# Bioaugmentation of biogas production by a hydrogen-producing bacterium

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## HIGHLIGHTS

- Addition of $H_2$ producing *Enterobacter cloacae* augmented biogas production.
- *E. cloacae* became a stable member of the biogas producing microbial community.
- Addition of *E. cloacae* significantly altered the community composition.
- Polymer degrading *Clostridiales* increased their abundance dramatically.
- A syntrophic relationship between polymer degradation and in situ $H_2$ generation is suggested.

## ARTICLE INFO

## ABSTRACT

The rate-limiting nature of the hydrogen concentration prevailing in the anaerobic digester has been recognized, but the associated alterations in the microbial community are unknown. In response to the addition of *Enterobacter cloacae* cells in laboratory anaerobic digesters, the level of biogas production was augmented. Terminal restriction fragment length polymorphism (T-RFLP) and real-time polymerase chain reaction (Real-Time PCR) were used to study the survival of mesophilic hydrogen-producing bacteria and the effects of their presence on the composition of the other members of the bacterial community. *E. cloacae* proved to maintain a stable cell number and to influence the microbial composition of the system. Bioaugmentation by a single strain added to the natural biogas-producing microbial community was demonstrated. The community underwent pronounced changes as a result of the relatively slight initial shift in the microbiological system, responding sensitively to the alterations in local hydrogen concentration.

## 1. Introduction

Among the renewable energy production technologies, biogas generation offers outstanding benefits. In a biogas reactor, various organic wastes and by-products can be converted to a useful energy carrier and the product biogas can be utilized in any application where fossil natural gas is employed today. The biogas industry is developing rapidly in terms of both engineering and biotechnology. Biogas, a mixture primarily of $CO_2$ (35–40%) and $CH_4$ (55–60%) is produced by a specialized microbial consortium. The composition of this sophisticated microbial food chain is complex, comprising several hundreds of microbial species in varying abundances. Through classical microbiological approaches, such communities can be studied only superficially. Molecular biological tools can help identify the species involved and provide an insight into the intricate metabolic pathways (Cater et al., 2013).

The attainment of improvements in biogas production technology is a challenging endeavor with the aim of achieving an understanding of the operational mechanism of the microbial community, whereby the competitiveness of this energy carrier relative to the fossil fuels can be enhanced. Several methods have been suggested for the intensification of biogas productivity, e.g. the facilitation of biomass decomposition by means of various pretreatments, modification of the composition of the microbial community, or conduction of the various anaerobic digestion (AD) phases in separate reactors (Divya et al., 2015; Miah et al., 2005; Weiß et al., 2010).

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Hydrogen ($H_2$) plays important regulatory roles in the AD microbial community (Demirel and Scherer, 2008). It is evolved by the acetogenic members of the community, but its accumulation inhibits their biological activity via product inhibition (Dong et al., 1994). Hydrogenotrophic methanogens remove the $H_2$ from the system, leading to additional $CH_4$ formation directly or in collaboration with the action of syntrophic acetate oxidizers (Rivera-Salvador et al., 2014). In this way hydrogenotrophic methanogens maintain the fermentative activity of the acetogens and contribute to an enrichment of the $CH_4$ in the gaseous final product. A sensitive balance between the $H_2$ producers and consumers is critical for the optimal operation of the system. In natural biogas-producing consortia, $H_2$ generation is most probably downregulated in order to reinforce the stability of the microbiological system.

The assumed regulatory role of $H_2$ in the overall process of AD (Bagi et al., 2007) is an intriguing scientific challenge. There are also important practical consequences of opening up this bottleneck in AD. $H_2$ can be produced at large scale in both photosynthetic and fermentative technologies (Azwar et al., 2014). The storage and distribution network of this novel clean energy carrier is, however, globally underdeveloped and will require huge investments. Conversion of $H_2$ to $CH_4$ offers an easy approach to store and transmit $H_2$ energy via the existing pipelines and utilization technologies for natural gas. Similarly, the discontinuous methods to generate renewable electricity, e.g. photovoltaic or wind, can be handled in the "power-to-gas" concept by transforming electricity to $H_2$ by electrolysis and generating $CH_4$ in AD with accompanying $CO_2$ mitigation as a surplus.

To test the validity of this assumption and to examine whether $H_2$ is indeed one of the bottlenecks in the overall process, an experimental approach was employed in which a pure culture of $H_2$-producing bacteria was added to the biogas-producing community under mesophilic or thermophilic conditions, and the augmentation of the process was observed in batch AD with the use of various biomass sources (Bagi et al., 2007). It was established in a separate study that the degree and duration of the augmentation effect in thermophilic AD in continuously stirred AD reactors depended on the organic loading rate (Kovács et al., 2013). The fate of the added $H_2$ producer strain was followed in these investigations by selective PCR and sequencing of the amplified DNA fragments, but the potential rearrangement of the remaining members of the community was not examined.

In the present study, we determined whether detectable changes took place within the biogas-generating microbial community following the addition of a $H_2$-producing strain to the AD reactors under mesophilic conditions. An acetogenic bacterium, close relatives of which are normally present in the natural community (Li et al., 2013), was selected. The fate of the added *Enterobacter cloacae* cells was monitored by means of Real-Time PCR. Terminal restriction fragment length polymorphism (T-RFLP), a semiquantitative molecular fingerprinting technique which is robust, cost-effective and reproducible (Liu et al., 1997), was applied to follow the changes in the eubacterial taxonomic groups.

# 2. Methods

## 2.1. Anaerobic fermentation reactor set-up

Anaerobic digestions were carried out in identical 5-L continuously stirred tank reactors (Kovács et al., 2013) in fed-batch operational mode. The inoculum (TS 6.75% and oTS 4.92%) was from the mesophilic industrial biogas plant operated by Zöldforrás Ltd., Szeged, Hungary, involving the treatment of a mixture of maize silage, sweet sorghum and pig manure. After a 4-week start-up phase, with maize silage (TS 36.5%, oTS 34.05%) as substrate at a feeding rate of $6 \, g \, oTS \, L^{-1}$, biogas production stabilized. Thereafter, one set of reactors was inoculated with the $H_2$-producing strain, while all the other operational and feeding parameters were kept constant. *E. cloacae* DSM 16657 was purchased from DSMZ (Braunschweig, Germany). Cultivation was carried out in sterile Luria–Bertani growth medium inoculated with 5 v/v% bacterium. The Erlenmeyer flasks were held at 30 °C overnight. Cells were collected by centrifugation of $OD_{600} = 1.57$ culture and were suspended in a small volume of the liquid phase temporarily removed from the AD reactor. $10^9$ CFU of *E. cloacae* per mL reactor volume was dosed at the start of data collection. Liquid samples were withdrawn weekly in order to assess some of the operational parameters and to purify genomic DNA for further analysis.

## 2.2. Gas chromatographic analyses

The $CH_4$, $CO_2$, $H_2$ and $O_2$ levels in the gas phases were measured by gas chromatography. An Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a thermal conductivity detector and an Agilent HP-Molsieve column (length 30 m, diameter 0.320 mm, film 12.0 μm) was used in splitless mode. Linde HQ argon 5.0 gas was used as carrier gas. The temperatures of the injector, thermal conductivity detector and column were maintained at 150 °C, 160 °C and 60 °C, respectively. The column pressure was 47.618 psi. The flow rate of the column was $12 \, mL \, min^{-1}$. Samples of 50 μL were analyzed. Three biological replicates were used for the measurements. Calibration curves for each component were established by serial dilutions of the pure gases in 25-mL gas-tight vials, and data from three replicates were averaged for the calibration curve.

## 2.3. Determination of fermentation parameters

### 2.3.1. Organic dry mass (oDM)
The dry matter content was quantified by drying the biomass at 105 °C overnight and weighing the residue. Further heating of this residue at 550 °C provided the organic dry mass content.

### 2.3.2. Density measurement
Density was measured with an automatic density-meter (Grabner Instruments, MINIDENS, Wien, Austria).

### 2.3.3. C/N
To determine C/N, an Elementar Analyzer Vario MAX CN (Elementar Analysensysteme, Hanau, Germany) was employed. This works on the principle of catalytic tube combustion under an oxygen supply at high temperatures (combustion temperature: 900 °C, post combustion temperature: 900 °C, reduction temperature: 830 °C, column temperature: 250 °C). The desired components were separated from each other with the aid of specific adsorption columns (containing Sicapent, in C/N mode) and determined in succession with a thermal conductivity detector. Helium served as flushing and carrier gas.

### 2.3.4. $NH_4^+$-N
For the determination of ammonium ion content, the Merck Spectroquant Ammonium test (1.00683.0001) was used.

### 2.3.5. VOAs/TAC
5 g of fermenter sample was taken for the analysis and diluted to 20 g with distilled water. The subsequent process was carried out with a Pronova FOS/TAC 2000 Version 812-09.2008 automatic titrator.

### 2.3.6. Conductivity

The conductivity of the fermentor liquid was assessed with a WTW inoLab® 740 automated device (WTW Wissenschaftlich-Technische Werkstätten GmbH, Germany).

### 2.4. DNA extraction

1.5 mL samples, withdrawn weekly from the reactor liquid phase, were used for DNA extraction. A modified cetyltrimethyl-lammonium bromide (CTAB)-based method was employed to extract the whole genomic DNA content. Samples were centrifuged at 13,000 rpm for 10 min to pellet the cells, suspended in sucrose–EDTA–Tris (SET) buffer supplemented with lysozyme (15 mg mL$^{-1}$) and incubated at 37 °C for 2 h with continuous agitation (Ács et al., 2013). The samples were then centrifuged as before, the supernatant was discarded, and the pellet was suspended in 400 µL of SET buffer. 200 µL of 10% SDS buffer was added to the mixture to disrupt the cell wall, and to liberate the genomic DNA. The samples were placed in a water bath at 65 °C for 30 min to maximize the effect of the detergent. This was followed by another centrifugation step at 13,000 rpm for 5 min. 150 µL of 5 M NaCl and 100 µL of 10% CTAB were added to the supernatant, and the mixture was incubated in a water bath at 65 °C for another 20 min. Chloroform:isoamyl alcohol (24:1) purification was employed twice and the genomic DNA was precipitated with isopropanol. The pellet was washed with 70% chilled ethanol and dissolved in sterile bidistilled water. Its concentration was determined spectrophotometrically (NanoDrop ND-1000 Technologies, Washington, DC, USA), and its integrity was assessed by agarose gel-electrophoresis.

### 2.5. PCR amplification

All PCR reactions were carried out in an ABI 9600 Fast Thermal Cycler (Life Technologies, Carlsbad, CA, USA), targeting an approximately 500 bp region of the 16S rRNA gene by using the primer pair F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and URP519 (5′-GTATTACCGCGGCTGCTG-3′). For the T-RFLP experiments, the forward primer was replaced with a primer labeled with the fluorescent dye tetrachloro-6-carboxyfluorescein. The oligomers were purchased from Sigma (Sigma–Aldrich Co, St. Louis, MO, USA). The reaction mixture contained 1× DreamTaq reaction buffer, 200 µM of each dNTP, 0.25 µM forward and reverse primers each, 1.5 U of DreamTaq DNA polymerase and approximately 30 ng of template DNA in a final volume of 30 µL. Chemicals and enzymes were purchased from Thermo Scientific (Thermo Fisher Scientific Inc., Waltham, MA, USA). The thermal profile of the reaction was as follows: initial denaturation at 96 °C 3 min, followed by 26–30 cycles of denaturation at 96 °C for 30 s, annealing at 52 °C for 30 s and elongation at 72 °C for 1 min. A 10-min final extension step was added in order to allow the polymerase to finish any incomplete DNA strands.

The PCR products were separated by electrophoresis on 1% agarose gel (100 V, 26 min) using Tris/acetate buffer, and visualized with ethidium-bromide under UV light (Green and Sambrook, 2012). When necessary, the PCR product was purified with a PCR clean-up kit (Viogene, Taiwan), following the recommendations of the manufacturer, eluted in 30 µL, and stored at −20 °C.

### 2.6. Real-Time PCR

Appropriate PCR primer pairs were designed (HycE_F2 5′-TGT TGCCGCGCAGCATGTAG-3′ and HycE_R2 5′-TGACCGGCGACAACCA GAAG-3′), with the use of Primer Express® 2.0 software (Life Technologies, Carlsbad, CA, USA) to amplify an approximately 100-bp product from the large subunit of the hydrogenase III gene of *E. cloacae*.

Sensitivity studies were performed to determine the lowest amount of DNA that can be used as positive control. The sensitivity measurements were carried out with pure genomic DNA, creating serial dilutions.

All PCR reactions were performed in a 7500 Fast Real-Time system, with Power SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA). The original concentration of the standard genomic DNA was 150 ng µL$^{-1}$. Five dilutions were made; each of them was attenuated 10 times relative to the previous one. Real-Time PCR plates included non-template controls, standards, positive controls and various samples in three parallel measurements. The reactions contained the primers (1.5 pg µL$^{-1}$), the template in various amounts (1.5–200 ng µL$^{-1}$), SYBR® Green PCR Master Mix (12.5 µL), and water to a final volume of 25 µL. The temperature profile was as follows: 10-min initial enzyme activation at 95 °C, followed by 40 consecutive cycles at 95 °C for 15 s, and termination at 60 °C for 1 min.

### 2.7. Clone library preparation

Parallel clone libraries were produced with the help of the pGEM-T vector system (Promega Corporation, Madison, WI, USA), following the recommendations of the manufacturer. The first library was acquired before the inoculation (W0), and the second was obtained after 6 weeks of AD (W6). The PCR reaction for the ligation step was as previously described, with the difference that the primer pair was replaced with a nonfluorescent phosphorylated one, and the final extension step was prolonged to 30 min to allow enough time for the incorporation of the extra adenine nucleotide at both ends of the PCR product. NovaBlue chemical competent cells (NovaGene, Billerica, MA, USA) were utilized for the transformation. Cells were plated on Luria–Bertani agar plates supplemented with IPTG, X-GAL and ampicillin (Green and Sambrook, 2012). More than 200 individual white colonies were selected from each clone library for further screening after overnight incubation. First, the colonies were individually picked and transferred onto identical selective plates with the help of sterile toothpicks. The remaining cells from the toothpick were rinsed into 30 µL of distilled water. The cells were then held at 95 °C for 5 min. The cell debris was collected by centrifugation at 12,000 rpm for 5 min. The supernatant was the template for the verification PCR step. Vector-specific M13 forward and reverse primers were used to amplify the insert from the plasmid as described above. Clones carrying an insert of correct size were identified by agarose gel electrophoresis and marked for further analysis; others were discarded.

### 2.8. T-RFLP analysis and screening

Nested PCR was performed with 100-fold diluted PCR products from the previous PCR step of the preselected clones as template, using fluorescent forward primer. The amplicons were verified as described above. Restriction digestions were carried out with the endonucleases RsaI and MspI (Thermo Fisher Scientific Inc., Waltham, MA, USA). The digestion mixture contained 4–8 µL of the PCR product (∼100 ng), 2 µL of Tango buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA), 3.3 U of the enzymes RsaI and MspI and bidistilled water to a final volume of 20 µL. The reaction was carried out in a heating block at 37 °C for 3 h. The enzymes were inactivated by incubation at 80 °C for 20 min, followed by ethanol precipitation. 2–5 µL of the digested DNA was mixed with 0.3 µL of GeneScan™ 500 LIZ™ size standard and Hi-Di formamide (Life Technologies, Carlsbad, CA, USA) to a final volume of 15 µL. The lengths of the terminal restriction fragments (T-RFs) were

determined by comparison with the size standard in an automated ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in GeneScane mode with the help of the GeneMapper® 3.1 analysis software (Applied Biosystems, Foster City, CA, USA). Both the mixed PCR products and the cloned amplicons were processed as described above. The restriction pattern of the community PCR was compared with the fragments of the positive clones through use of the GeneMapper 3.1 program. Every run was conducted in triplicate in order to minimize the bias during electrophoresis. The T-REX software (http://trex.biohpc.org/) helped generate the restriction fragment size patterns. The T-RF lengths corresponding to the capillary electrophoresis peaks were adjusted to round numbers and noise-filtered. Peak intensities with an area lower than 2% of the total fluorescence were excluded. The cloned fragments that could be matched with the predominant peaks in the restriction pattern were selected for sequencing and the DNA sequences of the entire inserts were determined.

### 2.9. Sequencing

The clones for sequencing were selected after determination of their unique T-RF, and evaluation of their restriction pattern. Sänger sequencing was performed on plasmids purified from the selected clones with the help of the GeneElute plasmid miniprep kit (Sigma–Aldrich Co, St. Louis, MO, USA) according to the manufacturer's instructions. The sequences were then compared with GenBank entries with the BLAST tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned against the entries of the Ribosomal Database Project (RDP Release 10, Update 29; http://rdp.cme.msu.edu/).

## 3. Results and discussion

### 3.1. Reactor performance

Crucial fermentation parameters were monitored continuously or measured periodically. Redox potential, pH and temperature were registered nonstop by the process control equipment. The values of the parameters were in the normal range during the start-up period of 4 weeks when the reactors were fed with maize silage, and throughout the experiment in the subsequent 6 weeks. The $NH_4^+$ content (around 1.5 g $L^{-1}$) was constantly below the limiting threshold. The measured ratios of the volatile organic acids and buffer capacity (VOAs/TAC) were of great importance because of the relatively high organic loading rate. The results presented in Fig. 1 show the constant values in the start-up phase, with a noteworthy peak in the bioaugmented fermentor after inoculation (F2, W1). In the next stage of the experiment, the ratio VOAs/TAC demonstrated a moderate increase. To avoid process failure, the daily loading of the reactors was reduced by 30% after week 6. It is noteworthy that the VOAs/TAC values in the inoculated fermentor were generally lower than those for the control and the system recovered more rapidly. These findings support our assumption of a hydrolyzing efficiency enhancement caused by the $H_2$-producing bacteria. The $CH_4$ content of the produced biogas was around 50–55% throughout the fermentation. The redox potential, pH, conductivity and density remained balanced during the experiment at $<-450$ mV, $\sim7.83$, 20.5 mS $cm^{-1}$ and 1,040 kg $m^{-3}$, respectively. Due to the dosage of the feedstock, the levels of total solids (TS) and organic total solids (oTS) in the fermentors were relatively high, at around 7.82% and 6.23%, respectively. The cumulative daily gas production values indicate parallel operation before the inoculation with the $H_2$-producer strain on day 24. Shortly after the introduction of the E. cloacae cells, the inoculated reactor exhibited a pronounced increase in biogas yield. The effect persisted during the remainder of the fermentation period. The overall gas production rate of the control reactor was 595 L $(kg \, oTS)^{-1}$, while that for the reactor inoculated with extra E. cloacae reached 718.5 L $(kg \, oTS)^{-1}$ after 6 weeks of operation, which is considerably (more than 20%) higher than that for the non-inoculated control. In this respect, it should be noted that the substrate maize silage is a lignocellulose-rich material (C/N = 45) that is not ideal for E. cloacae, which does not possess carbohydrate polymer-degrading enzymes.

### 3.2. Real-Time PCR quantification of E. cloacae

In order to establish conclusively that the observed intensification of biogas productivity was related to the addition of E. cloacae,
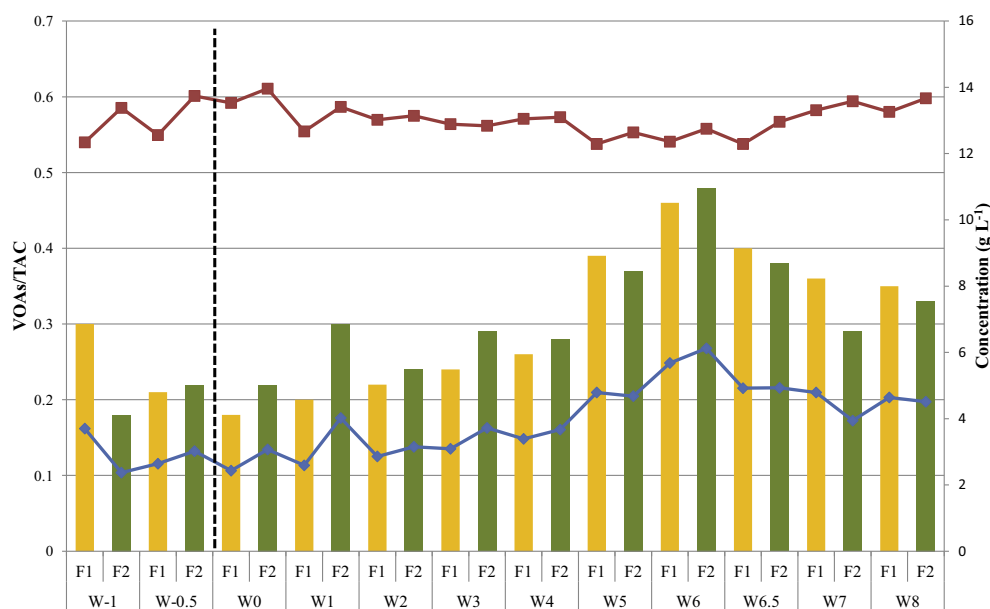


**Fig. 1.** Measured VOAs/TAC values of the control (F1) and E. cloacae-inoculated (F2) reactors. The blue line indicates the total concentration of volatile fatty acids (VOAs), and the red line the total buffer capacity (TAC). The orange and green columns indicate the F1 (control) and F2 (inoculated) fermenters, respectively. The dotted vertical line marks the start of the experimental period after the start-up of the reactors.
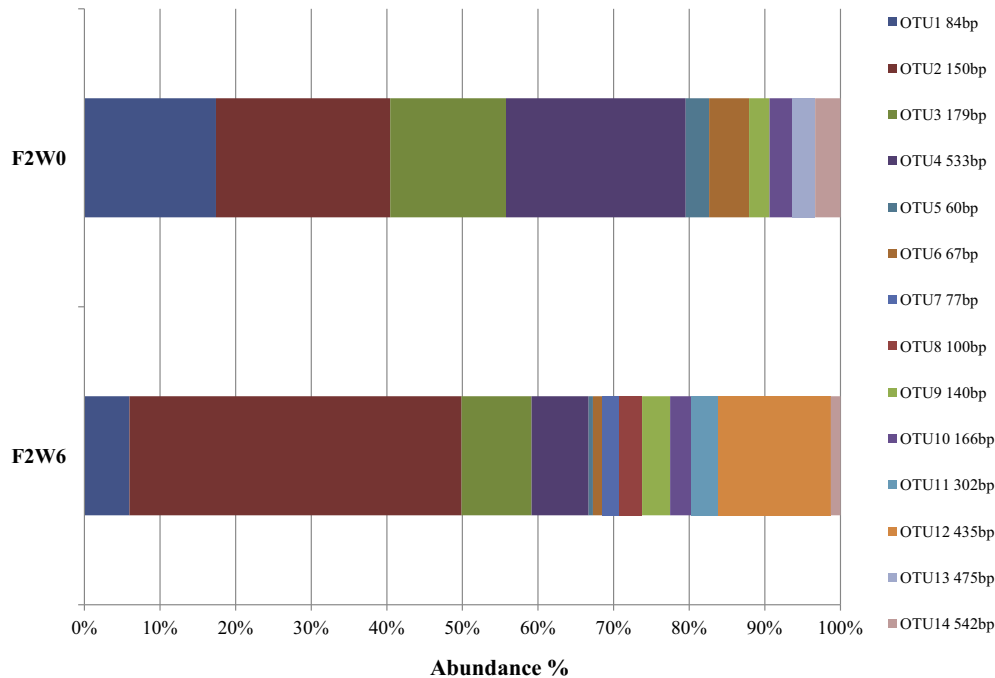
**Fig. 2.** Restriction fragment distribution of the inoculated fermentor (F2) before (W0), and 6 weeks after (W6) the inoculation. The relative abundances of the various OTUs are indicated in different colors.

it was imperative to demonstrate that the added bacteria remained present in the microbial community of the reactor and were not washed out of the system during the 6-week duration of the experiment. The number of inoculated *E. cloacae* cells (around $1 \times 10^9$ CFU mL$^{-1}$), determined by Real-Time PCR technique, indicated an initial moderate decrease but this was followed by a stabilization of the *E. cloacae* specific DNA content within the community (about $6 \times 10^7$ CFU mL$^{-1}$), this level continuing until the end of the examined period. Members of the family *Enterobacteriaceae* are usually present in biogas reactors fed with substrates of agricultural origin. *Escherichia coli* has been detected frequently (Wirth et al., 2012), but *E. cloacae* has been found in noticeable abundance only in solid-state AD (Li et al., 2013). *E. cloacae* clearly did survive in the environment provided by this experiment and multiplied together with the rest of the community.

### 3.3. Eubacterial community analysis by T-RFLP

#### 3.3.1. Operational taxonomic units (OTUs) predominating in the community

The eubacterial community structures at the beginning of the experiment, i.e. before the addition of the *E. cloacae* cells, and 6 weeks after the inoculation were compared through the use of 16S rRNA gene sequences and clone libraries. Several restriction endonucleases were tested, both alone and in combination, for

the generation of the most diverse restriction patterns. Enzymatic fragmentation of the PCR products with the restriction endonucleases MspI and RsaI gave promising restriction patterns (Fig. 2). The T-RFLP fingerprints of the bacterial 16S rRNA gene fragments revealed a total of 14 terminal restriction fragments, which were organized into Operational Taxonomic Units (OTUs). 10 of them were already present in the samples taken at the start of the experiment, i.e. before addition of the *E. cloacae* cells (W0), while sampling after 6 weeks of AD by the augmented microbial community (W6) yielded a diversified group of eubacteria comprising 13 OTUs. Four taxonomic groups (Table 1) were most abundant in both samples, accounting for 79% and 67% of the whole population in the W0 and W6 samples, respectively. Others are listed in Table 2.

The cell number (colony forming units) of the inoculated *E. cloacae* ($10^9 \times$ CFU mL$^{-1}$) quickly stabilized at $5$–$7 \times 10^7$ CFU/mL. The average cell density in biogas reactors is $10^9$–$10^{10} \times$ CFU mL$^{-1}$ (Langer et al., 2013) and the community is composed of several hundred strains in various abundances. *E. cloacae* therefore did not predominate in the microbial community and its abundance was not high enough to reach the sensitivity threshold of the T-RFLP method, although its presence was readily detectable by Real-Time PCR.

The ostensible difference between the two sets of results stems from the distinct nature of the applied methods. Real-Time PCR

**Table 1**
Sequence data of the predominant eubacterial groups. The OTUs displaying a decrease in abundance are indicated in blue, and those showing an increase are in red in the last column.

| OTU | Closest known specie | Similarity (%) | Number of clones | Abundance (%) | |
|---|---|---|---|---|---|
| | | | | W0 | W6 |
| **OTU-1 (84 bp)** | *Proteiniphilum acetatigenes* | 87 | 4 | **17** | 6 |
| **OTU-2 (150 bp)** | Unknown *Clostridiales* | 100 | 5 | **23** | 44 |
| **OTU-3 (179 bp)** | *Desulfotomaculum salinum* | 85 | 4 | **15** | 9 |
| **OTU-4 (533 bp)** | *Streptococcus bovis/equinus* | 99 | 3 | **24** | 7.5 |

**Table 2**
Sequence data of the less dominant eubacterial groups. The OTUs displaying a decrease in abundance are indicated in blue, and those ones showing an increase are in red in the last column. The underlined species showed remarkable increase in abundance.

| OTU | Closest species | Similarity (%) | Abundance (%) | |
|---|---|---|---|---|
| | | | W0 | W6 |
| **OTU-5 (60 bp)** | *Anaerobaculum mobile* | 94 | **3** | 0.56 |
| **OTU-6 (67 bp)** | Unknown *Bacteroidetes* | 100 | **5.3** | 1.2 |
| **OTU-7 (77 bp)** | Unknown *Clostridiales* | 93 | **0** | 2.3 |
| **OTU-8 (100 bp)** | *Halocella cellulosilytica* | 89 | **0** | 2.9 |
| **OTU-9 (140 bp)** | *Clostridium caenicola* | 92 | **2.7** | 3.7 |
| **OTU-10 (166 bp)** | Unknown *Clostridiales* and *Bacteroidales* | 100 | **3** | 2.8 |
| **OTU-11 (302 bp)** | *Defluviitalea saccharophila* | 89 | **0** | 3.6 |
| **OTU-12 (435 bp)** | <u>*Clostridium pasteurianum*</u> | 94 | **0** | 14.8 |
| **OTU-13 (475 bp)** | *Thermoanaerobacter tengcongensis* | 81 | **3** | 0 |
| **OTU-14 (542 bp)** | *Enterococcus cecorum* | 99 | **3.4** | 1.3 |

involves the use of specific primers and its sensitivity exceeds that of the end-point PCR with the universal primers employed in T-RFLP at least 100-fold. Given the nature of the multitemplate PCR, the various fragments compete for the components (primers, enzyme, etc.) and this could easily result in discrimination against the template originating from the inoculated strain.

To investigate this issue further, we conducted several Real-Time PCR and T-RFLP experiments with various amounts of *E. cloacae* cells added to the liquid phase of AD reactors that had no previous contact with the bacterium. The results corroborated that the samples with the closest threshold cycle ($C_t$) values to W6 in Real-Time PCR displayed similar behavior in the T-RFLP fingerprinting, i.e. the T-RFLP peak corresponding to *E. cloacae* did not significantly emerge from the background noise. Nevertheless, the community clearly responded to the introduction of the novel $H_2$-producing strain; the changes are discussed in the next section.

*3.3.1.1. OTU-1 (84 bp).* The smallest identified predominant terminal restriction fragment (TR-F) was 84 bp long. The abundance of this strain was reduced from an initial 17% to 6%, suggesting that this strain had limited ability to adapt to the altered microbial environment. Four clones were sequenced, two from each clone library. The result of the Ribosomal Database Project (RDP) on-line similarity search gave a straightforward result, identifying the species in question as a member of the family *Porphyromonadaceae*. The closest matching strain in the NCBI BLAST database was *Proteiniphilum acetatigenes*, though the confidence value was rather low (87%). This bacterium was found in a biogas-producing consortium using denaturing gradient gel electrophoresis for identification (Pobeheim et al., 2010). *P. acetatigenes* was first isolated from an upflow anaerobic sludge blanket-type anaerobic digester and was described as a new lineage within the *Cytophaga-Flavobacterium-Bacteroides* group. In co-culture with its syntrophic propionate degrading partners *Syntrophobacter sulfatireducens* and *Methanobacter formicicum*, *P. acetatigenes* effectively converted propionate to acetate and $CH_4$ (Chen and Dong, 2005).

*3.3.1.2. OTU-2 (150 bp).* This taxonomic unit displayed the highest representation in both examined samples, indicating is important role in the AD process. The abundance of the strain at the beginning of the test (W0) was around 23%; this increased considerably, to 44%, 6 weeks after addition of the *E. cloacae* cells under otherwise identical conditions. Four clones were investigated in this OTU group. The search results indicated the order *Clostridiales*, but more precise identification of the bacterium was ambiguous due to the lack of adequate sequence data in the databases. This observation underlines the need for good-quality specific entries into the databases from newly characterized bacteria. The

*Clostridiales* are well-known cellulose-degrading bacteria, and hence their high abundance in AD reactors fed with maize silage is not surprising (Hanreich et al., 2013).

*3.3.1.3. OTU-3 (179 bp).* This OTU also belonged in one of the predominant groups. The representation of this strain decreased slightly following the introduction of *E. cloacae*, with abundances of ∼15% and 9% in the W0 and W6 samples, respectively. Four clones were examined in detail and another member of the order *Clostridiales* was indicated. The closest species identified was *Desulfotomaculum salinum*, albeit with a rather low confidence value (85%). Dissimilatory sulfate reducers are commonly found in AD communities, competing with methanogens for the reducing power. *Desulfotomaculum* species harbor very active [FeFe] hydrogenases and use electrons from lactate and acetate to reduce metals in anaerobic respiration (Wirth et al., 2012). They have been implicated in foam formation in AD reactors fed with cattle manure (Kougias et al., 2014) recently. Their relatively high representation in a reactor fed with plant biomass was unexpected.

*3.3.1.4. OTU-4 (533 bp).* This group, similarly to OTU-1, lost its abundance as the AD process with added *E. cloacae* progressed. The strain comprising this OTU was present initially at a level of 24%, which dropped spectacularly to 7.5% in the W6 sample. The reduction in cell number could be brought about by either of two effects: the lack of an appropriate adaptive capability, or the rapid growth and predominance of other strains, e.g. OTU-2.

The sequencing results were identical in all four clones selected, identifying the clones as belonging in the order *Lactobacillales*. The NCBI BLAST search tool indicated two possible strains as closest relatives, both belonging in the genus *Streptococcus*. A distinction between *Streptococcus bovis* and *Streptococcus equinus* was not possible because of the high similarity of the 16S rDNA sequences of these strains (Hinse et al., 2011). It has even been proposed that the two strains should in fact be regarded as one in view of upon the high level of similarity (99%) between their 16S rDNA sequences (Schlegel et al., 2003). *Lactobacilli* are the predominant taxa in the ensilaging process, and their frequent occurrence in maize silage is therefore not surprising. The reactors were fed with maize silage daily, with the concomitant introduction of *Lactobacillales*, and the significant loss in their richness in the W6 sample is therefore difficult to rationalize.

*3.3.2. Less frequent OTUs*
*3.3.2.1. OTU-5 (60 bp) and OTU-6 (67 bp).* These two units are discussed together because of their similar behavior in the digester. They were present only in the W0 sample, in rather low abundances, i.e. 3% and 5.3%, respectively. Both OTUs had disappeared by week 6 (W6 sample). An on-line search gave an exact hit in

the case of OTU-5. *Anaerobaculum mobile*, a member of the family *Synergistaceae*, was identified. Synergistic acetate oxidation is recognized as playing an increasingly important role in biogas formation (Menes and Muxí, 2002; Müller et al., 2012). OTU-6 corresponded to an unknown representative of the phylum *Bacteroidetes*.

*3.3.2.2. OTU-7 (77 bp).* This TR-F was apparently not present in the restriction pattern after noise filtering in the W0 sample, i.e. at the start of the experiment, but exhibited a 2.3% share of the total fluorescence in the W6 sample. The species providing this TR-F is an unknown *Clostridiales*.

*3.3.2.3. OTU-8 (100 bp).* This OTU appeared only in the W6 sample with an abundance of around 3%. The sequencing results indicated yet another species belonging in the class *Clostridia*, with 89% similarity to *Halocella cellulosilytica*. This strain has an outstanding cellulose-degrading capacity and produces ethanol and $H_2$ from lignocellulosic substrates (Roush et al., 2014). It has been described as a salt-tolerant species in the order *Halanaerobiales*, which can apparently thrive at the relatively low salt concentration of the maize silage-fed AD reactor.

*3.3.2.4. OTU-9 (140 bp).* The representation of this unit changed only slightly during the AD of maize silage with or without *E. cloacae*, i.e. abundances of 2.7% and 3.7% in the W0 and W6 samples, respectively. All 4 clones harboring OTU-9 were sequenced, and all sequences identified the species as *Clostridium caenicola*. This Clostridium was first isolated from a biogas-producing microbial community digesting cellulose and/or cellobiose (Shiratori et al., 2009). It is a moderately thermophilic strain with high affinity for cellulose as a substrate.

*3.3.2.5. OTU-10 (166 bp).* The abundance of this OTU in the examined samples was about 3%. Both libraries contained clones with the corresponding TR-F, but the sequencing results were inconsistent. This could mean that the separation of the TR-Fs was not successful in all cases, despite all efforts, or it could be a result of a false restriction digestion. This was the only OTU in which apparently mixed groups were hit upon.

*3.3.2.6. OTU-11 (302 bp) and OTU-12 (435 bp).* These two units are also discussed together because of their similar behavior. Neither was detectable in the reactors before the addition of the *E. cloacae* cells (W0), but after 6 weeks they demonstrated appreciable abundances: 3.6% (OTU-11) and 14.8% (OTU-12). Thus, OTU-12 became one of the predominant members in the W6 community. Sequencing results indicated that both strains were affiliated with the often encountered class *Clostridia*, but they are distinct from the previously detected members of this taxon. The closest relative to OTU-11 was *Defluviitalea saccharophila*, with relative low confidence (89%). This thermophilic, anaerobic, Gram-positive, spore-forming bacterium was first isolated from a filter of slaughterhouse wastewater (Jabari et al., 2012). A precise taxonomic hit in the case of OTU-12 identified this strain as *Clostridium pasteurianum*. *C. pasteurianum* has been thoroughly studied because of its excellent fermentative $H_2$-producing capability and its [FeFe] hydrogenases and cellulase activity (Masset et al., 2012).

*3.3.2.7. OTU-13 (475 bp).* This strain was found only in the W0 sample, i.e. before inoculation with *E. cloacae*, in a relative abundance of 3%. The sequencing data indicated an additional Clostridium, *Thermoanaerobacter tengcongensis*, a strain frequently found in both completely stirred and solid-state biogas producing facilities (Li et al., 2013; Schlüter et al., 2008).

*3.3.2.8. OTU-14 (542 bp).* The abundance of this group was rather low, i.e. 3.3% and 1.3% in the W0 and W6 samples, respectively. The sequencing results suggested that another *Lactobacillus*, *Enterococcus cecorum*, was the closest relative of the species. Similarly to OTU-4, this Lactobacillus decreased in abundance during the AD with *E. cloacae*, although the reactors were regularly supplied with maize silage, the likely source of lactic acid bacteria in the system. *E. cecorum* is commensal in the gut of numerous mammals and has also been implicated in the induction of occasional pathogenic disease outbreaks among broiler chickens (Kense and Landman, 2011; Stalker et al., 2010). The AD reactors did not come into contact with chicken manure or residues, and it is therefore best assumed that this strain was introduced with the maize silage substrate, together with the other lactic acid bacteria ensilaging the green biomass.

## 4. Conclusions

*E. Cloacae* was expected to boost the biogas generation if the local $H_2$ concentration was a rate-limiting factor. The data confirmed that this was indeed the case, at least to a certain degree.

After 2 weeks the cell number of *E. cloacae* stabilized according to Real-Time PCR. The resolution of the T-RFLP was not sufficient for the detection of the added *E. cloacae*.

*Lactobacillales* did not fit well into the biogas-producing microbial community. A group of unknown *Clostridiales* and a close relative of *C. pasteurianum* increased in abundance spectacularly; this also suggests a key role of the polymer-degrading step. It seems that a syntrophic relationship developed between the polymer-degrading and $H_2$-producing Clostridia and the principally acetogenic *E. cloacae*.

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