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Changes in the Archaea microbial community when the biogas fermenters are fed with protein-rich substrates

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HIGHLIGHTS

- Laboratory-scale CSTR AD was done with casein or pig blood as sole substrate.
- The mesophilic methanogenic microbial community acclimated to these substrates.
- T-RFLP and sequencing of *mcrA* and 16S rRNA gene identified methanogenic Archea.
- The methanogens responded to the change in substrate.

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ABSTRACT

Terminal restriction fragment length polymorphism (T-RFLP) was applied to study the changes in the composition of the methanogens of biogas-producing microbial communities on adaptation to protein-rich monosubstrates such as casein and blood. Specially developed laboratory scale (5-L) continuously stirred tank reactors have been developed and used in these experiments. Sequencing of the appropriate T-RF fragments selected from a methanogen-specific (*mcrA* gene-based) library revealed that the methanogens responded to the unconventional substrates by changing the community structure. T-RFLP of the 16S rDNA gene confirmed the findings.

1. Introduction

The scarcity of cheap fossil energy carriers and the global environmental changes associated with their extensive consumption are posing a threat to the maintenance of sustainable living conditions and giving rise to economic and social crises on a global scale. This situation has led to the initiation of worldwide efforts with the goal of the replacement of fossil resources with renewable and environmentally friendly ones. Biogas, a product of the anaerobic degradation (AD) of organic material, is one of the most promising renewable energy carriers, in part because of the wide range of substrates that can be utilized in the AD process (De Paoli et al., 2011; Ferreira et al., 2012). This is the only biotechnological approach that effectively combines the treatment and elimination of organic waste streams with direct energy production. The process is carried out by a microbial community involving several hundred individual species. The biogas product of AD is a mixture of CH_4, CO_2 and traces of other gaseous components. Biogas is either utilized directly for heat generation through burning of the raw gas, or is partially purified before conversion to heat and electric power. In addition, there is enormous potential in using biomethane, the thoroughly purified biogas, as a biofuel (Murphy et al., 2004; Osorio and Torres, 2009).

Biogas is formed under strict anaerobic conditions by a unique microbial consortium that can be divided into three functional groups (Gerardi, 2003).

The microbial food chain leading to biogas production is very complex, a multitude of methods are therefore used to follow and understand the relationships among the members of this community. In everyday practice, this includes the measurement of

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various chemical parameters (pH, redox potential, FOS/TAC, conductance, etc.), which indirectly reflect the functional status of the biogas reactor. Cultivation-independent molecular biological tools have been developed including denaturing gradient gel electrophoresis (Boon et al., 2002), automated ribosomal intergenic spacer analysis (Pecchia et al., 1998) and single strand conformation polymorphism (Quéméneur et al., 2010). Terminal restriction fragment length polymorphism (T-RFLP) is one of the molecular biological approaches that can be used to identify the most predominant microbes in a biogas producing digester (Liu et al., 1997; Osborn et al., 2000). This technique was chosen for the present study, because it is widely employed for the semiquantitative detection of microbes from environmental samples (Horz et al., 2000; Schütte et al., 2008), including those from biogas reactors (Collins et al., 2003; Klocke et al., 2007; Kobayashi et al., 2009).

T-RFLP is a fingerprinting tool mainly targeting the small subunit (16S and 18S) ribosomal RNA genes from the total DNA of the community. Polymerase chain reactions (PCRs) have been developed, wherein one or both primers are labeled with a specialized fluorescent dye. The PCR product is then digested with restriction enzymes, which have four base-pair recognition sites, and the fragments are visualized, e.g. in a capillary gel electrophoresis system. The nascent short DNA sequences, often called T-RFs (terminal restriction fragments) represent the dominant species in the examined sample. Typically, T-RFLP analysis requires a clone library for identification of the assortment of DNA sequences. The members of the clone library are screened and those that yield a substantial individual T-RF are sequenced via the Sanger method.

In this set of experiments the changes in the composition of the biogas-producing microbial community after the biogas fermenter was fed solely with a protein-rich substrate were investigated. Protein-rich materials, which frequently accumulate as waste by-products to be disposed of in food processing, are generally considered toxic for the biogas fermentation process because of the high level of ammonia released upon protein degradation. We have recently demonstrated that the microbial community acclimates to a high concentration of protein without any additional co-substrate, and the modified community is capable of an elevated level of biogas production (Kovacs et al. to be published). The changes in the microbial composition involved practically all of the genera participating in biomass degradation. The present study focuses on the methanogenic Archaea by targeting a part of the methyl coenzyme-M reductase gene (*mcrA*) with universal primers designed earlier (Luton et al., 2002). The results are complemented with an analysis of the V3–V5 variable region of the 16S rRNA gene, with the use of universal primers (Baker et al., 2003).

2. Methods

2.1. Fermentation conditions

All anaerobic fermentations were carried out in 5-L continuously stirred tank reactors (CSTRs) (Kovacs et al. Journal of Biomedicine and Biotechnology, in press) designed and constructed by Biospin Ltd., Hungary, and installed at the Department of Biotechnology, University of Szeged. Experiments were run in triplicate. Two protein-rich substrates were tested: casein (C) is a by-product of milk processing, while pig blood (B) is a waste material from slaughterhouses. The adaptation was started with an inoculum from an operational biogas plant, in which substrates of agricultural origin, i.e. mixtures of pig manure and maize silage, are treated. Steady-state biogas production was attained after 4–6 weeks of operation and daily feeding. From this time point on the fermenters were fed only with the protein-rich substrate, an increasing amount of the material being supplied daily (Kovacs et al. to be published and HU patent P1100510). Temperature was maintained at 37 ± 1.0 °C. The pH was kept between 7 and 8, and the redox potential was <−500 mV. Gas volume was measured with thermal mass flow devices (DMFC, Brooks) attached to each gas exit port.

2.2. DNA extraction

Two milliliter of samples was withdrawn from the fermenters after the first week of the acclimation to the protein-rich substrates (C1 and B1) and after 5 weeks (C5 and B5). A cetyltrimethylammonium bromide-based buffer was used to extract the total genomic DNA (gDNA) (Minas et al., 2011). Phenol: chloroform (1:1) purification was employed (Roose-Amsaleg et al., 2001). gDNA concentration was determined spectrophotometrically (NanoDrop ND-1000 Technologies, Washington, USA). DNA purity was assessed by agarose gel-electrophoresis.

2.3. PCR amplification of the partial mcrA and 16S rRNA gene

In order to amplify the approximately 500 bp fragment of the methyl coenzyme-M reductase coding gene, a fluorescent labeled forward primer (TET-mcrA_F) and a reverse primer (mcrA_R) were used (Luton et al., 2002). An approximately 550 bp fragment of the 16S rRNA gene was amplified by using the primer pair F344 and R934 (Baker et al., 2003; Raskin et al., 1994), in this system the forward primer was also labeled with the same fluorescent dye. PCR reactions were carried out in a volume of 30 μL, containing approximately 50 ng of gDNA, 1X DreamTaq reaction buffer, 100 μmol of dNTP, 3 mM $MgCl_2$, 10 μM of each primer (Sigma, St. Louis, MI, USA) and 1.5 U of DreamTaq DNA polymerase. Chemicals, excluding the primers, and enzymes were purchased from Fermentas (St. Leon-Rot, Germany). The PCR reactions were performed in an ABI 9600 Fast Thermal cycler (Applied Biosystem, Foster City, CA, USA), where the reaction profile was as follows: initial denaturation at 96 °C for 3 min, followed by 30 cycles of denaturation at 96 °C for 30 s, annealing at 52 °C for 30 s and elongation at 72 °C for 1 min. To avoid amplification of the host 16S rRNA gene, a nested PCR reaction was used in the case of the clones carrying the partial 16S rDNA. In the first step, the vector specific primer pair (M13F and M13R) was applied to multiply the insert. In the second step, the product of the previous reaction was used as template for the primer pair F344 and R934. The PCR protocol was as described, except that the annealing temperature was raised to 58 °C in the case of the primer pair F344 and R934, due to the different T_m of the oligonucleotides. A final extension step for 10 min was added in order to allow the polymerase to finish incomplete PCR products.

The PCR products were separated by electrophoresis in 1% agarose gel using TRIS-acetate buffer (Green and Sambrook, 2012), and visualized with ethidium bromide under UV light. The DNA fragments were purified by using the PCR clean-up kit (Viogene-Biotek, New Taipei, Taiwan) following the recommendations of the manufacturer; the recovered DNA was eluted in 30 μL, and stored at −20 °C.

2.4. Generation of the mcrA and 16S rDNA clone libraries

For the preparation of the clone library, the pGEM-T vector system was employed (Promega, Madison, WI, USA). The PCR product was used for the ligation, except that non-fluorescent forward primers were employed. NovaBlue chemical competent cells (NovaGene, Billerica, MA, USA) were utilized for the transformation. Cloned inserts were amplified by PCR as above, using vector-specific M13F (5′-TGT AAA ACG ACG GCC AGT-3′) and M13R (5′-CAG GAA ACA GCT ATG ACC-3′) primers. Clones carrying an insert of correct size were identified by agarose gel electrophoresis.