Systematic and Applied Microbiology 35 (2012) 404–413

Contents lists available at SciVerse ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Hydrogenotrophic methanogens dominate in biogas reactors fed with defined substrates

K. Kampmann[a,*], S. Ratering[a], R. Baumann[a], M. Schmidt[b], W. Zerr[b], S. Schnell[a]

[a] Justus-Liebig-University Gießen, Institute of Applied Microbiology, IFZ, Heinrich-Buff-Ring 26-32, 35392 Gießen, Germany
[b] Landesbetrieb Hessisches Landeslabor (LHL), Standort Bad Hersfeld, Schloss Eichhof, 36251 Bad Hersfeld, Germany

ARTICLE INFO

Article history:
Received 24 February 2012
Received in revised form 15 July 2012
Accepted 16 July 2012

Keywords:
Methanogenic diversity
Methanogenic community
Biogas
Hydrogenotrophic *Archaea*
Acetoclastic methane production
mcrA gene analysis

ABSTRACT

Methanogenic communities in 200 L biogas reactors containing liquid manure were investigated for 33 d. The reactors were consecutively fed with casein, starch and cream. Real-time PCR with primers targeting the gene for methyl coenzyme-M reductase (*mcrA*) resulted in copy numbers of up to 2.1×10^9 g dry mass^{-1}. Single strand conformation polymorphism (SSCP) analysis revealed a stable community consisting of few hydrogenotrophic methanogens. One of the two most abundant species was closely related to *Methanospirillum hungatei*, whereas the other one was only distantly related to other methanogens, with *Methanopyrus kandleri* being the closest cultivated relative. Most probable number (MPN) cultivations were accomplished with a sample from a 600 m^3 reactor from which all manures used in the experiments originated, and equal cell counts of ca. 10^9 g dry mass^{-1} were found for cultivations with acetate, H_2 and methanol. SSCP analysis of these samples and sequencing of the DNA bands identified different hydrogenotrophic methanogens in all samples, and acetoclastic methanogens closely related to *Methanosarcina mazei* in the samples cultivated with acetate and methanol. As the acetoclastic species were not found in any other SSCP sample, it was supposed that the ammonia values in the manure of the laboratory biogas reactor, which ranged from 2.48 to 3.61 g NH_4-N L^{-1}, inhibited the growth of the acetoclastic methanogens.

Introduction

The methanogenic *Archaea* are the only microorganisms capable of producing large amounts of methane as a major product of their energy metabolism. As the shortage of fossil fuels has increased the interest in renewable resources, such as biogas, a lot of research on this microbial group has been undertaken in recent years.

Methanogenic *Archaea* can utilize three different types of substrates: (I) Methane is produced via reduction of CO_2 with H_2 or from formate. Almost all known methanogens are hydrogenotrophic, meaning that they are capable of using H_2 as an electron donor. (II) Some methyl compounds, such as methanol or methane thiol, are formed by the second group of substrates. (III) The third group contains acetate, which is cleaved into CH_4 and CO_2. Only a few of the isolated species are acetoclastic, with all of them belonging to the genera *Methanosarcina* and *Methanosaeta* [4,45]. Since they are strict anaerobes, the natural habitats of methanogenic *Archaea* are anoxic biotopes, such as flooded soil, sediments or gastrointestinal tracts [45]. Methanogens are difficult to isolate because of their often long growth times and dependence on syntrophic bacterial partners [4]. Hence, for the characterization of methanogenic communities, cultivation-independent molecular methods are mostly used. Many of these methods require well-designed primer pairs, such as in the construction of clone libraries, real-time PCR or PCR-based fingerprint methods like denaturing gradient gel electrophoresis (DGGE) or single strand conformation polymorphism (SSCP) analysis. In many studies concerning methanogenic communities in anaerobic digesters, primers detecting 16S rRNA gene sequences from all *Archaea* or from certain methanogenic taxa have been used [1,8,12,18,19,37]. Another possibility is the use of primers binding to the *mcrA* gene which encodes a subunit of the methyl coenzyme-M reductase. This enzyme catalyzes the final reaction step in methanogenesis and is only found in methanogens [20], and therefore specific PCRs can be used. Analysis of methanogenic communities in biogas reactors using *mcrA*-detecting primers has also been carried out [15,24,25,29]. Comparisons of clone libraries constructed with the help of 16S rRNA gene-detecting primers and *mcrA* gene-detecting primers have revealed similar results [15,25] but, for some genera, a higher diversity was found with *mcrA* primers [25]. In many studies, methanogenic communities found in biogas reactors mainly

Abbreviations: SSCP, single strand conformation polymorphism; MPN, most probable number; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence in situ hybridization; dm, dry mass.

* Corresponding author. Tel.: +49 641 99 37350; fax: +49 641 99 37359.
E-mail address: kristina.kampmann@umwelt.uni-giessen.de (K. Kampmann).

0723-2020/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.syapm.2012.07.002

consisted of hydrogenotrophic methanogens. In a mesophilic 60 L biogas reactor treating pig manure and maize silage, the predominant orders identified were *Methanobacteriales* and *Methanomicrobiales* [1]. In a production-scale reactor fed with maize silage, green rye and small amounts of chicken manure, Krause et al. [14] and Schlüter et al. [31] applied metagenomic methods and found *Methanoculleus*-related species to be the main methane producers. This result was confirmed by analysis of clone libraries based on archaeal 16S rRNA gene sequences and *mcrA* sequences [15]. Dominant methanogens in a mesophilic full-scale biogas reactor treating pig manure were closely related to *Methanoculleus bourgensis*, *Methanosarcina barkeri* and *Methanospirillum hungatei*, as revealed by sequencing of a clone library [19]. *Methanoculleus*-related species were also the most abundant methanogens (78.4%) in a thermophilic full-scale biogas plant treating biowastes and liquid manure [39]. Methods such as fluorescence in situ hybridization (FISH) and real-time PCR applied on samples from six full-scale biogas plants fed with different liquid manures and renewable raw materials revealed a dominance of hydrogenotrophic *Methanomicrobiales* [26]. In contrast to these findings, a different study using FISH showed that the dominating methanogens in biogas plants with manure or with wastewater sludge were members of the *Methanosarcinaceae* and *Methanosaetaceae*, respectively, which are acetoclastic [11]. However, in all these studies, bioreactors fed with maize silage, biowastes or other renewable materials, along with slurry or manure, were investigated. Moreover, in most cases, sampling was only conducted either once or with periods of several weeks occurring between the sampling points. The composition, development and stability of methanogenic communities during degradation of different defined substrates or pure substances, including frequent sampling and the use of pilot-scale reactors, has not been reported yet. In the present study, the degradation of manure supplemented with casein, starch and cream in 200 L pilot-scale digesters was monitored for the first time over a time of up to one month. These model substrates represented the basic components of organic material, such as protein, carbohydrates and fat, and they were added at concentrations found in complex substrates like maize silage. The identification of key methanogenic organisms and the observation of the methanogenic community's stability were accomplished with the help of SSCP and subsequent sequencing. Real-time PCR with *mcrA*-targeting primers [20] was used to quantify methanogenic *Archaea*. Additionally, methanogens from the reactor from which all manures used in this study originated were diluted 10-fold up to a dilution of 10^{-8} and enriched with different substrates directly in the dilution tubes, and they were then analyzed by SSCP and sequencing. In this way, the most abundant producers of methane from the typical methanogenic substrates hydrogen, acetate and methanol were identified.

Materials and methods

Reactor operation and sampling

Anaerobic reactor experiments were carried out at the Eichhof in Bad Hersfeld, Germany. All liquid manures for laboratory experiments were taken from a full-scale biogas reactor with a volume of 600 m^3 and initially consisted of liquid cattle manure (70%) and liquid pig manure (30%) with a dry mass of 3–4%. This reactor was regularly fed with maize silage and bruised grain and was kept at a temperature of 36–38 °C. For the experiments, pilot-scale reactors of 200 L were used with anchor stirrers that reached the bottom of the tanks. Stirring was conducted for 15 min per hour and the temperature was maintained at 39 °C. In a preliminary test, laboratory-scale reactors of 20 L were used that were built up and operated in the same way. Gas production was measured in a drum-type gas meter (Type 1/6, Ritter, Bochum, Germany) and gas was collected into bags. Methane contents and short-chain fatty acids were determined as described in Kampmann et al. [10]. After each of the experiments, the manure from the parallel reactors was interchanged and mixed with each other in order to make sure that potential community changes in subsequent experiments only resulted from the substrate added. Before starting the laboratory experiments, liquid manure was incubated until no further gas formation could be observed, and new experiments were started when no further gas formation from the last substrate could be detected. This resulted in rest periods of up to six weeks between the experiments. During this time, heating and stirring of the reactors were conducted as before. The following defined substances were added as substrates: casein (95%, Merck KGaA, Darmstadt, Germany), starch (Toffena potato starch, Südstärke GmbH, Schrobenhausen, Germany) and Rama cream (33% percentage fat, Unilever, Rotterdam, Netherlands). In a preliminary experiment, two parallel-running 20 L reactors were fed with 8 g L^{-1} casein. The following five experiments were carried out in 200 L reactors. In the first experiment (A1), 2.45 g L^{-1} casein was added to two parallel-running 200 L reactors. In a second experiment (A2), after two weeks of rest, 8 g L^{-1} casein was added. This was followed by a third fermentation (B) after six weeks of rest with 4.8 g L^{-1} starch and, at a second feeding time point after 184 h, 6.6 g L^{-1} starch was added. In this case and in the following experiments, a third reactor was set up as a control that was not fed with any substrate. After another rest period of one week, a fourth experiment (C) with continuous starch feeding was carried out (0.6 g L^{-1} d^{-1}) and lastly, after one week of rest, fermentation (D) of cream containing 33% fat (2.2 g L^{-1} fat) was carried out. Feeding of the reactors was conducted twice, with a second feeding step after 382 h with the same amount of cream. The substrate concentrations chosen reflected the approximate concentrations of protein (in the case of 8 g L^{-1} casein), starch and fat found in typical biogas substrates such as maize silage. In this way, experiments were assumed to be similar to the actual conditions in full-scale agricultural biogas reactors.

Samples were taken in sterile 50 mL plastic centrifugation tubes (Falcon, Greiner Bio-One, Frickenhausen, Germany) via a valve that was situated at one third height of the tank reactor and immediately frozen at −20 °C. They were transported to Giessen in a freezer box to prevent thawing and stored again at −20 °C. Samples that were taken at distinct points in the course of substrate degradation were chosen for molecular analyses, and always included one sample taken immediately before substrate addition.

Enumeration of methanogens with the most probable number method and enrichment of methanogens

In order to provide the methanogens from the biogas samples with trace elements and organic growth factors, growth media were supplemented with an extract from the biogas reactors prepared as follows. Samples from both parallel 200 L reactors during casein fermentation were taken in sterile 15 mL plastic centrifugation tubes (Falcon, Greiner Bio-One, Frickenhausen, Germany) and centrifuged for 25 min at 39,000 × *g* and 4 °C. The supernatant was applied to a folded filter (grade 602 h ½, Schleicher and Schuell, Dassel, Germany) and the filtrate was collected in a sterile glass bottle (50 mL with screw caps, Ochs, Bovenden, Germany) and autoclaved for 40 min at 121 °C. The extract was stored at 4 °C and added to media in a concentration of 1% (v/v).

For cultivation of the methanogens in the MPN dilution tubes, an anoxic, sulfide reduced, bicarbonate buffered medium, supplemented with trace element solution and vitamins, as described by Widdel and Bak [46], was used. As electron donors, sterile solutions of acetate (10 mM) or methanol (100 mM) were added or the tubes