a kinetic advantage in the enrichments. Further research using different inocula, applying different conditions to the enrichments series and/or studying more in detail the inocula and the obtained cultures with the isolation and identification of other microorganisms, will lead us to better understand the effect of CO on mixed cultures. The isolation of high yielding syngas/CO-rich waste gas degrading bacteria is necessary for successful commercialization of syngas fermentation technology.
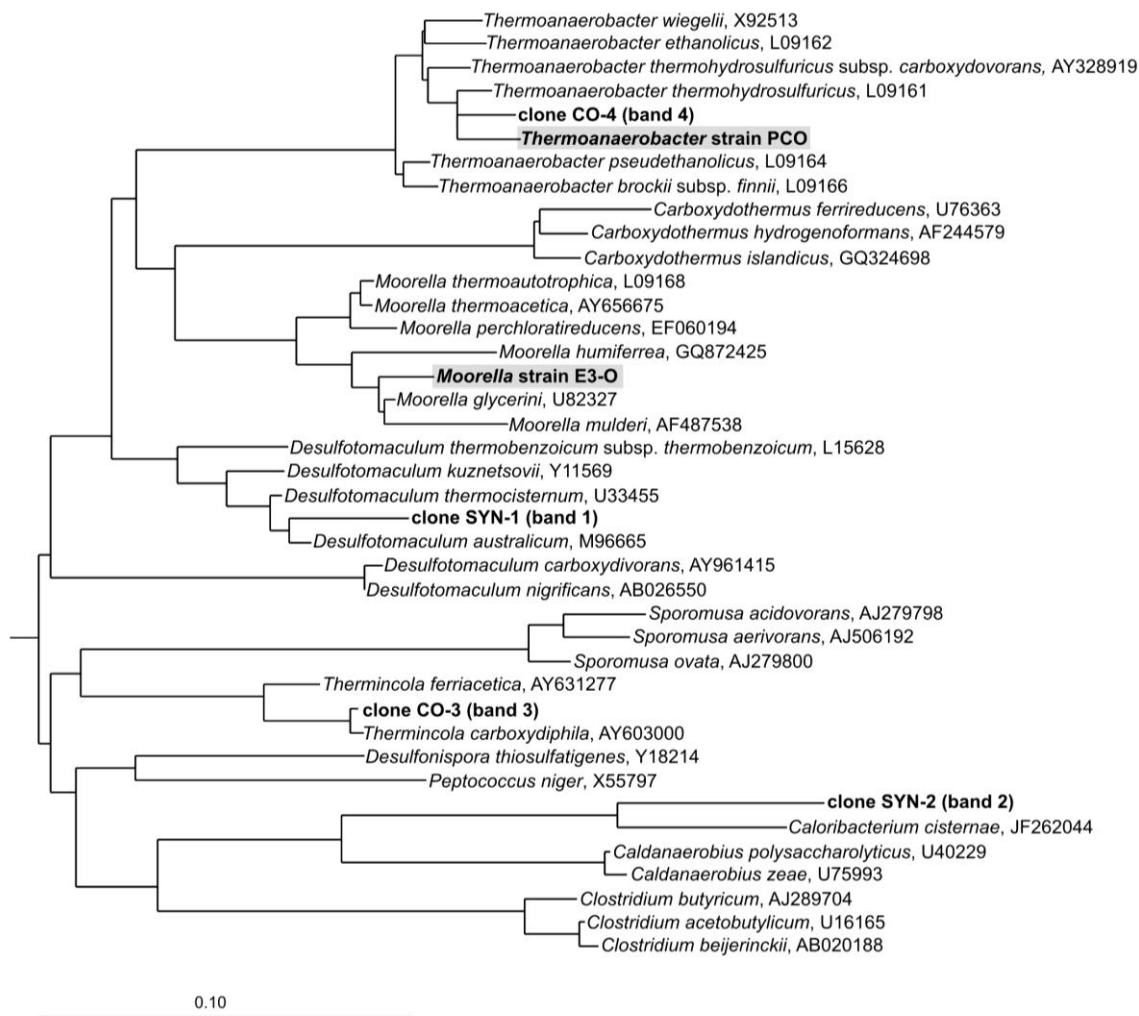
Figure 3. Phylogenetic tree of 16S rRNA gene sequences showing the position of all the clones from the enriched cultures, and the two isolated strains - strain E3-O^T and strain PCO^T - relative to other selected reference sequences of bacteria. Bar, 10% sequence divergence.

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