Anaerobic Digestion of Renewable Biomass: Thermophilic Temperature Governs Methanogen Population Dynamics

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Beet silage and beet juice were digested continuously as representative energy crops in a thermophilic biogas fermentor for more than 7 years. Fluorescence microscopy of 15 samples covering a period of 650 days revealed that a decrease in temperature from 60°C to 55°C converted a morphologically uniform archaean population (rods) into a population of methanogens exhibiting different cellular morphologies (rods and coccoid cells). A subsequent temperature increase back to 60°C reestablished the uniform morphology of methanogens observed in the previous 60°C period. In order to verify these observations, representative samples were investigated by amplified rRNA gene restriction analysis (ARDRA) and fluorescence in situ hybridization (FISH). Both methods confirmed the temperature-dependent population shift observed by fluorescence microscopy. Moreover, all samples investigated demonstrated that hydrogenotrophic Methanobacteriales dominated in the fermentor, as 29 of 34 identified operational taxonomic units (OTUs) were assigned to this order. This apparent discrimination of acetoclastic methanogens contradicts common models for anaerobic digestion processes, such as anaerobic digestion model 1 (ADM1), which describes the acetotrophic Euryarchaeota as predominant organisms.

The replacement of fossil fuels by renewable energy sources such as agricultural crops is gaining momentum internationally as a means to decrease emissions from conventional fuel sources impacting global warming (39). Thereby, biogasification using energy crops is the only fuel-producing process with sources impacting global warming (39). The production of biogas from plant waste or other organic materials is a feasible strategy in view of both ecology and economy (63). Fodder beet was chosen as the renewable biomass source for a thermophilic biogas fermentor because the European Union decreased the regulatory price for sugar beets in 2006, and therefore many farmers are looking for an alternative use. Furthermore, fodder beet was considered an attractive renewable energy crop due to its high methane yield per hectare (67), as well as the ideal ensiling conditions enabling the storage of beet silage for many years. Furthermore, the sugar beet was only recently identified as one of the most sustainable energy crops with regard to its water footprint when used for biofuel production (22).

A long-term experiment was started on 4 July 2001 (see reference 48 for startup details), and the same biogas fermentors are still running stable due to the use of fuzzy logic control (16, 48). During the conversion of biomass to methane, four different microbial processes can be distinguished: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (17, 69). Population changes might therefore impact the entire community by triggering an imbalance that is reflected in the bioreactor performance via accumulation of intermediates such as volatile fatty acids (mainly C2 and C3), via pH changes, or via reduced efficiency (52). This work focused on the methanogens which directly reduce CO2 to CH4 or use acetate or methylated C1 compounds as the main substrate to yield methane (35). However, about 65 to 70% of methane produced by methanogens is assumed to originate from acetate (4, 5), and the so-called acetoclastic Euryarchaeota are also dominant in many biogas fermenters used for anaerobic wastewater treatment and sewage sludge digestion (17, 24, 30, 53).

Our results seem to contradict these assumptions, as they clearly demonstrate that hydrogenotrophic methanogens can dominate during a thermophilic fermentation process with renewable biomass (16, 49–51). It appears that temperature has a decisive influence on the type of archaean morphotypes present, as rod-like methanogens dominated at 60°C periods, whereas different morphotypes of methanogens appeared when 55°C conditions were enabled. However, studies elucidating the population dynamics of both acetotrophic and hydrogenotrophic methanogens during the anaerobic digestion of particulate solid biomass for biogas production are rather scarce. These population processes remain somewhat of a "black box" (12) due to the lack of data concerning the microbial consortia involved therein. Molecular biological techniques such as those targeting the 16S rRNA gene represent a valuable addition to culture-based techniques for studying the biodiversity and structure of complex microbial communities.
By targeting methanogens, this study aimed to improve our insight into the poorly understood population dynamics of anaerobic digestion processes and how they are linked to operating conditions such as temperature.

MATERIALS AND METHODS

Sampling and operation characteristics. The automated biogas fermentor employed has been running continuously since the start of the fermentation on 4 July 2001 (day 1) (50). The total fermentor volume was 6 liters, whereof 5.7 liters was used as the operating volume. At startup, the fermentor was inoculated once with a mixture consisting of 1/3 swine manure (swine manure plus maize from a biogas plant in Nordhausen, Germany), 1/3 sewage sludge (from a wastewater treatment plant [WWTP] in Geesthacht, Germany), and 1/3 hot-compost suspension (compost from grass and garden greens, without solids). After a couple of months, the surplus ammonium derived from the original inoculum had been washed out to a measured constant ammonium level of 250 to 300 mg/liter (50). These values reflect a stable fermentation and are typical for monosubstrate fermentation processes of energy crops with a nitrogen content of <1% of the dry matter and without any addition of manure or similar supplements to it. In order to minimize a potential inoculation effect and to safeguard defined reactor conditions, the first sample was taken more than 600 days after inoculation. Acetate and propionate concentrations were measured fortnightly. Methanogens were specifically detected by fluorescence excitation at 420 nm and verified by 0.7% agarose gel electrophoresis (AppliChem, Darmstadt, Germany). 10 ng/ml methylammonium bromide (CTAB; 2% [wt/vol]) solution, with 6 mg each of l-lysine and protease K added. The cells were then dispersed by two passages through a French pressure cell (20) at 1,000 lb/in². After cell lysis, debris was separated from the aqueous phase containing DNA by performing a phenol-chloroform extraction, but the prior incubation step for 20 min at 65°C described by Rheims and Stackebrandt was not performed due to the high ionic strength of the fermentor. After extraction, precipitation with sodium acetate and cold isopropanol was performed as reported previously (45), in addition to a column purification step (Genomic DNA Wizard purification kit; Promega) performed according to the manufacturer’s instructions. The purified DNA was stored at −20°C in Tris buffer (10 mM pH 9).

PCR amplifications were performed with a Biometra T-Gradient 96 cycler (Biometra, Göttingen, Germany). The PCR mix consisted of 1× PCR buffer, 1.5 mM MgCl₂, a 0.2 μM concentration (each) of forward and reverse primers, a 2.5 mM concentration of each deoxynucleoside triphosphate (dNTP), 0.8 U hot-start Taq DNA polymerase (Peqlab, Erlangen, Germany), and 10 ng of template DNA (determined using a Helios Omega spectrophotometer; Fisher Scientific GmbH, Schwerte, Germany).

For amplification of 16S RNA, the following specific primers were used:

- ARCH 69F-Mod, 5′-YGAYTACGCTATGCRAGT-3′ (33; modified from reference 57); and Arch 915 R, 5′-TCGTCCCCCGGAATCTCCT-3′ (59).

The PCR protocol used included an initial DNA denaturation for 5.0 min at 94°C; 30 cycles of 94°C for 30 s, annealing at 53°C for 30 s, and elongation at 70°C for 1 min; 10 min at 70°C (final extension); and incubation at 4°C until samples were processed further. The length (850 kb) of the amplified 16S rRNA fragment was confirmed by gel electrophoresis (Applied Biosystems 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA) and the purified extracts were sequenced in Tris-acetate-EDTA (TAE) buffer (pH 8.0) and staining with ethidium bromide under UV light, using an appropriate size marker (1-kb ladder; Fermentas GmbH, St. Leon-Rot, Germany).

Cloning of PCR amplicons. Purified (E.Z.N.A. Cycle Pure kit; Peqlab, Erlangen, Germany) PCR products were cloned into the pGEM-T Easy plasmid (Promega, Mannheim, Germany) and subsequently transformed into competent cells of Escherichia coli JM109 according to the manufacturer’s guidelines (Promega, Mannheim, Germany). Among the recombinant plasmids (approximately 2,500 clones per library), a randomly selected subset of 102 to 115 clones was picked (Table 1), followed by an enrichment step in 5 ml LB medium with ampicillin (50 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG) (60 μg/ml; Carl Roth GmbH & Co., Karlsruhe, Germany), and 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal) (60 μg/ml; AppliChem GmbH, Darmstadt, Germany). The purification of extracted plasmids was performed by using a commercial kit (E.Z.N.A. plasmid miniprep kit; Peqlab, Erlangen, Germany). Recombinant plasmids were characterized with a double restriction digest (NcoI/SalI) according to the manufacturer’s protocol (Fermentas, St. Leon-Rot, Germany).

ARDRA. Inserts of the expected length (850 bp) were reamplified for PCR as described above, using primers SP6 and T7. The PCR conditions were as follows:

- Denaturation for 1 min at 94°C; 30 cycles for 5 min at 94°C, 30 cycles for 5 min at 55°C; 10 min at 72°C (final extension); and incubation at 4°C until samples were processed further.
Phylogenetic and diversity analysis. A 98% similarity threshold was used for operational taxonomic unit (OTU) assignment (25, 58). To generate the most reliable phylogenetic tree, the ARB software (37) (version 2007) was used. The alignment of selected sequences was performed by the SILVA aligner (41; http://www.arb-silva.de/aligner/), and different phylogenetic trees were constructed by the maximum parsimony, maximum likelihood, and neighbor-joining methods (46), using resampling for 600 replicates to verify tree topologies. The resulting 542 sequences was about 850 bp. An impact of chimera formation upon the results can be excluded, as all sequences were checked for chimera artifacts using the Chimera Check tool (Ribosomal Database Project II [RDP]). In addition, resulting 542 sequences was about 850 bp. An impact of chimera formation upon the results can be excluded, as all sequences were checked for chimera artifacts using the Chimera Check tool (Ribosomal Database Project II [RDP]). In addition, all sequences were compared with small-subunit (SSU) sequences deposited with the RDP database (http://rdp.cme.msu.edu/) in order to synchronize the SILVA database (version 2008).

Method validation for ARDRA. To verify at which cell densities species present can be detected by ARDRA, a spiking and recovery experiment was performed. Thus, randomly allocated cell numbers of precultivated pure cultures of Methanosarcina barkeri, Methanococcus vannieli, Methanobacterium formicicum, Methanosaeta concilii, and Methanospirillum hungatii, within a range of 2.6 × 10^2 to 2.5 × 10^6 cells/ml, were added to autoclaved fermentor matrix to simulate various environmental cell densities. One hundred ten clones were analyzed as described previously (16).

Analytical methods. Methane, volatile fatty acids (VFAs), alkalinity, and pH were analyzed as described previously (16).
Methanosarcina were not detected by phase-contrast or fluorescence microscopy. The majority of Methanosarcina spp. were present as single coccoid cells—a rarely encountered cell status of Methanosarcina cultivated in vitro (1). However, ARDRA and FISH confirmed the presence of both Methanosarcinales and Methanomicrobiales during the 55°C period. While one clonal sequence (HAW–R60–A–727d–F) was assigned to Methanobacterium formicicum (Fig. 1), Methanomicrobiales organisms were present at about 9.17% among the dominating hydrogenotrophic Methanobacteriales (81.65%) (Table 1). Two OTUs (HAW–R60–A–727d–I and HAW–R60–A–727d–E) were assigned to the genus Methanothrix (Fig. 1; Table 1), known to exhibit coccoid shapes (7, 35). Only 1.88% of clonal sequences analyzed revealed a relationship to the family Methanosarcinaceae. Within the order Methanosarcinales, 9.17% of identified OTUs were allocated to the coccoid species Methanosarcina thermophila (HAW–R60–A–727d–D), again—as reported above—apparently present in the unusual form of single cells (1).
The presence of *Methanomicrobiales* was confirmed, at a proportion of 2.09% (Table 2).

On fermentor day 745 (when fodder beet silage was replaced by freshly squeezed fodder beet juice) (Fig. 2), *Methanobacteriales* dominated (98.2%), while merely 1.8% of organisms were assignable to the order *Methanosarcinales* (Table 1). Within the order *Methanobacteriales*, only one OTU (HAW–R60–A–745d–H) among the clones investigated was closely related to *Methanothermobacter wolfeii*. As opposed to the case on previous fermentor days 609 and 727, no clones exhibited a close relationship to *Methanobacterium formicicum*. The remaining 5 OTUs formed a single cluster within the order *Methanobacteriales*. Only 1 OTU (HAW–R60–A–745d–A) belonged to the *Methanosarcinales*.

After the temperature was again increased to 60°C (day 924) (Fig. 2), the archaeal population resembled that occurring on day 609 (Table 1; see Fig. S1c in the supplemental material). ARDRA and FISH revealed that all OTUs detected belonged to the order *Methanobacteriales* (Tables 1 and 2). In contrast to the case for day 609, *Methanothermobacter wolfeii* and *Methanothermobacter thermautotrophicus* were now the most abundant representatives (Table 1).

On fermentor day 1249, organisms exhibiting a rod-like shape and therefore perceived as hydrogenotrophic methanogens dominated again in the samples. The proportion of *Methanothermobacter thermautotrophicus* organisms increased from 8.82% (on day 745) to 27.19%. The abundance of *Methanosarcina* spp. was low (3.51%), and *Methanothermobacter wolfeii* was not detected in these samples. FISH showed that representatives of the *Methanosarcinaceae* were present in rather small numbers (3\times10^7 cells/ml) (Table 2; see Fig. S2 in the supplemental material), confirming the results derived by ARDRA (Table 1).

To verify whether the sample size for the determination of diversity within the 16S rRNA clone libraries was sufficient, rarefaction analyses were performed and indices of diversity were calculated (Table 1; Fig. 3). The rarefaction curves for fermentor days 745, 924, and 1249 indicated that the majority of ARDRA profiles present in the samples were covered by the analysis, which was confirmed by the nonparametric corrected

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**TABLE 2. Quantitative results for FISH analysis of four representative fermentor days**

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Cell count (cells/ml [% of total count]) on reactor day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>609</td>
</tr>
<tr>
<td>Total(^a)</td>
<td>3.20E+10 (100)</td>
</tr>
<tr>
<td>Bacteria(^b)</td>
<td>2.65E+10 (82.81)</td>
</tr>
<tr>
<td>Archaea(^c)</td>
<td>5.50E+09 (17.19)</td>
</tr>
<tr>
<td><em>Methanobacteriales</em></td>
<td>5.50E+09 (17.19)</td>
</tr>
<tr>
<td><em>Methanosarcinales</em></td>
<td></td>
</tr>
<tr>
<td><em>Methanosarcinaceae</em></td>
<td></td>
</tr>
<tr>
<td><em>Methanomicrobiales</em></td>
<td></td>
</tr>
<tr>
<td><em>Methanomicrobiales</em></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Established by DAPI counts.

\(^b\) *Bacteria* were quantified by calculating the difference between the total cell number obtained by DAPI staining and the cell count for *Euryarchaeota* cells identified by FISH.

\(^c\) Representatives of the family *Methanosetaeaceae* and of *Methanococcic* were not detected.

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**FIG. 2.** Overview of process parameters and samples analyzed during the investigated fermentation period. On fermentor day 757, automated feeding was started. *, fermentor period from days 734 to 755—freshly squeezed fodder beet juice was used instead of fodder beet silage. Ten additional sample days (days 599, 626, 629, 686, 791, 793, 940, 952, 1207, and 1221) analyzed by fluorescence microscopy are not indicated in the graph for the sake of clarity (see Table 3).
Chao-1 estimation (Fig. 3), demonstrating that sampling was sufficient to characterize the diversity of the methanogens. The steeper curves for reactor days 609 and 727 indicated that the clones analyzed had only partially uncovered the diversity in the population. This is not an unusual result, as it is rather difficult to establish a total species census for an environmental sample, as there are always undiscovered species left in every molecular inventory (44). With a Shannon index of 1.73 and a Simpson’s index of diversity of 0.88, the diversity of the methanogenic Archaea population was highest on fermentor day 924, while the same data calculated for fermentor day 745 indicated a lower level of diversity, with corresponding values of 0.86 and 0.47 (Table 1).

In order to validate the apparent absence of hydrogenotrophic Methanococcus spp., a representative spiking and recovery experiment was performed (Fig. 4). Methanococcus vanniellii was recovered from 110 clones analyzed to validate the ARDRA technique, but this was not directly correlated to the number of M. vanniellii cells added. This limited recovery might be due to unknown matrix effects or inherent genus-specific

![Figure 3: Species accumulation (rarefaction) curves for all fermentor samples investigated, illustrating the relationship between the number of OTUs (phytype richness) and the number of clones collected. The corresponding corrected Chao-1 calculation values are presented on the right. All calculations were performed using EstimateS software.](image3.png)

![Figure 4: Validation of the applied ARDRA technique. To validate the ARDRA method, 5 different strains of Euryarchaeota were added at various concentrations to the autoclaved fermentor matrix. One hundred ten clonal sequences were analyzed in order to determine the recovery rate. By using different cell densities, various cell concentrations occurring in natural habitats were simulated. For a better presentation of the data obtained, the ordinate values were scaled logarithmically.](image4.png)
TABLE 3. Fluorescence microscopic analysis of methanogen morphotypes present during 55 and 60°C periods for 10 additional sample days not evaluated by ARDRA and FISH

<table>
<thead>
<tr>
<th>Reactor days</th>
<th>Methanogens detected by image analysis*</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>599, 626, 629, 686</td>
<td>Only rod-shaped methanogens of 3 to 8 μm; no other morphotypes</td>
<td>60</td>
</tr>
<tr>
<td>793, 791</td>
<td>Mixed methanogenic population of irregular cocc (mostly single cells) plus bacillus-like methanogens of 3 to 8 μm; some cells partly in chains (&lt;5%); no Methanosaeta-like shapes</td>
<td>55</td>
</tr>
<tr>
<td>940, 952, 1207, 1221</td>
<td>Only rod-shaped methanogens of 3 to 8 μm; no other morphotypes</td>
<td>60</td>
</tr>
</tbody>
</table>

* Five microscopic fields were analyzed by fluorescence microscopy at a wavelength of 420 nm for each sample.

properties but might explain why we failed to detect Methanococcus spp. by ARDRA. However, it seems that, in principle, a high cell density in the spiked fermentor sample also enabled recovery by ARDRA. Apparently, the detection limit for ARDRA is about 10⁵ cells per ml, which is similar to the detection limit established for epifluorescence microscopy (10⁵ cells/ml).

DISCUSSION

Analysis of the molecular inventory of methanogens in a thermophilic fermentor showed an all-time dominance of hydrogenotrophic Euryarchaeota, although the inocula used typically comprise both acetoclastic and hydrogenotrophic methanogens (40). The dominance of H₂-oxidizing Methanobacteriales organisms might be due to their higher specific growth rate than that of acetate-utilizing methanogens (70). In fact, even in the absence of acetotrophic Methanosetaeaceae, anaerobic acetate oxidation to H₂ and CO₂ by syntrophic acetate-oxidizing bacteria can be the major pathway enabling dominance of Methanobacteriales and Methanomicrobiales over Methanosarcinales (31, 35, 69). An increased H₂ partial pressure typical for thermophilic fermentors (69) and the fact that thermophilic temperatures are considered unfavorable for homocacetogenesis (2) imply that acetate formation from H₂ and CO₂ can be neglected. Thermophilic conditions might favor hydrogenotrophs, as these are known to be more capable of adapting to higher temperatures than acetoclastic methanogens (10) and seem to have the ability to adapt to new and untypical habitats, being more resistant to oxic shock or desiccation (19, 32, 61). Even mesophilic hydrogenotrophs were detected under thermophilic fermentor conditions (60°C), which was reported earlier for thermophilic habitats (62). However, an outstanding result was that temperature governed microbial population dynamics (Tables 1 to 3). Morphotype diversity in fermentor samples explicitly depended on the temperature employed, as observed when a temperature shift from 60 to 55°C took place, inducing the emergence of a morphologically mixed methanogenic population (see Fig. S1 in the supplemental material). Although samples from days 745 and 727 were taken during the same 55°C period and the proportion of sarcina-like cells on day 745 was lower than that on day 727 (Table 1), fluorescence microscopy performed on days not assessed by FISH and ARDRA clearly demonstrated that temperature governs the diversity in methanogen morphotypes (Table 3). The change from beet silage to freshly squeezed beet juice, lacking intermediates of ensiling processes, such as acetate, might be the cause for that decrease (Fig. 2). The establishment of a morphologically uniform culture upon a rise in temperature from 55 to 60°C in a medium rich in organics is rather exceptional. So far, a monotype culture of methanogens in Australian sheep fed with oat hay or lucerne was reported, with >90% of OTUs belonging to Methanobrevibacter (68). Similarly, the majority of rumen Archaea were identified as hydrogenotrophs (28), resembling the dominance by hydrogenotrophic Methanobacteriales during our fermentation. Common to all these studies was a high OLR, as also observed for the anaerobic digestion of waste (17).

Consistent with other, rumen-based studies (18, 60), we failed to detect Methanococcales during the fermentation process. Although microbial diversity established by analysis of 16S rRNA genes can be influenced by several experimental factors, these were minimized because in addition to ARDRA, PCR-independent FISH and epifluorescence microscopy confirmed the DNA/RNA-based results. The additional spiking experiment (Fig. 4) showed the reliability of the method. It is currently still assumed that about 70% of methane produced by Archaea originates from acetate (7), with acetotrophs dominating in many thermophilic biogas fermentors (23, 30, 38, 56, 66). Fermentors employed in wastewater purification typically have low acetate concentrations, enabling domination by acetoclastic Methanosaeta due to their high substrate affinity (29). However, hydrogenotrophic methanogens dominated during the formation of methane in our thermophilic fermentor in the presence of low concentrations of acetate (<7 mM) and ammonia (<300 mg/liter), contradicting other reports (24, 30, 66). This study might therefore help to expand the currently recognized model for anaerobic digestion (anaerobic digestion model 1 [AD model]), which is based on early reports exclusively dealing with sewage sludge (5). By elucidating the black box of microbial diversity (12) in a continuously running thermophilic fermentor, our understanding of fermentation stability will be improved by considering the microbial community component frequently neglected by biogas engineers.

ACKNOWLEDGMENTS

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