Journal of Bioscience and Bioengineering
VOL. 117 No. 6, 730–736, 2014

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# Assessment of a biogas-generating microbial community in a pilot-scale anaerobic reactor

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Received 19 July 2013; accepted 18 November 2013
Available online 10 January 2014

**In this work bacteria and methanogenic archaea utilizing agricultural wastes in a pilot-scale biogas reactor were examined using sequencing and terminal restriction fragment length polymorphism analysis. Based on the analyses of 16S rRNA genes, *Clostridia* represented the most diverse group in the digester. Of the *Clostridia*, unclassified *Clostridiales* and the members of the genera *Anaerotruncus* and *Tissierella* were detected at high abundances. The representatives of the bacterial phyla *Bacteroidetes* and *Proteobacteria* were also defined, but in minor proportions, and were assigned to non-dominant communities. Within the phylum Euryarchaeota, the members of the orders *Methanosarcinales* and *Methanomicrobiales* were found at high levels. Methanogenic archaea were analyzed using both 16S rRNA and *mcrA* genes. Actually good results were received using both approaches; however, the rRNA gene method missed the non-dominant order *Methanobacteriales*.**



[**Key words:** Biogas; Agricultural wastes; 16S rRNA genes; *mcrA* genes; Terminal restriction fragment length polymorphism]

Many research works have been devoted to the development of new clean and renewable alternative energy sources due to the increase of gas and oil prices, the depletion of energy resources, and the necessity to protect the environment from global warming. One of the perspective alternative energy sources is biomass. Large amounts of organic wastes, such as agricultural and municipal wastes, become valuable sources of energy. In this connection, it is necessary to create new processing and recycling technologies, including biotechnology for microbial conversion of organic residues with biogas production, which is considered as one of the most efficient and environmentally attractive methods (1–3).

Anaerobic treatment of different organic wastes by microbes, involving the members of the Bacteria and Euryarchaeota, appears to be the effective biotechnological method to convert biowastes into bioenergy. Intensive process of anaerobic digestion of biowastes requires the maintenance of certain optimal microbial processes. As a consequence, many researchers have focused their works on the production of biogas from biodegradable materials (e.g., agricultural, municipal, and industrial wastes as well as sewage sludge) and have made successful efforts on the investigation of the structure and dynamics of biogas-producing microbial associations in various bioreactors (2,4–10).

Except for the cultured collection of bacterial and methanogenic strains, in the last years a large group of uncultured microorganisms was discovered. Since it is difficult to study anaerobic microorganisms with culture-based methods, culture-independent molecular methods were intensively developed to investigate complex bacterial and archaeal communities in the environment. These methods allow the studying prokaryotic communities diversity based on 16S rRNA gene and some functional genes. Methanogens can be effectively analyzed based on α subunit of methyl coenzyme M reductase (*mcr*A) gene, molecular metabolic marker of methanogenesis (11–14). Methyl coenzyme M reductase, which is peculiar to methanogenic archaea, catalyzes the reduction of methyl coenzyme M with coenzyme B to heterodisulfide and methane under anaerobic conditions (15). Molecular biology techniques developed for the characterization of microbial populations, which carry out the anaerobic treatment of biomass, allow researchers to monitor microbial interactions and ultimately will help to improve the efficiency of the whole anaerobic digestion process.

Within the main research topics, to achieve a more stable anaerobic digestion process and to avoid its failure, should be the selection of well-adapted microbial populations based on the substrate composition as well as the investigation of the key biochemical pathways for various organic compounds degradation with biogas production. Scientific advances in the establishment of highly active and well-adapted members of biogas-producing microbial communities should be a breakthrough in solving the problem of biowastes disposal and, at the same time, should provide a scientific basis for regulation of the anaerobic process with biogas generation. Recently we investigated bacteria and archaea involved in anaerobic digestion of multifarious organic waste materials in lab-scale digesters (10,16), and in this research we set a goal to determine bacterial and methanogenic archaeal community diversity in a pilot-scale biogas reactor. The feedstock for the bioreactor was composed primarily of cattle manure and plant biomass. The diversity of the bacterial community involved in this anaerobic process was assessed by creating clone libraries for the bacterial 16S rRNA genes and by terminal restriction fragment

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1389-1723/$ – see front matter 
http://dx.doi.org/10.1016/j.jbiosc.2013.11.013

length polymorphism (T-RFLP) analysis. For methanogenic archaea composition, the community structure was analyzed using both 16S rRNA and *mcrA* genes.

## MATERIALS AND METHODS

**Operation of the biogas reactor and analytical techniques** The agricultural biogas reactor is located in a dairy farm in the Buinsky municipal district, the Republic of Tatarstan, Russia. This is the first pilot-scale continuous stirred tank reactor constructed in the Republic of Tatarstan in 2010. The anaerobic digester with a working volume of 25 $m^3$ was continuously fed with agricultural wastes, primarily with cattle manure and plant biomass, and operated stably at mesophilic regime (38–39°C). The reactor had been running stably for more than 6 months when the research was started. Samples for analyses were taken in October, 2011 and March, 2012 and then analyzed as described in our recent publications (10,16). The biogas yield varied in the range of 304–331 L $kg^{-1}$ volatile solids and the methane content was in the range of 52–55% during the reactor operation. The pH value was kept at 7.6–7.8 in the digester. The concentrations of organic acids and ammonium in the first sample achieved 1.1 ± 0.06 g $L^{-1}$ and 0.71 ± 0.02 g $L^{-1}$ values, respectively, while the organic acids and ammonium amounts in the second sample were 1.3 ± 0.08 g $L^{-1}$ and 0.85 ± 0.03 g $L^{-1}$, accordingly.

**DNA extraction and purification** Samples for microbial community analyses were collected into sterile 15 mL Falcon tubes and used immediately for DNA extraction and purification. DNA was eluted and purified from 0.25 mL of the sample with a PowerSoil DNA Isolation Kit (MO BIO, USA), checked by 1.5% agarose gel electrophoresis, and quantified with a NanoVue Plus UV–Vis spectrophotometer (GE Healthcare, USA).

**16S rRNA and *mcrA* genes amplification, cloning, and sequencing** Bacterial 16S rRNA gene fragments were PCR-amplified using the primers UniBac27F (5′-GAG TTT GAT CMT GGC TCA G-3′) and Univ1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′), whereas archaeal 16S rRNA gene fragments were amplified with the forward primer UniArc21F (5′-TTC YGK TTG ATC CYG SCR G-3′) and the reverse primer UniArc931R (5′-CCC GCC AAT TCC TTT HAG-3′) as described previously by us (10,16). *McrA* genes were amplified by using a combination of the primers mcrA-mlas (5′-GGT GGT GTM GGD TTC ACM CAR TA-3′) and mcrA-rev (5′-CGT TCA TBG CGT AGT TVG GRT AGT-3′) as described by Steinberg and Regan (12).

Amplified 16S rRNA and *mcrA* genes were checked in 1.5% gel electrophoresis and then purified using a QIAquick PCR Purification Kit (Qiagen, Germany). Purified genes were cloned using InsT/Aclone PCR Cloning Kit (Fermentas, Lithuania) according to manufacturer's recommendations. The presence of the appropriate inserts of bacterial and archaeal 16S rRNA genes as well as *mcrA* genes in positive clones was analyzed using the vector-specific M13 primers. 1 μl of the obtained amplicons were further treated with the restriction enzyme *Hae*III (New England Biolabs, Germany) and separated electrophoretically. The restriction patterns were analyzed using a Phoretix 1D software (Nonlinear Dynamics, UK). Representative clones from all clusters were chosen for partial 16S rRNA or *mcrA* sequencing, which was performed in Syntol Labs (Moscow). The received 16S rRNA data were compared to the NCBI database by using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST) and taxonomically assigned in appliance with the RDP Classifier (http://rdp.cme.msu.edu). *McrA* sequences were analyzed using BLASTX program (http://www.ncbi.nlm.nih.gov/BLAST). Data were checked for chimeric sequences with Bellerophon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl). The partial sequences of 16S rRNA and *mcrA* genes received in this work were deposited in the GenBank database (accession numbers KF419189–KF419207).

**T-RFLP analysis** T-RFLP analysis was performed in accordance with our previous works (10,16). Bacterial 16S rRNA genes were PCR-amplified with UniBac27F-FAM and Univ1492R, archaeal 16S rRNA genes using the primers UniArc21F-FAM and UniArc931R, and *mcrA* genes with the primers mcrA-mlas and mcrA-rev-FAM. Amplicons were then cleaned up with a QIAquick PCR Purification Kit (Qiagen, Germany). Bacterial 16S rRNA genes were then subject to restriction enzyme digestion with the restriction endonucleases *Hae*III and *Rsa*I (New England Biolabs), archaeal 16S rRNA genes were cut with *Hae*III and *Mse*I (New England Biolabs), and *mcrA* genes were digested with *Hae*III and *Msp*I (New England Biolabs).

GeneScan 500 ROX and GeneScan 1200 LIZ Size Standards (Applied Biosystems, USA) were used to obtain the molecular size of restriction patterns. Fluorescently labeled T-RFs were sized on a genetic analyzer in Syntol Labs, and T-RFLP fingerprint patterns were analyzed using Peak Scanner Software v1.0 (Applied Biosystems). T-RFs of archaeal 16S rRNA genes that were <35 bp and >900 bp in size were excluded from further analyses; T-RFs of bacterial 16S rRNA genes and *mcrA* genes <50 bp and >500 bp were also removed from subsequent analyses. T-RF values of the sequenced clones were received by T-RFLP analysis of corresponding 16S rRNA and *mcrA* clones. Noise removal, peak binning to account for inter-run differences in T-RF size and normalization of signal intensity were performed using R script (R version 2.12.2; http://www.r-project.org) and using a cutoff value of six times the standard deviation to remove background noise (17).

## RESULTS AND DISCUSSION

**Bacterial community composition** The bacterial diversity in the biogas reactor was assessed by constructing 16S rRNA gene clone libraries at two distinct sampling times. 288 clones were collected in total and screened by restriction fragment length polymorphism analysis. Table 1 illustrates the sequencing results of representative clones, the next BLAST relatives (to cultured strains only), the taxonomic affiliation of the clones based on the RDP Classifier as well as the terminal restriction fragment (T-RF) lengths. Based on the RDP Classifier results and the T-RF values, 9 operational taxonomic units (OTUs) were defined. The discovered bacterial phyla were the *Firmicutes* comprising 6 OTUs, the *Bacteroidetes* comprising 2 OTUs, and *Proteobacteria* with 1 OTU detected. Of the *Firmicutes*, *Clostridia* was the most diverse class and comprised members of unknown *Clostridia* and the genera *Anaerotruncus*, *Tissierella*, and *Saccharofermentans*. Two clones were affiliated with unknown *Bacteroidetes* and one clone belonged to the phylum *Proteobacteria* (*Castellaniella* sp.).

Bacterial community composition was compared with T-RFLP analysis with two different restriction enzymes, *Hae*III and *Rsa*I. Fig. 1 shows the results received after using of *Hae*III in T-RFLP analysis. T-RFs were assigned to OTUs based on the sequence data shown in Table 1. Since one restriction enzyme cannot resolve all OTUs because of the same T-RF lengths of a few phylotypes, for the taxonomic assignment also the T-RFLP profiles generated with *Rsa*I were considered (data not shown). T-RFLP allowed the recognition of about 17–22 different profiles in the reactor. In spite of the fact that some T-RFs could not be taxonomically assigned as they did not match with any OTUs, most of T-RFs with significant relative abundances were identified and, therefore, bacteria playing the key role in the anaerobic digestion of cattle manure and plant biomass were established.

The most abundant bacterial phylum detected in the samples from the biogas reactor was *Firmicutes*, within it *Clostridia* represented the most diverse group. Within all *Clostridia*, OTU 3 dominated in our reactor and comprised up to 40% of the total T-RF peak

**TABLE 1.** Sequencing results of representative bacterial 16S rRNA gene clones and experimentally determined terminal restriction fragments (T-RF).

| OTU | Clone (bp) | Acc. no. | Closest affiliation[a] (acc. no.)/% similarity | Taxonomic affiliation according to RDP 10 | *Hae*IIIT-RF (bp) | *Rsa*IT-RF (bp) |
|---|---|---|---|---|---|---|
| OTU 1 | bac_B1 (322) | KF419195 | *Thermoanaerobacter uzonensis* JW/IW_A615 (HM182375)/88% | *Clostridia* | 159 | – |
| OTU 2 | bac_D5 (425) | KF419199 | *Desulfotomaculum kuznetsovii* strain 17 (AY036903)/91% | *Clostridia* | 153 | 304 |
| OTU 3 | bac_G4 (590) | KF419202 | *Clostridium* sp. 6-31 (FJ808611)/86% | *Clostridiales* | 234 | 471 |
| | bac_D3 (403) | KF419198 | *Clostridium* sp. 6-31 (FJ808611)/87% | *Clostridiales* | 235 | 471 |
| OTU 4 | bac_A5 (471) | KF419194 | *Anaerotruncus colihominis* strain S6 (KC206033)/89% | *Anaerotruncus* sp. | 295 | 100 |
| OTU 5 | bac_B5 (542) | KF419196 | *Tissierella creatinophila* strain Kre4 (NR_037028)/93% | *Tissierella* sp. | 297 | 61 |
| OTU 6 | bac_A4 (544) | KF419193 | Rumen bacterium NK4A65 (GU324373)/92% | *Saccharofermentans* sp. | 308 | 71 |
| OTU 7 | bac_F4 (636) | KF419201 | *Flexibacter aggregans* strain: IFO 15974 (AB078038)/85% | *Bacteroidetes* | 68 | 309 |
| OTU 8 | bac_E1 (541) | KF419200 | *Bacteroidales* bacterium RM68 (AB730709)/86% | *Bacteroidetes* | 255 | 469 |
| OTU 9 | bac_C4 (602) | KF419197 | *Castellaniella* sp. MJ05 (GQ250433)/97% | *Castellaniella* sp. | 226 | 126 |

[a] Uncultured/environmental sample sequences were excluded from the analyses.