Microbial diversity in a biogas-producing co-fermentation of maize silage and bovine manure

Khadidja Souidi[1,2], Jan Mumme[1,2], Kerstin Mundt[1], Edith Nettmann[1,3], Ingo Bergmann[1,3], Bernd Linke[1,2] and Michael Klocke[1*]

[1] Leibniz-Institut für Agrartechnik Potsdam-Bornim e.V., Max-Eyth-Allee 100, D-14469 Potsdam-Bornim, Germany

[2] Humboldt-Universität zu Berlin, Landwirtschaftlich-Gärtnerische Fakultät, Institut für Pflanzenbauwissenschaften, Fachgebiet Agrartechnik, Philippstrasse 13, D-10115 Berlin, Germany

[3] Technische Universität Berlin, FG Umweltmikrobiologie, Sekretariat FR 1-2, Franklinstrasse 29, D-10587 Berlin, Germany

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Abstract - Kurzfassung

The efficiency of biogas reactors strongly depends on the substrate utilization by the microbial biocoenosis. Up to now, the structure of this microbial community has not been sufficiently characterized. In this study, an overview of the most abundant species within a biogas-producing, completely-stirred tank reactor (CSTR) during the co-fermentation of maize silage and bovine manure was surveyed. The analysis based on two 16S rDNA libraries each enriched for methanogenic *Archaea* (81 clones) and fermentative *Bacteria* (118 clones). The CSTR showed a huge diversity within the methanogenic *Archaea*. The order *Methanomicrobiales* was present with eight operational taxonomic units (OUT), the order *Methanosarcinales* with three OTU and the order *Methanobacteriales* with two OTU. The order *Methanococcales* was not detected. One OTU could not be assigned to any order. Most OTU (71 %) were affiliated to hydrogenotrophic methanogens like *Methanoculleus bourgensis*, *Methanobrevibacter ruminantium*, *Methanospirilllum hungatei* and *Methanocorpusculum parvum*. Acetate-utilizing Archaea were detected with 3 OTU related to *Methanosarcina barkeri* and *Methanosaeta concilii*. Within the domain *Bacteria* a rich diversity was found within the members of the phyla *Firmicutes* (27 OTU) and *Bacterioidetes* (13 OTU). Also certain OTU of the phyla *Proteobacteria* (4 OTU), *Spirochaetes* (2 OTU), *Fibrobacteres* (1 OTU) and *Chloroflexi* (1 OTU) were found. Ten OTU could not be grouped with any cultivated type strain. The dominance of OTU related with H_2-consuming methanogens in the *Archaea*-specific 16S rDNA library and their rich diversity point to major formation of methane by oxidation of CO_2.

Keywords: 16S rDNA sequence analysis, biogas reactor, co-digestion, maize silage, microbial community

Mikrobielle Diversität in einer biogas-produzierenden Kofermentation von Maissilage und Rindergülle

Die Effizienz von Biogasreaktoren hängt in wesentlichem Maße von der Substratverwertung durch die beteiligte Mikroflora ab. Die Zusammensetzung der mikrobiellen Gemeinschaft ist jedoch bislang nur oberflächlich charakterisiert. In dieser Studie wird daher eine Übersicht über die mikrobielle Diversität in einem biogas-produzierenden Rührkesselreaktor (CSTR) während der Kofermentation von Maissilage und Rindergülle gegeben. Die Analyse erfolgte anhand zweier 16S rDNA Bibliotheken jeweils spezifisch für methanogene *Archaea* (81 Klone) und fermentative *Bacteria* (118 Klone). Der CSTR wies eine hohe Diversität innerhalb der methanogenen *Archaea* auf. Aus der Ordnung *Methanomicrobiales* wurden acht OTU nachgewiesen, für die Ordnung *Methanosarcinales* drei OTU und für die Ordnung *Methanobacteriales* zwei OTU. Eine weitere OTU wies eine deutliche genetische Distanz zu den bekannten taxonomischen Ordnungen auf. Die Ordnung *Methanococcales* wurde nicht detektiert. Die meisten OTU (71 %) konnten in Verwandtschaft zu hydrogenotrophen Methanbildnern wie *Methanoculleus bourgensis*, *Methanobrevibacter ruminantium*, *Methanospirilllum hungatei* und *Methanocorpusculum parvum* gesetzt werden. Acetatverwertende *Archaea* wurden mit 3 OTU verwandt zu *Methanosarcina barkeri* und *Methanosaeta concilii* detektiert. Innerhalb der Domäne *Bacteria* wurde eine hohe Diversität unter den Vertretern der Phyla *Firmicutes* (27 OTU) und *Bacterioidetes* (13 OTU) festgestellt. Ebenso wurden mehrere OTU der Phyla *Proteobacteria* (4 OTU), *Spirochaetes* (2 OTU), *Fibrobacteres* (1 OTU) und *Chloroflexi* (1 OTU) detektiert. 10 OTU konnten nicht taxonomisch eingeordnet werden. Die Dominanz von OTU verwandt mit H_2-konsumierenden Methanbildnern in der *Archaea*-spezifischen 16S rDNA Bibliothek sowie deren große Diversität sind Indizien für eine verstärkte Bildung von Methan durch Oxidation von CO_2.

Schlüsselwörter: 16S rDNA Sequenzanalyse, Biogasreaktor, Kofermentation, Maissilage, Mikrobielle Gemeinschaft

* Corresponding author. Tel: ++49 (0) 331 5699 113; Fax: ++49 (0) 331 5699 849; E-mail: mklocke@atb-potsdam.de

1 Introduction

After the first oil crisis in the 1970s, several European research organizations started to evaluate alternative renewable energy resources as substitutions for fossil fuels and nuclear power. Influenced by the rapidly increasing cost of fuels throughout the world, the application of biogas emerged. In 2005, biogas was used to generate 2.9 TWh of electric power in Germany alone. The total potential for energy production from biogas in Germany is estimated at 752 Petajoule per year corresponding to 209 TWh (Fachverband Biogas 2005).

Biogas is one of the major products of a naturally occurring biological process, the anaerobic degradation of biomass by a complex microbial biocoenosis. Generally, this process proceeds in two steps: first, the high-molecular organic compounds are catabolized by fermentative bacteria to short-chain carbon acids (e.g. acetate, butyrate), alcohols (e.g. ethanol) and carbon dioxide. These intermediates are the energy donor but also C-source for certain methanogenic *Archaea* like *Methanosaeta* sp. (Garrity 2001).

Alternatively, the volatile fatty acids (VFA, C >2) can be utilized by certain *Bacteria* e.g. *Clostridia* sp. with acetate as product. In this metabolic pathway, hydrogen serves as final acceptor for free electrons. During the biogas fermentation hydrogen escapes as gas, or is utilized by certain hydrogen-consuming *Archaea* as electron donor for catabolizing carbon dioxide to methane. Hydrogenotrophic *Archaea* are often associated with VFA-degrading *Bacteria* in syntrophic communities facilitating an interspecies hydrogen transfer (Stams 1994). Predominantly, hydrogenotrophic *Archaea* are found within the orders *Methanobacteriales*, *Methanomicrobiales* and *Methanococcales* (Liu et al. 2002). Typically, biogas contains 50-75 % methane, 25-50 % carbon dioxide, 0-1 % hydrogen and 0.005-0.6 % hydrogen sulphide.

For utilization of liquid substrates such as waste water the upflow anaerobic sludge bed (UASB) reactor was developed. In a UASB reactor effective degradation is obtained through support of the formation of microbial granules, which ensure the close spatial orientation of syntrophic fermentative *Bacteria* and methanogenic *Archaea* (Lettinga 1995).

UASB reactors are inapplicable for utilization of energy-rich solid substrates like silages. Such co-fermentations are normally conducted in more simply constructed, completely stirred tank reactors (CSTR). Resulting from the large amounts of solid plant material and the continuous stirring, no visible granules similar to those in UASB reactors are formed in CSTR. However, detailed knowledge of the microbial biocoenosis involved and the resulting microbial conversion processes is crucial for optimizing the operation of CSTR.

The microbial ecology within CSTR has been the object of several studies. This topic was recently reviewed by Hofman-Bang et al. (2003). First analyses described the microbiology in CSTR during utilization of organic remnants like dung and slurry from livestock farming or communal sewage (e.g. Raskin et al. 1995, Hansen et al. 1999). Recently, also a first report was published on the microbial community in CSTR degrading renewable carbohydrate rich "energy" plants like fodder beet silage as sole substrate (Klocke et al. 2007). The microbial community structure during metabolization of other common energy plants like maize has not been the subject of publications up to now.

A comprehensive analysis of total bacterial and archaeal communities in biogas reactors requires culture-independent methods for parallel detection of many hundreds or even thousands of species. A common approach is the analysis of microbial DNA sequences e.g. of the gene for the 16S rRNA (16S rDNA). This approach includes (1) the purification of total microbial DNA from an environmental sample, (2) the amplification of a selected region of microbial DNA by polymerase chain reaction (PCR), (3) the construction of a plasmid-based 16S rDNA library in *Escherichia coli*, (4) screening of the 16S rDNA library with amplified rDNA restriction analysis (ARDRA), (5) estimation of the DNA sequence responsible for an individual ARDRA pattern, (6) comparison with gene bank entries and estimation of phylogenetic relationships. This procedure has already been successfully applied for the analysis of a broad range of environmental samples (e.g. Raskin et al. 1995, Godon et al. 1997, Sekiguchi et al. 1998, Merkel et al. 1999, Fernandez et al. 1999).

In this study an analysis of the microbial diversity in a biogas-producing CSTR for the utilization maize silage and bovine manure in a co-digestion process is published for the first time. For this purpose, two different 16S rDNA libraries, each specific either for *Archaea* or *Bacteria,* were constructed and subsequently characterized with ARDRA and sequence analysis.

2 Material and methods

2.1 Operation of CSTR and sampling

Samples for microbial community analysis were taken from a CSTR (batch volume 3 l) loaded semi-continuously with maize silage. The CSTR was started with the effluent from another laboratory-scale CSTR (characteristic values of the inoculum: pH 7.7, total solids (TS) 5.49 % whereof 74 % volatile solids (VS), volatile fatty acids (VFA) 0.12 g l^{-1}. A constant temperature of 35 °C (i.e. mesophilic conditions) was maintained by a water jacket heated by a thermostat.

The CSTR was mixed occasionally with a glass stirrer. The biogas was stored in gas bags and analyzed automatically once a day with a gas meter (Ritter, Bochum, Germany) and a biogas analyzer (Pronova, Berlin, Germany).

At the start of the experiment the reactor was filled with cattle slurry. After one week maize silage was additionally used as co-substrate. The daily loading rate as referred to the volatile solids was kept constant at 1 g l^{-1} d^{-1} (cattle slurry) and 2 g l^{-1} d^{-1} (maize silage) respectively.

Chemical characteristics of the maize silage determined according to Linke & Mähnert (2005): TS at 105 °C 88.5 % fresh matter, VS at 550 °C 92.4 % TS, pH 4.3, VFA 3.3 g kg^{-1} acetic acid equivalent, nitrogen (N) 1.7 % TS, carbon (C) content 46.3 % TS, crude fat (XL) 30.57 g kg fresh mass (FM), saccharose 4.98 % TS, crude fiber (XF) 25.52 % TS. For cattle slurry the chemical values were determined as TS (105 °C) 8.3 % fresh matter, VS (550 °C) 80.6 TS, pH 7.3, VFA 7.3 g l^{-1} acetic acid equivalent. Gas volume and composition, pH and VFA in the effluent were measured daily. TS, VS, N in the effluent were determined weekly.

Samples (volume 50 ml) for DNA preparation were taken 3 weeks after the start of the experiments. The total microbial DNA was isolated according to Klocke et al. (2007).

3 Analysis of microbial diversity

In view of the huge phylogenetic distance between methanogenic *Archaea* and fermentative *Bacteria* these microbial domains were analyzed by two different molecular approaches.

The archaeal diversity was estimated based on analysis of an *Archaea*-specific 16S rDNA library constructed as follows: all PCR amplifications were performed with a Biometra T gradient 96 (Whatman Biometra, Göttingen, Germany). The reaction mixture was set up on ice as follows: 100 ng template DNA, 1x PCR buffer, 1.5 mmol l^{-1} $MgCl_2$, 0.2 µmol l^{-1} forward primer, 0.2 µmol l^{-1} reverse primer, 0.2 mmol l^{-1} of each dNTP, 0.8 U *Taq*-DNA-Polymerase (recombinant), ad 20 µl final volume bi-distilled H_2O. All chemicals and enzymes were provided by Fermentas (St. Leon-Rot, Germany).

For the PCR the following primer pair specific for the archaeal 16S rDNA gene were used: forward primer Arch16S-Forw2 5'-YGAYTAAGCCATGCRAGT-3' (15 - 32 bp *Escherichia coli* position) modified after Fernandez et al. (1999), reverse primer Univ16S-Rev5 5'-TGC TCCCCCGCCAATTCCT-3' (915 - 934 bp *E. coli* position) modified after Giovannoni et al. (1990). The size of the amplicon is approximately 900 bp. The PCR conditions were: (1) initial denaturation at 94 °C for 120 s, (2) cycle denaturation at 95 °C for 30 s, (3) cycle annealing at 46 °C for 60 s, (4) elongation at 72 °C for 60 s, (5) final extension at 72 °C for 300 s. Steps (2) to (4) were repeated 34 times. PCR products were stored at 4 °C.

For additional analysis of the bacterial diversity a second 16S rDNA library was constructed according to the previously used protocol with following modifications: for PCR the bacteria specific primers 16S-Forw 5'-AGAGTTTGATCMTGGCTCAG-3' (8 - 27 bp *E. coli* position) (Lane 1991) and 16S-Rev 5'-TACGGYTACCTTGTTACGACTT-3' (*E. coli* position 1,490 - 1,512 bp) modified after Lane (1991). Expected size of the PCR product is approx. 1,500 bp. Due to the altered primers, an altered PCR program was applied: (1) initial denaturation at 94 °C for 120 s, (2) cycle denaturation at 94 °C for 30 s, (3) cycle annealing at 53 °C for 30 s, (4) elongation at 70 °C for 60 s, (5) final extension at 72 °C for 900 s. Steps (2) to (4) were repeated 30 times.

The purification of PCR amplicons was conducted according to Klocke et al. (2007). The purified amplicons were cloned into the pGEM-T plasmid (Promega, Mannheim, Germany) via TA cloning and subsequently transformed into *E. coli* str. Novablue (Promega) competent cells (Promega) according to manufacturer's guidelines. Recombinant cells were separated on agar plates. Clones were denominated as ATB-KS and a consecutive number. Subsequent purification of plasmids was conducted as published previously (Klocke et al. 2006). For discrimination of duplicates, the 16S rDNA libraries were screened by amplification rDNA restriction analysis (ARDRA) performed with the restriction enzymes *Bsu*RI and *Hin*6I as described in Klocke et al. (2007) in detail.

3.1 Phylogenetic analysis of 16S rDNA sequences

After a visual comparison of ARDRA patterns, clones harboring 16S rDNA sequences representative for individual ARDRA patterns were selected. Each 16S rDNA was sequenced using the T7 promoter region as starting point. All sequencing reactions were performed by MWG Biotech (Ebersberg, Germany). The length of resulting sequences was about 800 up to 900 bp. Sequences were used as operational taxonomic units (OTU) and denominated accordingly to the clone harboring the 16S rDNA. All sequences were checked for chimeric artifacts by the Chimera Check software tool (Cole et al. 2003). A rarefaction analysis was performed to estimate the adequacy of the sample size for the determination of the diversity within the 16S rDNA clone library (Holland 2003). Sequences were compared with NCBI GenBank (Benson et al. 2006) entries using the nucleotide-nucleotide

BLAST (Altschul et al. 1990). Alignment of clonal sequences and sequences from selected reference species or uncultured clones from the NCBI GenBank were performed with the software ClustalW (Higgins et al. 1994) using standard settings. This software was also applied for calculating similarity values between two sequences. Phylogenetic trees were constructed by the neighbor-joining method (Saito & Nei 1987). Bootstrap resampling analysis (Felsenstein 1985) for 1,000 replicates was performed to estimate the confidence of tree topologies. Except for alignment, all phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (Kumar et al. 2004). All estimated sequences were deposited in the NCBI GenBank database with following accession numbers: EF686879 - EF686999. The taxonomic system of Bergey's Manual was used as base for all phylogenetic analyses (Garrity et al. 2001). All NCBI GenBank entries used as references were cited in brackets.

4 Results

4.1 Biogas production from the CSTR

Samples for molecular analysis were taken after two weeks of continuous CSTR operation with daily feeding with cattle slurry and maize silage (constant loading rate 3 g VS l^{-1} d^{-1}). At this point in time, the biogas production was 1.5 l_{biogas} $l_{fermenter\ volume}^{-1}$ d^{-1}. Referred to the applied VS, the biogas production was 0.46 l kg_{VS}^{-1} equivalent to 0.44 l kg_{FM}^{-1} FM. The biogas yield contained 56.1 % methane (CH_4), 40.7 % carbon dioxide (CO_2), 411 ppm hydrogen (H_2), 1,981 ppm sulfur hydrogen (H_2S) (average of 7 days).

Within the two weeks prior to sampling, acetic acid was the exclusive short chain carbon acid (C_2-C_6 FA) observed in the effluent of the biogas reactor. The content of acetic acid ranged between 0.12 und 0.41 g l^{-1}. The pH value ranged from 7.71 to 8.06. Additionally, the effluent was characterized by TS 5.87 % FM, VS 74.80 % FM, NH_4-N 2.28 g l^{-1}, total N 3.87 g l^{-1}.

4.2 Overall phylogenetic analysis

For analysis of the diversity within the domain *Archaea* and *Bacteria* two domain specific 16S rDNA libraries were constructed and subsequently analyzed with ARDRA and sequence analysis.

Members of the domain *Archaea* were exclusively detected within the *Archaea* specific 16S rDNA library. In this library, a total of 81 clones were analyzed, which were representative of 24 OTU as indicated by individual ARDRA patterns (Table 1). From those, 14 OTU were assigned to the domain *Archaea* representing 68 clones (83.9 %). Interestingly, beside the archaeal OTU also 10 bacterial OTU were amplified with the applied PCR assay. After estimation of the DNA sequence, these OTU were determined to be related to different unclassified bacterial species, predominantly to unclassified members of the order *Clostridia* (10 clones).

Within the *Bacteria* specific 16S rDNA library 118 clones were analyzed in total. The ARDRA resulted in 58 individual ARDRA patterns (Table 2). In the subsequent phylogenetic analysis the DNA sequences of these fingerprints were grouped in certain orders within the domain *Bacteria*.

A rarefaction analysis was conducted to estimate the adequacy of the sample size for determination of the diversity within the 16S rDNA library. As shown in Fig. 1, the calculated rarefaction curves did not reach a clear saturation. Thus, it can be concluded, that the analysis of 81 archaeal and 118 bacterial clones in total had only partially covered the whole microbial diversity. However, the large number of analyzed clones should be well suited for detection of dominant species within the biogas producing biocoenosis.

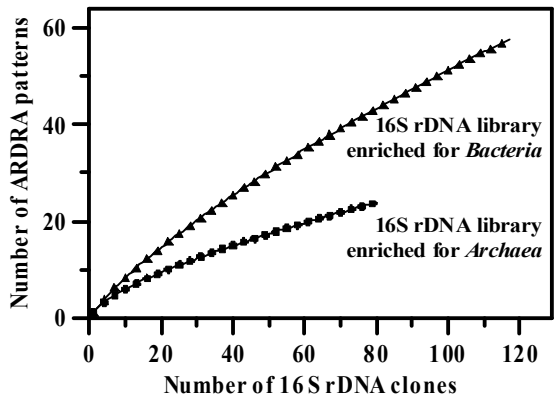

Fig. 1: Rarefaction curve for the different ARDRA patterns of 16S rDNA clones of the two libraries constructed in this study (Holland 2005).

4.3 Domain Archaea

All detected archaeal DNA sequences were assigned to the phylum *Euryarchaeota*. Within this phylum, all detected OTU were related to certain methanogenic species (Table 1, Fig. 2).

Most of the archaeal OTU were affiliated to the order *Methanomicrobiales*. In total 8 OTU representative of 58 clones were grouped into this order. Within the order *Methanomicrobiales* the genus *Methanoculleus* exhibited the largest diversity within the biogas reactor. Six different OTU showed a close relationship to the reference species *Methanoculleus bourgensis* DSM 3045 [AY196674]. One of these clones, ATB-KS-076, was representative of 22 clones and therefore

Table 1: Distribution of OTU and corresponding clones within the 16S rDNA library enriched for sequences from *Archaea* (percentages on the level of order refer to number of OTU or clones assigned to the phylum)

	OTU	16S rDNA clones
Domain *Archaea*		
Phylum *Euryarchaeota*	14 (58.3 %)	68 (83.9 %)
Order *Methanomicrobiales*	8 (57.1 %)	58 (85.2 %)
Order *Methanosarcinales*	3 (21.4 %)	6 (8.8 %)
Order *Methanobacteriales*	2 (14.2 %)	3 (4.4 %)
Order *Methanococcales*	ND	ND
Unclassified *Euryarchaeota*	1 (7.1 %)	1 (1.4 %)
Domain *Bacteria*		
Phylum *Firmicutes*	9 (37.5 %)	10 (12.3 %)
Unclassified *Bacteria*	1 (4.1 %)	3 (3.7 %)

of 25.9 % of all analyzed clones of the *Archaea* specific 16S rDNA library.

The OTU grouped within the genus *Methanoculleus* also possessed a close relationship to a broad range of other uncultured clones derived from a molecular analysis of certain anaerobic environmental samples such as specimens from Chinese landfill leachate (clone GZK7 [AJ576208]) or from mesophilic protein digesters (clones BSA2A-01 [AB175346] and BSA2A-05 [AB175350]).

Beside the family *Methanomicrobiaceae,* members of the families *Methanospirillaceae* and *Methanocorpusculaceae* were also found. One OTU, ATB-KS-085, representing 28.2 % of the archaeal 16S rDNA library was assigned with the reference *Methanospirillum hungatei* JF1 [AY196683] with a sequence similarity of 92 %. Also the family *Methanocorpusculaceae* was present with one OTU, ATB-KS-221, which showed highest similarities to *Methanocorpusculum parvum* DSM 14092 [M59147].

The order *Methanosarcinales* was detected with three OTU. Two of them, each representing one clone within the archaeal 16S rDNA library, were assigned to *Methanosarcina barkeri* DSM 800 [AY012094], the type strain of the genus *Methanosarcina*. Related organisms were detected by 16S rDNA analysis in landfill leachate in France (clone 221 [AJ801161]) and in anaerobic thermophilic solid waste digesters in Japan (clone MANA01 [AB114309]). The third OTU, ATB-KS-077, was affiliated with members of the family *Methanosaetaceae*. This OTU possessed a sequence identity of 90 % with *Methanosaeta concilii* Opfikon [X16932] and was found in four clones of the 16S rDNA library.

The third order *Methanobacteriales* was present with two OTU, ATB-KS-152 and ATB-KS-155. OTU ATB-KS-152 was assigned to the type strain *Methanobrevibacter ruminantium* DSM 1093 [AY196666] with a sequence similarity of 91 %. A higher sequence similarity was given to the uncultured organisms ARC52 [AF029182] and ARC23 [AF029175] detected in the bovine rumen by their 16S rDNA sequence. In the phylogenetic analysis, also OTU ATB-KS-155 was clustered with these members

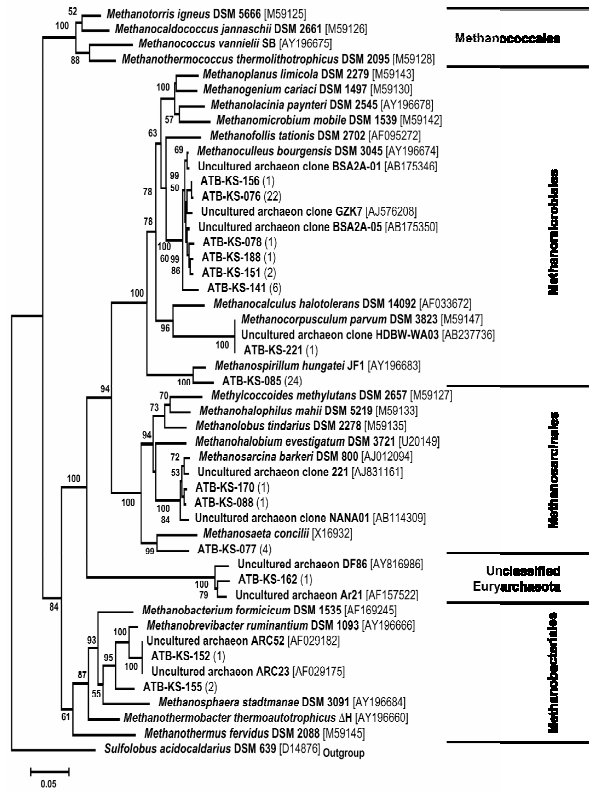

Fig. 2: Phylogenetic tree of the clones among the phylum *Euryarchaeota* (domain *Archaea*) based on neighbor-joining analysis (Saito & Nei 1987) of partial 16S rDNA sequences. Numbers behind the OTU derived from the *Archaea*-specific 16S rDNA library (ATB-KS-) indicate the number of clones of which this OTU is representative. Numbers at nodes represent bootstrap values (Felsenstein 1985) for the nodes in percent (1000 times resampling analysis). Only bootstrap values above 50 % were displayed. Numbers in brackets indicate the corresponding NCBI GenBank accession number If obtainable, type strains were used as reference. *Sulfolobolus acidocaldarius* DSM 639 was used as the outgroup.

Table 2: Distribution of OTU and corresponding clones within the 16S rDNA library enriched for sequences from *Bacteria*

	OTU	16S rDNA clones
Domain *Bacteria*		
Phylum *Firmicutes*		
Class *Clostridia*	26 (44.8 %)	55 (46.6 %)
Class *Mollicutes*	1 (1.7 %)	1 (0.8 %)
Phylum *Bacteroidetes*	13 (22.4 %)	17 (14.4 %)
Phylum *Proteobacteria*		
Class *α-Proteobacteria*	1 (1.7 %)	1 (0.8 %)
Class γ-*Proteobacteria*	2 (3.4 %)	2 (1.6 %)
Class ε-*Proteobacteria*	1 (1.7 %)	1 (0.8 %)
Phylum *Spirochaetes*	2 (3.4 %)	6 (5.0 %)
Phylum *Fibrobacteres*	1 (1.7 %)	1 (0.8 %)
Phylum *Chloroflexi*	1 (1.7 %)	1 (0.8 %)
Unclassified *Bacteria*	10 (17.2 %)	33 (27.9 %)

of *Methanobacteriales*. In contrast to OTU ATB-KS-152, the relationship of OTU ATB-KS-155 to the reference species was lower.

The fourth order *Methanococcales* was not detected within the archaea specific 16S rDNA library.

Interestingly, one OTU, ATB-KS-162, representative for one clone, could not be clustered within one of the four orders of *Euryarchaeota*. In the phylogenetic analysis this OTU was grouped between the cluster of members of *Methanosarcinales* and *Methanomicrobiales*, respectively, and the cluster of the *Methanobacteriales*. However, several other uncultured clones isolated from certain environmental samples showed high 16S rDNA sequence similarities with this OTU. As an example, the clones DF86 [AY816986] and Ar21 [AF157522] detected in pig slurry and manure were included into the phylogenetic analysis.

4.4 Domain Bacteria - phylum Firmicutes

Within the domain *Bacteria*, the phylum *Firmicutes* was most abundant (Table 2, Fig. 3). The *Firmicutes* were represented by 56 clones of the bacterial 16S rDNA library. The corresponding 27 OTU were affiliated with the classes *Clostridia* and *Mollicutes*.

Members of the class *Bacilli* were not detected. From these 27 OTU, 15 OTU representatives for 34 clones were assigned to the family *Clostridiaceae* (Fig. 3).

Interestingly, these OTU showed limited similarities to cultured references like *Clostridium aldrichii* DSM 6159 [X71846], *C. jejuense* str. HY-35-12 [AY494606], *C. straminisolvens* str. CSK1 [AB125279] or *C. alkalicellum* str. Z-7026 [AY959944]. In most cases, higher similarities were found with 16S rDNA sequences derived from as yet uncultured organisms. As examples, several OTU detected in a Korean food waste digester (clone F117 [DQ232857]), an US-American digester for pharmaceutical waste (clone tbr1-10 [AF280825]) as well as in the gastrointestinal tract of pigs in Denmark (Klon p-5278-2Wa3 [AF371938]) were included in the phylogenetical analysis.

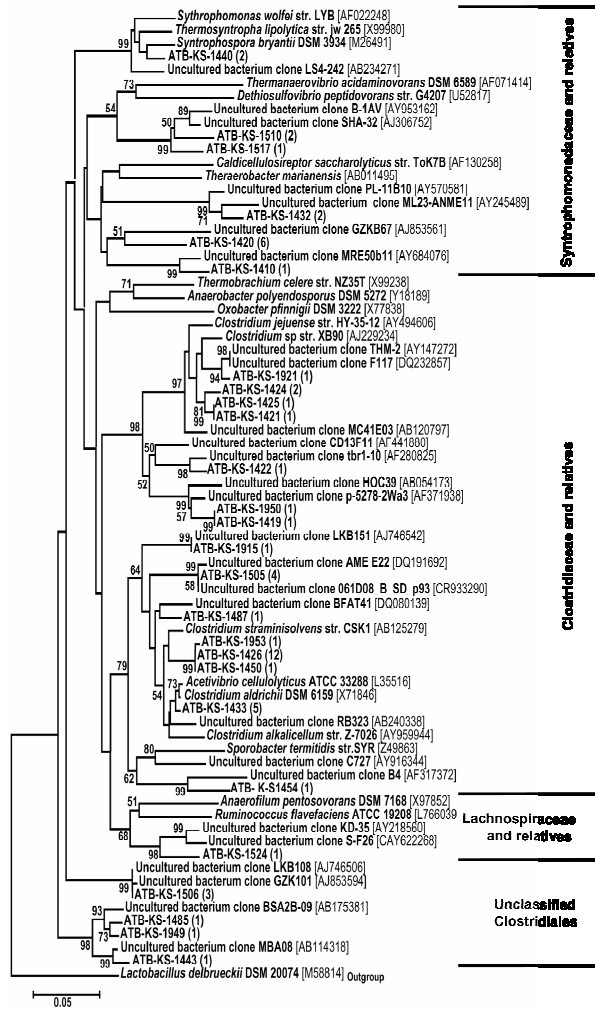

Fig. 3: Phylogenetic tree of the clones among the order *Clostridiales* of the domain *Bacteria* based on neighbor-joining analysis of partial 16S rDNA sequences. Numbers behind the OTU derived from the *Bacteria*-specific 16S rDNA library (ATB-KS-) indicate the number of clones of which this OTU is representative. *Lactobacillus delbrueckii* DSM 20074 was used as the outgroup. Further details are as given in Fig. 2.

Similarly, OTU assigned to further families within the class *Clostridia* also exclusively showed high similarities to uncultured micro-organisms. This was the case for six OTU assigned to the family *Syntrophomonadaceae* and the one OTU assigned to the family *Lachnospiraceae* (Fig. 3). The environments within which related micro-organisms were detected are sediments (clone LS4-242 [AB234271]), soils (clone S-F26 [AY622268]) or a sodium bicarbonate-enriched sea (clone ML23-ANME11 [AY245489]).

Four OTU could not be grouped with any taxonomic group within the class *Clostridia*. However, similarities were found to certain 16S rDNA sequences detected in landfill leachate clone (GZK101 [AJ853594]), mesophilic BSA digesters (clone BSA2B-09 [AB175381]) or thermophilic sewage reactors (clone MBA08 [AB114318]) (Fig. 3).

One OTU, ATB-KS-1431, representative of one clone within the bacterial 16S rDNA library, was affiliated to the class *Mollicutes.* Within this class, the OTU was grouped with *Holdemania filiformis* ATCC 51649 [Y11466].

4.5 Domain Bacteria - phylum Bacteroidetes

Another major group within the *Bacteria*-specific 16S rDNA comprising 13 OTU was constructed together with members of the phylum *Bacteroidetes*. Within the *Bacteroidetes*, most OTU were grouped with members of the classes *Flavobacteria* and *Bacteroides*.

Four OTU, each indicated by one single clone within the 16S rDNA library, were assigned to members of the class *Flavobacteria.* The OTU ATB-KS-1411 and ATB-KS-1934 showed similarities of 99 % and 93 % respectively to cultured references like *Ruminofilibacter xynalolyticum* str. S1 [DQ141183] and *Aequorivita antarctica* str. S4-8 [Ay771732].

The class *Bacteroides* was detected by five OTU representing 7 clones. Here, highest 16S rDNA similarities were detected to clones derived from certain environmental samples, e.g. clone LKB109 [AJ746507] derived from Chinese landfill, clone 009C01_B_SD_P15 [CR33168] derived from waste water samples in France and clone ET10-9 [DQ443965] derived from a sulfur-reducing digester in China.

As shown by the phylogenetic analysis, four OTU were clustered separately besides the conventional three classes. Three of these OTU showed only minor differences within their 16S rDNA sequences and were grouped in a separate cluster. No cultivated reference species could assigned to this cluster. However, certain uncultured organisms represented by their 16S rDNA sequences were grouped within this cluster like clone EUB50-2 [AY693829] isolated from anaerobic sludge from Thailand and the clones T20H80A09 [AB244036] and p3h06ct-1 [AY578592] derived from the bovine rumen biocoenosis. An affiliation of this cluster to the class *Sphingobacteria* remains questionable due to their large sequence dissimilarities.

4.6 Domain Bacteria - phylum Proteobacteria

Three of the five classes of the phylum *Proteobacteria* were detected within the bacteria-specific 16S rDNA library. Several OTU were assigned to members of the classes *α-*, *γ-*, *ε-Proteobacteria*, while *β-* and *δ-Proteobacteria* were not found.

The class *α-Proteobacteria* was represented by one OTU, ATB-KS-1444, which was affiliated with members of symbiotic root tuber bacteria of *Rhizobiaceae*. As an example, 95 % of the 16S rDNA sequence of *Xhantobacter agilis* str. SA35 [X94198] isolated from roots of certain legumes (Moreira et al. 1998) and out of soil samples (Vacca et al. 2005) was similar to the corresponding sequence of OTU ATB-KS-1444.

Two OTU from the class *γ-Proteobacteria* were detected, each representative of one clone within the bacterial 16S rDNA library. OTU ATB-KS-1494 was assigned to the clones GZKB12 [AJ853507] and GZKB48 [AJ853542] both detected in landfill leachate and waste water respectively. OTU ATB-KS-1435 was grouped with *Pseudomonas* sp. str. CL-2 [DQ337603] exhibiting a sequence similarity larger than 99 %.

The class *ε-Proteobacteria* was found to be present in the 16S rDNA library with one OTU, ATB-KS-1516. This clone was affiliated with the order *Campylobacterales* together with members of the genus *Arcobacter*. The references *Arcobacter* sp. 16695-3 [AY314754] and *Arcobacter cryaerophilus* [L14624] possessed sequence similarities of 97 and 93 % respectively with ATB-KS-1516. These strains were isolated out of animal excrements.

4.7 Domain Bacteria - phylum Spirochaetes

Two OTU, ATB-KS-1452 and ATB-KS-1434, representative of together six clones in the bacterial 16S rDNA library, were assigned to the phylum *Spirochaetes*. Highest sequence similarities were found with the reference *Spirochaeta zuelserae* [M88725] and with the uncultured clone LKB86 [AJ746489] derived from landfill leachate.

4.8 Domain Bacteria - phylum Chloroflexi

The phylum *Chloroflexi* was detected by one clone and OTU, respectively, ATB-KS-1492, within the 16S

rDNA library. The highest similarities of approximately 96 % were given to sequences of uncultured micro-organisms. Examples are the clone JN18A7F [DQ168648] isolated from sediments (USA) or the clones 261 und 955 [AY935659, AY935680] derived from a sample of fermented oil mill waste in Spain.

4.9 Domain Bacteria - phylum Fibrobacteres

The phylum *Fibribacteres* was present in the bacteria-specific 16S rDNA library with one OTU, ATB-KS-1923. This OTU was assigned with the cellulose-degrading bacteria *Fibrobacter succinogenes* str. FE [AJ496566]. The similarity of the both 16S rDNA sequences was about 89 %.

4.10 Domain Bacteria - unclassified bacterial micro-organisms

Interestingly, a huge number of OTU within the library enriched for bacterial 16S rDNA sequences could not be grouped directly with known taxonomic units in the phylogenetic analysis. This was the case for nine OTU representing 33 clones (i.e. 28 %) of the 16S rDNA library. However, these OTU possessed high sequence similarities of 95 to 97 % with certain 16S rDNA sequences of also yet uncultivated micro-organisms derived from various environmental samples. Together with these environmental clones, the nine OTU formed three distinct clusters. One cluster was formed by seven OTU found in this study and environmental clones like GZKB79 [AJ853573] isolated out of waste water, BSA2B-20b [AB175392] isolated from a mesophilic BSA-degrading bioreactor and BHB21 [AB248650] isolated from a mesophilic butyrat-degrading bioreactor. Out of these seven OTU the OTU ATB-KS-1409 was present in the 16S rDNA library with 24 clones (*i.e.* 20.3 %).

Another cluster was formed by OTU ATB-KS-1503 and ATB-KS-1929 and clones derived from saline milieus like QLS20-B20 [AY940536]. A third cluster was constructed from OTU ATB-KS-1446 and the uncultured clones PL-11B10 [AY570581] and clone MS149BH1062003_13 [DQ354746] isolated from a French oil reservoir and ground water in South-Africa, respectively. The 16S rDNA sequences within this cluster proved to be similar in approximately 93 % of the nucleotides analyzed.

5 Discussion

The co-fermentation of maize silage with bovine manure in a CSTR is known to enable a productive and stable digestion process. According to Linke & Mähnert (2005) the maximal VS-related biogas yield is 0.61 l g^{-1} for bovine manure and 0.92 l g^{-1} for maize silage, respectively. Given VS:substrate relation of 2:1 a potential biogas yield of 0.71 l_{biogas} $l_{fermenter\ volume}^{-1}$ g^{-1} can be calculated. The actual biogas yield of 0.46 l^{-1} g^{-1} measured in this experiment was equal to 64.8 % of the hypothetic potential. The determined content of methane of 56.1 % was also of a similar dimension as found previously in other experiments (Linke & Mähnert 2005). Both the comparatively low concentrations of acetic acid (in maximum 0.41 g l^{-1}) and the stable pH value (7.7 to 8.1) in the effluent of the reactor indicate that the milieu was advantageous for the production of methane within the analyzed CSTR.

The microbial biocoenosis within a biogas reactor depends highly on the substrate composition and the fermentation conditions. However, certain microbial groups with similar substrate requirements can be defined: (1) fermentative *Bacteria* catabolizing the oxidation of carbohydrates to VFA, acetate and H_2, (2) *Bacteria* converting VFA to acetate and H_2 by anaerobic oxidation, (3) anaerobes oxidizing acetate to H_2 and CO_2, (4) acetate-utilizing *Archaea* (i.e. acetotrophic methanogens) and (5) H_2-oxidizing *Archaea* (i.e. hydrogenotrophic methanogens) (Ahring 2003). For analysis of this complex microbial community, the culture-independent analysis of microbial 16S rDNA has been proven to be a versatile and effective tool.

In the analyzed biogas-producing fermenter a broad range of methanogenic *Archaea* was detected. Most of these *Archaea* (71 % of the OTU representing 90 % of the archaeal clones) were related with H_2-utilizing methanogens like *Methanoculleus bourgensis*, *Methanobrevibacter ruminantium*, *Methanospirilllum hungatei* and *Methanocorpusculum parvum*. Only 21 % of the detected archaeal OTU representing 9 % of 16S rDNA clones were assigned to known acetate-oxidizing methanogens like *Methanosarcina barkeri* or *Methanosaeta concilii*. Especially *Methanosaeta* sp. is regarded as being important for formation of granules (Griffin et al. 1998, Merkel et al. 1999). The low abundance may be a result of the high organic fraction within the biogas reactor, which prevents the formation of granules.

The abundance of OTU related to hydrogenotrophic methanogens within the *Archaea* specific 16S rDNA library as well as the rich diversity of these OTU may indicate the establishment of a large H_2-consuming *Archaea* population beside the acetate-utilizing *Archaea* within this biogas reactor. This is in contrast to other studies, which reveal a predominance of acetotrophic *Archaea*. McHugh et al. (2003) reported a strong abundance of OTU closely related to *Methanosaeta concilii* in a range of different wastewaters utilizing granular sludge biogas reactors. In CSTR without obvious granule formation, a majority of potential acetotrophic *Archaea* like *Methanosaeta* sp. and

Methanosarcina sp. were also found (Klocke et al. 2007).

A large population of hydrogenotrophic methanogens would result in a low and constant H_2 concentration, which would force the oxidation of VFA to acetate by syntrophic acetogenic *Bacteria* (Ahring 2003). However, an accumulation of acetate in the effluent of the reactor was not observed. This finding may be caused hypothetically by three effects: (1) the detected acetotrophic methanogens are able to compensate the accumulation of acetate with an enhanced metabolism and synthesis of methane, (2) an oxidation of acetate to H_2 and CO_2 occurs through other micro-organisms than acetotrophic methanogens, (3) the experimental design is not sufficient to reflect the real quantities of acetotrophic and hydrogenotrophic methanogens within the biogas reactor. To solve this question, further research needs to be done.

Within the fermentative *Bacteria*, members of the class *Clostridia* are the most abundant OTU in the *Bacteria* specific 16S rDNA library (45 % of detected OTU representing 47 % of analyzed clones). Certain of these OTU were assigned with anerobic *Clostridia* like *C. jejuense* (Hyunyoung et al. 2004) and *C. straminisolvens* (Kato et al. 2004), which degrade high-molecular carbon sources to short chain organic acids like pyruvate, lactate or acetate and hydrogen. *C. jejuense* prefers glucose as substrate, in contrast *C. straminisolvens* utilizes cellulose. Another detected clostridial OTU related to *Syntrophospora bryantii* (Zhao et al. 1990) is presumed to be a member of the syntrophic community associated with hydrogenotrophic *Archaea*, which metabolize glucose to acetate, propionate and hydrogen. Similar to the clostrial fermentative micro-organisms, several OTU related to fermentative organisms from other phyla like *Holdemania* sp. (Willems et al. 1997) or *Fibrobacter* sp. (Montgomery et al. 1988, Bera-Maillet et al. 2004) were detected. However, the huge diversity of these acid-producing *Bacteria* demands further efforts in molecular analysis of this complex microbial community.

In conclusion, the molecular approach has proved to be useful for the characterization of the complex microbiology of plant-degrading CSTR. It provides insights not only into the participating microbial species. Furthermore, this approach allows an overview of possible metabolic substrate fluxes. However, this work provides no more than a first insight into this complex biocoenosis. Much work remains to be done on the microbiology and the operation of plant-degrading biogas reactors. A deeper understanding of the microbial conversion processes within biogas-producing digesters will facilitate optimization of recently applied technologies.

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