Biodegradation **11:** 359–364, 2000.

Acetate conversion in anaerobic biogas reactors: Traditional and molecular tools for studying this important group of anaerobic microorganisms

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Accepted 11 December 2000

Key words: CSTR reactor, methanogens, 16S rRNA hybridization, UASB reactor

Abstract

Different methods were applied to study the role of aceticlastic methanogens in biogas reactors treating solid waste and wastewater. We used traditional microbiological methods, immunological and 16S rRNA ribosomal probes for detection of the methanogens. Using this approach we identified the methanogenic spp. and their activity. In biofilm systems, such as the UASB reactors the presence of the two aceticlastic methanogens could be correlated to the difference in the kinetic properties of the two species. In biogas reactors treating solid wastes, such as manure or mixture of manure and organic industrial waste, only *Methanosarcina* spp. were identified. *Methanosarcina* spp. isolated from different plants had different kinetics depending on their origin. Relating the reactor performance data to measurement of the activity by conventional microbiological methods gave a good indication of the microbial status of specific trophic groups. 16S rRNA probing confirmed these observations and gave a more detailed picture of the microbial groups present.

Introduction

The importance of the acetate utilizing methanogens in the environment as well as their biotechnological potential in biogas production, waste and wastewater treatment is well recognized and has stimulated the attention for this group of microorganisms. During the anaerobic conversion of complex organic matter in such biogas systems, acetate is a key intermediate and the main precursor of methane production. To date, the only microorganisms participating in the direct conversion of acetate to methane are identified as the archaea of the genera *Methanosarcina* and *Methanosaeta* (Jetten et al. 1992; Zinder 1990). Representives of these two genera can be distinguished from each other both morphologically and physiologically and in their affinity for acetate. *Methanosaeta* spp. are filemantous organisms which are known to grow only on acetate. *Methanosarcina* spp. usually grow in large aggregates up to 1 to 3 mm in diameter. These aggregates consist of single cells surrounded by a thick wall. However, under certain conditions, such as high cation concentration, it can grow as single cells (Ahring et al. 1991). *Methanosarcina* spp. use several methanogenic substrates such as acetate, methanol, methylamines and sometimes also H_2/CO_2. Differences in their acetate kinetics, reflected by lower maximum specific growth rates (μ_{max}), lower half saturation constants K_S and lower threshold to acetate of *Methanosaeta* spp. comparing to *Methanosarcina* spp., is one of the reasons why *Methanosarcina* spp. are often found in environments with acetate concentration higher than 1 mM, while *Methanosaeta* spp. are

numerous in environments with acetate concentration below 1 mM.

The role and function of microorganisms have been studied in various natural and engineered ecosystems by several culture-based methods. Typically, these methods have estimated the functional biodiversity by testing the capability of the microbial population in a sample to utilize an array of different substrates. Alternatively, the study can be based on immunological methods. However, several methods have, in recent years, been developed utilizing molecular methods to circumvent the bias introduced by cultivation-based diversity analyses. Most of these methods are built upon analysis of the diversity of 16S RNA/rDNA of total extractable RNA/DNA from the investigated environment (Raskin et al. 1994; Zheng et al. 1996). In recent years, different molecular approaches of characterization of microbial activity have been developed. These methods are based on reverse transcription of mRNA coding for functionally specific enzymes/genes, or reported gene systems to measure promoter activity of specific genes (Hobson et al. 1995; Holmstrøm et al. 1999). In this way, it is possible to detect whether microorganisms possessing a specific gene are active with respect to a specific function.

In the present paper we will compare the different methods available for studying the conversion of acetate to methane in biogas reactors treating solid waste and waste water. By use of the traditional microbiological tests, immunological probes and 16S rRNA probes we characterized the acetate utilizing methanogenic microorganisms in the reactors and measured their activity.

Materials and methods

Microorganisms

Methanosarcina thermophila TM-1^T (DSM 1825^T) and *Methanosarcina mazei* S-6^T (DSM 2053^T) were originally obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, FRG). *Methanosarcina* sp. V-1P (DSM 11434) was from our culture collection. The strains TM-1^T, V-1P and S-6 were grown in an anaerobic medium containing 50 mM sodium acetate and 1 g/l yeast extract as previously described, under N_2/CO_2 (80% : 20%) atmosphere at temperature of 50, 54 and 37 °C, respectively (Mladenovska & Ahring 2000).

CSTR reactor experiment

The substrate used in the reactor experiment was cattle manure containing 52 g l^{-1} total solids (TS), 45 g l^{-1} volatile solids (VS) and 1.8 g NH_4-N l^{-1}. When needed, lipids in form of glycerol trioleate, GTO, (65–70%, technical quality, Sigma, St. Louis, USA) were added in amount 5% (w/w) of the manure.

The reactor set up was as previously described (Angelidaki & Ahring 1993). Two lab-scale biogas reactors, R1 and R2, each with a working volume of 3 l operated at 55 °C with a hydraulic retention time of 15 days. Both control reactor R1 and test reactor R2 were inoculated with digested cattle manure. Reactor R2 was in addition inoculated with cell suspension of *Methanosarcina* sp. V-1P, harvested from a volume of three liters culture broth. In the first part of the experiment (Part 1) the reactors were fed with cattle manure and in the second part of the experiment (Part 2), the mixture of cattle manure with GTO was used. Both substrates were used for feeding in 80 days.

UASB reactors

The substrate used in the UASB reactor experiment was BA medium with acetate as carbon source (Angelidaki et al. 1990). The reactor set up was as previously described (Schmidt et al. 1991). One UASB reactor with a working volume of 0.2 l was operated at 37 °C and a hydraulic retention time of 6 hours. The acetate concentration in the effluent was changed in steps from 10 mM to 70 mM. Granular sludge was sampled from the reactor under the different conditions when steady state was achieved.

Microbiological tests

The measurement of specific methanogenic activity (SMA) using 50 mM sodium acetate was done according to the method described previously (Sørensen & Ahring 1993). The counts of microbial populations present in the reactor were estimated by the three-tube MPN technique according to the standard method (de Man 1975). The substrate used was 50 mM sodium acetate. Both tests were done in Part 1 and Part 2 of the experiment.

Immunological methods

The slide immunoenzymatic assay constellation was used for the immunological identification and enumeration of methanogens as previously described. Methanogens were identified by the antigenic fingerprinting

method. Reference methanogens were used as controls and as reference morphotypes in each tests. All experiments were run at least three times (Schmidt et al. 1992).

16S rRNA oligonucleotide probes

The oligonucleotide probes complementary to the regions of 16S rRNA molecules used in this study were: Universal probe 5′-GACGGGCGGTGTGTACAA-3′ (Zheng et al. 1996), archaeal domain-specific probe 5′GTGCTCCCCCGCCAATTCCT3′ (Maidak et al. 1999), family *Methanosarcinaceae*-specific probe 5′-CTCACCCATACCTCACTCGGG-3′, genus *Methanosarcina*-specific probe 5′-CGCCATGCCTGACACCTAGCGAGC-3′ (Raskin et al. 1994) and strain *Methanosarcina thermophila* TM-1^T specific probe 5′-GAAAGCCTAACGGTTGAGCCGT-3′ (this study). Probes were synthesized commercially (DNA Technology, Århus, Denmark) and were subsequently 5′′ end-labeled with γ-^{32}P-isotope using T4 polynucleotide kinase (Pharmacia, Allerød, Denmark) as recommended by the manufacturer. The labelled probes were purified using Bio Gel P-6 microspin columns (Cat. No. 732-6201, Bio Rad, Copenhagen, Denmark). The dissociation temperature for each probe was determined as described by Zheng et al. (1996).

RNA extraction and hybridization

Aliquots of 1.5-ml manure were withdrawn from the reactor and kept frozen at −80 °C until analyzed. Total ribosomal RNA from the manure was extracted according to the published procedure (Ibrahim & Ahring 1999). The RNA concentration was adjusted spectrophotometrically. A detailed description of the hybridization conditions is published elsewhere (Ahring et al. 2000). The hybridization signal intensity was measured using Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA). The abundance of archaeal RNA was related to the total rRNA identified by universal probe. The amount of RNA of family *Methanosarcinaceae*, genus *Methanosarcina* and strain *Methanosarcina thermophila* TM-1 were related to the amount of archaeal RNA.

Analytical methods

The analysis of total solids, volatile solids and NH4-N was performed according to standard methods (Greenberg et al. 1992). Methane and volatile fatty acids (VFA) were measured by gas chromatography according to the described method (Angelidaki et al. 1990).

Table 1. The ratio between *Methanosarcina* spp. and *Methanosaeta* spp. in granules grown under different conditions. The methanogens were identified with the IIF-methods

Influent concentration of acetate (mM)	Effluent concentration of acetate (mM)	Ratio *Methanosarcina* spp./*Methanosaeta* spp
10	0.1	4.2
30	2.4	5.4
70	4.7	6.4

Results and discussion

Acetate degrading methanogens in UASB reactors

The IIF method was used to identify acetate utilizing methanogens in granules grown under different condition. The results are shown in Table 1. When the acetate concentration in the effluent was low the highest amount of *Methanosaeta* spp. was detected while the ratio between *Methanosarcina* spp. and *Methanosaeta* spp. increased when the acetate concentration in the effluent increased. This indicated that the presence of the two methanogens in the granules was correlated to the kinetics of the two methanogens.

Acetate degrading methanogens in CSTR reactors

Using immunological probes, we identified acetate utilizing methanogens in samples of organic waste treated in different thermophilic biogas plants (Mladenovska 1997). Independent of whether the reactors operated on manure alone, or on mixtures of manure and industrial organic waste, the only recognized acetate utilizing methanogens were *Methanosarcina*-related microorganisms. These strains were present in two morphological forms, both as single cells and as aggregates. No cells with a relation to the genus *Methanosaeta* could be seen in the samples (Mladenovska 1997).

Methanosarcina spp. isolated from different biogas plants distinguished from each other in growth

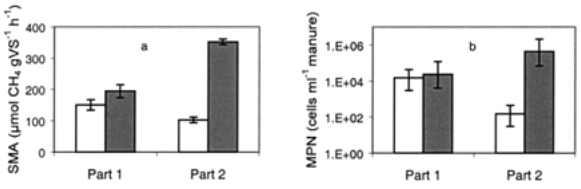

Figure 1. Specific methanogenic activity (a) and MPN counts (b) of the acetate-utilizing methanogens in control reactor (white bar) and seeded reactor (shadowed bar). The values are means of triplicates with standard deviations.

kinetics on acetate. The strains isolated from reactors operating on mixtures of manure and other types of organic waste had better affinity for acetate than strains isolated from reactors operated on manure alone (Mladenovska & Ahring 2000).

To further elucidate the role of *Methanosarcina* spp. in the digestion process, the performance of control reactor R1 and reactor R2 seeded with *Methanosarcina* sp. V-1P was compared. Table 2 summarizes the main reactor performance parameters.

When the reactors received manure as the sole substrate, no significant difference in the methane yield and level of volatile fatty acids were monitored. After the introduction of lipids, the control reactor R1 became unstable with constantly decreasing methane production and increasing the level of volatile fatty acids over time. On the contrary, the seeded reactor R2 operated stable, with a constant biogas production and constant levels of volatile fatty acids in the reactor. The degradation of organic matter in this reactor was significantly higher when feeding with the mixture of substrates. The biomass of reactors was tested by the MPN method and SMA test. Figure 1 compares the results from this experiment. When the reactors received manure as the sole substrate, no significant difference in MPN counts and SMA of the acetate utilizing methane producers was observed. After the introduction of lipids, a significant decrease in MPN numbers and SMA of acetate utilizing methanogens was observed in the control reactor R1, while an increase in MPN counts and enhancement of SMA were found in the reactor R2.

Oligonucleotide probing of 16S rRNA provided more detailed information about the activity of selected methanogenic populations in the reactors. The probes applied for the detection of the family *Methanosarcinaceae*, the genus *Methanosarcina* and the strain *Methanosarcina thermophila* TM-1^T revealed that all these populations are present in both systems, but some differences in their distribution were observed (Table 3).

The *Methanosarcinaceae*-targeting probe covers the methylotrophic methanogens grouped in genera *Methanosarcina*, *Methanococoides*, *Methanolobus*, *Methanohalophilus* and acetotrophic methanogens of *Methanosaeta* (Raskin et al. 1994). In the biogas reactors, the genus *Methanosarcina* seemed to be the most important member of the family *Methanosarcinaceae* because the amount of RNA identified by the genus specific probe represented a significant fraction of the RNA detected by the family targeted probe. Furthermore, the difference in the amount of RNA identified for the genus *Methanosarcina* and the strain *Methanosarcina thermophila* TM-1^T, indicated that two or more *Methanosarcina* strains might have been active in the reactors. During feeding with manure, the level of *Methanosarcinaceae* was very similar in both reactors, but a slightly higher concentration of the genus *Methanosarcina* and the strain TM-1^T was detected in the seeded reactor. During the second part of the experiment, the changes in the amount of targeted RNA reflected a shift in the distribution of population in reactor R1. A decrease in the family targeted RNA with an increased fraction of genus-targeted RNA reflected that the new conditions stimulated the activity of *Methanosarcina* and suppressed the activity of other genera within the family *Methanosarcinaceae.* The level of TM-1T population remained unchanged. This finding in combination with the fact that the activity of genus *Methanosarcina* was increased can only be explained by a stimulation of the activity of other *Methanosarcina* strains which are present in the system. The reactor R2 exhibited a parallel increase in the amounts of family-targeted RNA and genus targeted RNA, similarly to reactor R1, while the level of strain-targeted RNA remained constant. The amount of RNA identified as the family *Methanosarcinaceae* and the genus *Methanosarcina* was significantly higher in reactor R2 than in reactor R1.

The data obtained by molecular and conventional techniques and their relatedness to the performance of the reactors gives coherence between unstable operation of the reactor R1 and a lower activity of the methanogenic populations. The ability of the acetate converting methanogenic populations in reactor R1 and R2 might have been different and can probably be explained by different capacity of the participating *Methanosarcina* strains to convert acetate. Our data indicate that the population of the acetate degrading methanogens of the seeded reactor was more efficient

Table 2. Performance of two lab-scale biogas reactors operating at 55 °C

Parameter	Control reactor		Seeded reactor	
	Part 1	Part 2	Part 1	Part 2
Methane yield (ml g VS^{-1} d^{-1})	192 ± 26	decreasing	181 ± 25	432 ± 50
Total VFA (mg acetate l^{-1})	216 ± 74	increasing	176 ± 89	950 ± 256
Reduction in volatile solids (%)	26	decreasing	30	42

Table 3. Hybridization of rRNA from digested manure with specific probes. The results are given as percentage of the rRNA of the specific population in relation to the archaeal rRNA

Parameter	Control reactor		Seeded reactor	
	Part 1	Part 2	Part 1	Part 2
Methanosarcinaceae	7.2 ± 0.3	5.2 ± 0.2	8.0 ± 0.3	9.2 ± 0.2
Methanosarcina	3.1 ± 0.4	4.8 ± 0.1	6.1 ± 0.7	7.1 ± 0.3
Ms. thermophila TM-1^T	2.1 ± 0.2	2.1 ± 0.3	3.4 ± 0.1	3.2 ± 0.1

in conversion of the high concentration of acetate produced by degradation of lipids and this ability seems to be of significant importance for a proper and stable anaerobic degradation of lipid-rich waste.

Future direction

We have recently developed an *in situ* reverse transcription PCR technique for detection of heat shock-induced increases in the intracellular levels of the transcript of the universal stress gene dnaK (Lange et al. 2000). The model organism used was *Methanosarcina mazei* S-6 in the form of single cells. The seminested protocol was used for *in situ* reverse transcription PCR (Lange et al. 2000). The following three oligonucleotide primers which are specific for the *dna K* gene of *M. mazei* S-6 were designed: dnaKf (position 516 to 535 relative to the start codon; sense orientation: 5′-TGGAGGCGGAACCTTCGATG-3′), dnaKr (positions 1248 to 1267 relative to the start codon; antisense orientation: 5′-GGACTCCTGCCTGAATTGCTGC-3′) and dnaKi (position 1047 to 1066 relative to the start codon; antisense orientation: 5′-TTTACCTCTCCGCCCAGGACTC-3′, dnaKi was 5′ labeled with digoxigenin. The first step was a one-tube reverse transcription, followed by PCR amplification that was performed using the primers dnaKf and dnaKr. For the second PCR amplification primers dnaKf and dnaKi were used. The stressed, heat-exposed cells exhibited greater fluorescence than the non-treated cells and the increase corresponded to an increase in the level of *dnaK* transcript. As opposed to traditional mRNA analysis techniques *in situ* RT-PCR technique can reveal heterogeneous gene expression in microbial populations, providing a more detailed picture of the physiological state of the aceticlastic population in a biogas reactor.

Conclusion

Traditional microbiological tests give a fast and relatively good indication of the microbiological system and are the most common used methods to study the function of microorganisms. However, several major problems are associated with the use of the traditional culture dependent methods: 1. Only a minor percentage of the microorganisms in complex microbial samples are cultivable in a laboratory, and can therefore hardly be considered representative for the total microbial population. 2. The capability of metabolizing an array of substrates tells very little about what is actually metabolized under *in situ* conditions. 3. No information is gained on species diversity, since a few omnipotent organisms in principle can be responsible for the conversion of a large number of different substrates. However, these methods are often very simple and do not require special equipment etc.

To circumvent the bias introduced by cultivation-based diversity analyses, researchers have developed molecular methods for the characterization of microbial activity supplementing the traditional techniques for the detection of microorganisms in natural and engineered systems. One major disadvantage of many of these methods is that although species diversity of extractable nucleic acids is established, no distinction is obtained between active and dormant microorganisms, and no information is gained on functional diversity of the possessors of the extracted nucleic acids. However, with some of the methods (e.g., fluorescent *in situ* hybridization) it is not only possible to identify the microorganisms and determine their activity in environmental samples but also to find the spatial distribution of the cells in, e.g., biofilm or floc.

The *in situ* PCR techniques are a very promising method that can be a very valuable tool in the study of functional diversity in natural and engineered systems. In order to obtain a more detailed picture of the physiological state of a population, the *in situ* RT-PCR technique together with probing of 16S rRNA can be applied, providing the means of specific determination of which organisms express which genes under certain conditions. Further development of the technique with regard to use in complex biomass samples is necessary.

Acknowledgments

Dr. A. Ibrahim is acknowledged for the molecular work characterizing the specific population in the biomass. The works was supported by grant no. 16-5429-1 from the Danish Technical Research Council and grant no. 9502848 from the Danish National Research Council.

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