ISSN 0026-2617, Microbiology, 2006, Vol. 75, No. 6, pp. 683–688. © Pleiades Publishing, Inc., 2006.
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EXPERIMENTAL ARTICLES

Effect of Hydrogen Concentration on the Community Structure of Hydrogenotrophic Methanogens Studied by T-RELP Analysis of 16S rRNA Gene Amplicons

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Received January 16, 2006

Abstract—Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes was used to monitor the changes in the composition of the population of methanogens in enrichment cultures under high and low hydrogen concentrations. Hydrogen concentration was shown to determine the structure of a methanogenic community. High hydrogen concentration probably favors the hydrogen- and acetate-utilizing representatives of *Methanosarcinaceae,* while a more diverse methanogenic community is favored by low hydrogen concentrations.

DOI: 10.1134/S0026261706060105

Key words: T-RFLP analysis, hydrogenotrophic methanogens, syntrophy.

Methanogenic archaea are a highly specialized group of prokaryotes; they form methane as the only product of energy metabolism [1]. Methane is a greenhouse gas, second only to CO_2 in importance. Flooded areas, including rice paddy soil, are the main environments favoring methanogenic archaea; they are responsible for 25% of global methane emissions and therefore have a serious effect on planetary climate change [2]. Moreover, rice paddy soils are a convenient model for the study of such fundamental issues of microbial ecology as the diversity, structure, and dynamics of microbial communities and the structural and functional relationships between microbial groups.

In anaerobic rice field soils, methane formation is the result of an interaction of several groups within the complex microbial community, which includes, apart from methanogens, hydrolytic, fermentative, syntrophic, and homoacetogenic bacteria [3, 4]. Methane is primarily produced from acetate or from hydrogen and CO_2 [5]. Organic acids with longer carbon chains, such as propionate, must be converted to substrates suitable for methanogenesis by syntrophic bacteria in the presence of hydrogen- and formate-utilizing methanogens. Methanogens maintain extremely low concentrations of hydrogen and formate and thus make anaerobic propionate composition thermodynamically profitable [6].

A considerable portion of methane emission from rice paddy soil (from 20 to 45%) is produced by hydrogenotrophic methanogens of the families *Methanomicrobiaceae, Methanosarcinaceae*, and of rice cluster I (RCI), a new phylogenetic group within *Euryarchaeota*. Although none of the RCI organisms have yet been isolated in pure culture, this group of methanogens was found to possess a hydrogenotrophic phenotype [7, 8]. Information concerning the physiological characteristics which result in the preferential development of a certain hydrogenotrophic methanogen at the expense of others is scarce. While the representatives of *Methanomicrobiaceae*, *Methanobacteriaceae, Methanosarcinaceae*, and RCI are known to utilize H_2/CO_2 as a substrate, *Methanosarcinaceae* can also utilize acetate [1]. Different groups of hydrogenotrophic methanogens have been shown to dominate in different environments [9–11]. This domination can be the result of specific physicochemical conditions in a given environment, such as temperature, pH, or the concentrations of H_2, CO_2, formate, and acetate. The possible selective effect of a certain factor on the preferential development of a specific group of H_2-utilizing methanogens can be confirmed using several approaches. Of these, incubation of soil suspensions under specific conditions is the most widespread [4, 11–14]. This approach can be applied to assess the effect of temperature using an incubator. However, controlling the concentrations of H_2, CO_2, and acetate in a heterogeneous soil suspension (an environment where both their production and con-

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sumption occur simultaneously) seems problematic. Obtaining an enrichment culture and determination of the effect of environmental factors on the preferential development of specific groups of hydrogenotrophic methanogens is therefore more convenient. The method of T-RFLP (terminal restriction fragment length polymorphism) analysis, a modern semiquantitative molecular approach for the quick determination of the composition of microbial communities, is convenient for monitoring community structure [15, 16]. This method is based on amplification of a target gene (for example, the 16S rRNA gene) with a certain set of primers, one of which is fluorescent labeled, restriction of the amplification product, and subsequent determination of the relative amount of terminal restriction fragments.

The present work reports the results of T-RFLP analysis of the structural dynamics of the hydrogenotrophic methanogenic communities of enrichment cultures from rice paddy soil under high and low hydrogen concentrations.

MATERIALS AND METHODS

Soil samples, growth media, and culture conditions. The soil samples which were used to obtain enrichment cultures were collected in 1998 (after harvesting) from the rice fields of the Italian Rice Research Institute, Vercelli, northern Italy. Soil characteristics were described previously [11, 14]. The soil was air-dried, stored in the dark at room temperature, and prepared for the experiment according to the procedure described in [3]. Dry soil (20 g) and sterile oxygen-free distilled water (20 ml) were mixed under nitrogen in a hermetically sealed vial and were flushed with nitrogen several times. The soil suspension (3 ml) was then added to 47 ml of sterile, oxygen-free medium [4]; the mixture was incubated anaerobically for 15 days in 120 ml hermetically sealed vials. Incubation was performed in the dark at 25°C without stirring. Two experimental variants were applied, with high (80%) and low (0.005–0.05%) hydrogen concentrations. In the first case, the vessels were flushed for 10 min with H_2/CO_2 gas mixture (80%/20%, Messer-Griesheim, Frankfurt, Germany); the gassing was repeated after 4, 9, and 11 days of incubation. In the second case, the incubation was performed under N_2/CO_2, 80%/20%, Messer-Griesheim, Frankfurt, Germany; the low hydrogen concentration was maintained by endogenic syntrophic degradation of 8 mM propionate. Each experiment was performed in three repeats for every time interval (1, 3, 5, 8, and 15 days of incubation). The samples for the analyses of soluble organic compounds and for molecular studies were taken in triplicate from each series after 1, 3, 5, 8, and 15 days of incubation and stored at –20°C. The concentrations of produced gases (CH_4, H_2, and CO_2) were measured in enrichment cultures prior to sampling for T-RFLP analysis.

Analytical measurements. The concentrations of H_2, CH_4, and CO_2 were determined by gas chromatography (GC) [17] with a gas chromatograph equipped with a flame ionization detector (Shimadzu GC 8A, Kyoto, Japan). The concentration of CO_2 was determined after its conversion to CH_4 in a methane converter (Ni-catalyst, 350°C, Chrompack Middelburg, The Netherlands). Hydrogen concentration was determined with a thermal conductivity detector (Shimadzu GC 8A, Kyoto, Japan). The calibration curve was built using the standards (Messer-Griessheim, Frankfurt, Germany). The data were analyzed using the Peak Simple software package (SRI Instruments, Torrance, United States). The concentrations of organic compounds (fatty acids and alcohols) were determined by HPLC [12] with a refractometer and a UV detector with a 3 μmol detection limit (Sykam, Gilching, Germany). In the course of incubation, pH was determined with a glass electrode (Ingold, Steinbach, Germany).

DNA isolation from soil and enrichment cultures. The total DNA was isolated from 0.5 g of soil (in three repeats) and from 10 ml of enrichment cultures for every time interval. The FastDNA® Spin Kit for soils (Qbiogene, Heidelberg, Germany) was used according to the manufacturer's recommendations. To remove the humic acids, a stage of purification with 5.5 M guanidine thiocyanate was added.

PCR amplification of archaeal 16S rRNA genes. The archaeal 16S rRNA genes from each sample were PCR-amplified using the specific archaeal primers [18]. These primers enable amplification of 16S rRNA gene fragments from position 109 to 934 (positions for the *Escherichia coli* 16S rRNA gene). The 5'-end primer was labeled with 5-carboxyfluorescein. The reagents from Invitrogen (Karlsruhe, Germany) were used for amplification. Amplification was performed using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, United States), as previously described [19].

T-RFLP analysis. The fluorescently labeled amplicons of 16S rRNA genes were purified using the PCR purification kit (Sigma-Aldrich, Steinheim, Germany) according to the manufacturer's recommendations. The concentration of the purified 16S rRNA gene fragments was determined by standard UV spectrophotometry at 260 nm on a Biophotometer instrument (Eppendorf, Hamburg, Germany). Restriction of 30–50 ng of the amplicons of the16S rRNA gene was performed using *TaqI* restriction endonuclease (Promega, Mannheim, Germany) according to the manufacturer's recommendations, for 3 h at 65°C. Restriction products were purified on AutoSeq™ G-50 columns (Amersham Biosciences Europe, Freiburg, Germany). The purified restriction fragments (2 μl) were then mixed with 0.2 μl of single-stranded fluorescently labeled marker DNA MapMarker® 1000 (BioVentures, Murfreesboro, United States) and 12 μl of formamide Hi-Di™ (Applera, Darmstadt, Germany). The mixture was heated for 5 min at 95°C and analyzed with an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Darmstadt,

Germany). The relative frequency of the individual terminal restriction fragments in the total archaeal community was determined by the relative height of absorption peaks of each restrict, using the algorithm described in [15]. The methanogens were affiliated with specific phylogenetic groups according to the length of the terminal restriction fragments derived from the 16S rRNA genes cloned from Italian rice paddy soils [13, 14].

RESULTS

Effect of hydrogen concentration on methane production. Methane formation commenced on the first day of incubation of enrichment cultures both under high (80%) and low (0.005%) hydrogen concentrations (Table a). The rate of methane production was relatively low up to the eighth day and increased subsequently (Table b). However, by the end of the incubation, the amounts of methane accumulated under high hydrogen concentration were ~43 μmol/vial; under low hydrogen concentration, it was 24 times less (~1.8 μmol/vial).

T-RFLP analysis of the microbial community. The T-RFs (Terminal Restriction Fragments) with the lengths corresponding to RCI (*Euryarchaeota*, 388 bp) and to *Methanosarcinaceae* (190 bp) were predominant among the restricts of the T-RFs obtained from the native soil. Their relative abundance constituted 38 and 34% of the total archaeal number (Fig. 1; Fig. 2, day 0). The fragments belonging to other groups were present in smaller amounts; they corresponded to *Methanobacteriaceae* (90 bp) and *Methanosaetaceae* (283 bp). The restriction fragments present at low concentrations (less than 5% total) belonged to *Methanomicrobiaceae*, RCII and RCV (*Euryarchaeota*), RCIV and RCVI (*Crenarchaeota*), and to the organisms which were not affiliated with any known phylogenetic lineage. They were excluded from the analysis in the course of the normalization procedure [15]. The results of restrict analysis obtained in the course of rice paddy soil incubation under high and low hydrogen concentrations are presented in Fig. 2.

The results of T-RFLP analysis support the conclusion that the composition of the hydrogen-utilizing methanogenic community changed in the course of incubation both under high and low hydrogen concentrations (Fig. 2). In the enrichment culture incubated under high hydrogen concentrations (Fig. 2a), the relative amount of the T-RFs, corresponding to *Methanobacteriaceae, Methanosarcinaceae*, and RCI, did not change significantly up to the eighth day of incubation; after 24 h, the fragments corresponding to *Methanosa-*

Changes of methane concentration with time (a) and of the rates of methane production (b) by methanogenic communities under high and low concentrationa of hydrogen

a

Incubation time, days	CH_4^* μmol vial^{-1}	
	High H_2 concentration	Low H_2 concentration
0	0.006 ± 0.002	0.005 ± 0.001
1	0.014 ± 0.006	0.007 ± 0.001
3	0.070 ± 0.018	0.009 ± 0.001
4	0.156 ± 0.024	0.042 ± 0.006
5	0.269 ± 0.048	0.063 ± 0.006
8	0.898 ± 0.037	0.259 ± 0.020
9	2.828 ± 0.083	0.317 ± 0.020
15	43.090 ± 3.338	1.797 ± 0.187

b

Incubation time, days	Rate of methane production, μmol vial^{-1} day^{-1}	
	High H_2 concentration	Low H_2 concentration
0–1	0.008 ± 0.008	0.002 ± 0.002
1–3	0.026 ± 0.010	0.002 ± 0.001
3–5	0.100 ± 0.033	0.027 ± 0.004
5–8	0.210 ± 0.028	0.064 ± 0.010
8–15	6.027 ± 0.482	0.220 ± 0.029

Note: *, average value ± standard error for three independent repeats.

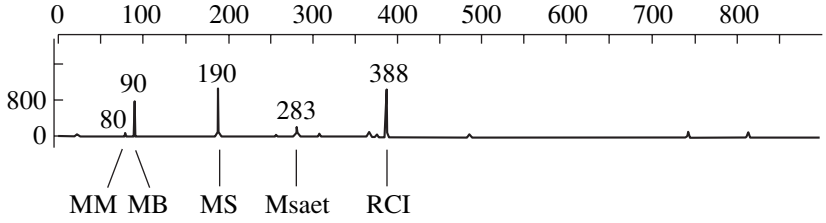

Fig. 1. A typical T-RFLP electrophoregram of the amplicons of archaeal 16S rRNA genes from Italian rice paddy soil (control). *X* axis—fragment length, bp. *Y* axis—relative intensity of fluorescence. MM, *Methanomicrobiaceae* (80 bp); MB, *Methanobacteriaceae* (90 bp); MS, *Methanosarcinaceae* (190 pb); Msaet, *Methanosaetaceae* (283 bp); RCI, rice cluster I (388 bp).

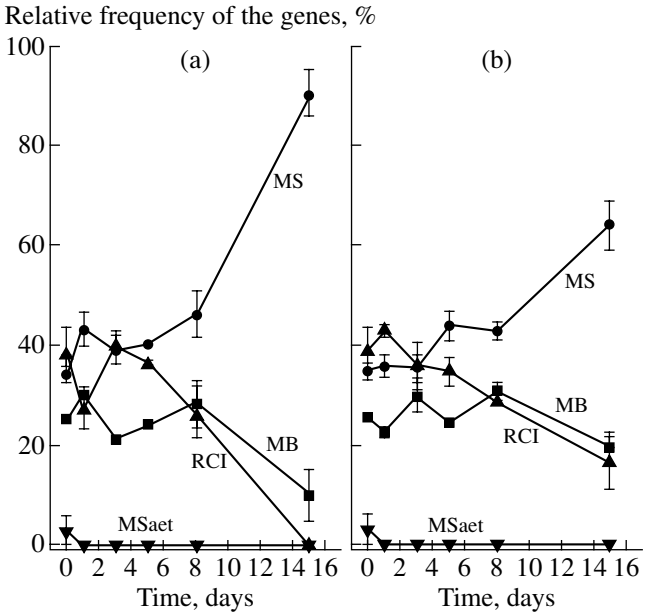

Fig. 2. T-RFLP analysis of the changes in the methanogenic populations in anaerobic enrichments from rice paddy soil incubated under high (a) and low (b) hydrogen concentrations. Msaet, *Methanosaetaceae*; MB, *Methanobacteriaceae*; MS, *Methanosarcinaceae*; RCI, rice cluster I.

etaceae were not recovered. During the second week of incubation, the number of fragments corresponding to *Methanosarcinaceae* increased, and those of *Methanobacteriaceae* and RCI decreased. Only *Methanobacteriaceae* and *Methanosarcinaceae* were revealed in the enrichment by the end of the incubation. The relative amount of *Methanosarcinaceae* by the end of incubation was ~90%. Acetate concentrations in the enrichments increased throughout the incubation period; the final concentration was ~2100 μmol/vial (data not shown). They exceeded the threshold level for *Methanosarcinaceae* (≥35 μM [1]) by the third day of incubation. Thus, after three days of incubation, *Methanosarcinaceae* were able to utilize acetate for methanogenesis.

In the enrichments incubated under low hydrogen concentrations ([6], Fig. 2b), the relative amount of the T-RFs corresponding to *Methanobacteriaceae* increased slightly (from 25 to 30%) during the first eight days of incubation and decreased during the subsequent week (from 30 to 19%). The relative number of fragments corresponding to RCI decreased throughout the incubation period from 43 to 16%; the number of *Methanosarcinaceae* increased from 33 to 62%, i.e., to a lesser extent than in the enrichments incubated under high hydrogen concentrations. Like the enrichments under high hydrogen concentration, *Methanosaetaceae* were not revealed after 24 h of incubation. The diversity of the methanogenic community after two weeks of incubation was much higher in the enrichments incubated under low hydrogen concentrations.

DISCUSSION

Analysis of the structure of the methanogenic community in the control (Fig. 1; Fig. 2, day 0) revealed the main phylogenetic groups described previously for the samples of Italian rice paddy soil collected in 1988 [11], i.e., *Methanosaetaceae, Methanosarcinaceae*, and *Methanobacteriaceae.* Our results differ significantly from the earlier ones for the same soils [11] in that high numbers of *Methanosaetaceae* were previously revealed, while according to our data this group constituted 2.9% on average. This difference may be due to the different sampling time. For our experiments, soil samples were taken after harvesting, while in [11] the analyses were performed on soil samples taken 28 days after the water came off. It should be mentioned that some of the T-RFs may belong to representatives of more than one phylogenetic lineage. The quantitative estimate of *Methanosarcinaceae* may therefore be biased, since the fragments of this group are also characteristic of some representatives of RCVI. Likewise, the *Methanosaetaceae* fragments may contain RCIV groups, and the RCI fragments, those of RCII [13, 14]. We believe, however, that since methanogenic enrichments were obtained, methanotrophic archaea predominated there.

T-RFLP analysis revealed that the structure of the hydrogenotrophic methanogenic community changed in the course of incubation under both high and low hydrogen concentrations (Fig. 2). Moreover, the community structure changed in a different way, depending on hydrogen concentration. By the end of incubation under high hydrogen concentration, the methanogenic community consisted mostly of *Methanosarcinaceae* (90%) and *Methanobacteriaceae* (10%), while under low hydrogen concentrations the community was more diverse and included RCI (16%). The relative abundence of the T-RFs affiliated to *Methanobacteriaceae* was higher (19%), while the portion of *Methanosarcinaceae* was lower (less than 62%).

The preferential development of homoacetogenic bacteria in the course of rice paddy soil incubation under hydrogen has been reported previously [20]. In the enrichments incubated under 80% hydrogen, high concentrations of acetate were detected (data not shown). Thus, after three days of incubation, the development of *Methanosarcinaceae* was possibly stimulated, since they can utilize both acetate and H_2/CO_2. Other groups of methanogens (*Methanobacteriaceae, Methanosaetaceae*, and RCI) can utilize only one substrate (either H_2/CO_2 or acetate); their growth was therefore suppressed by homoacetogenic bacteria and *Methanosarcinaceae.* The relative decrease of the other archaea with time is illustrated by Fig. 2. Although *Methanosaetaceae* are also capable of acetate utilization, they grow very slowly. This is possibly the reason why *Methanosaetaceae* could not compete with *Methanosarcinaceae* in our enrichments (Fig. 2a, 2b). The higher growth rate of *Methanosarcinaceae* in the pres-

ence of acetate in millimolar concentrations, compared to that of *Methanosaetaceae*, may therefore be another factor causing the preferential growth of the former group [21].

In enrichments with propionate (low hydrogen concentration), the amount of methane produced was ~25 times less than under high hydrogen concentrations, although methane production commenced on the first day of incubation. Moreover, the measurement of the total amount of extracted DNA indicated that the methanogenic community as a whole developed much more slowly under these conditions. Homoacetogenic bacteria cannot compete for hydrogen with methanogens at neutral pH, temperatures above 20°C, and limited hydrogen supply [6]. All the methanogenic groups revealed in our enrichments could therefore grow under low hydrogen concentrations. The threshold concentration of acetate for *Methanosarcinaceae* is ~0.5 mM and that of hydrogen is 4–8 μM for all hydrogenotrophic methanogens [1]. We believe therefore that *Methanosarcinaceae*, together with other methanogens, initially utilized the hydrogen produced in the course of syntrophic propionate degradation. In the initial period of incubation, their development followed parallel courses (Fig. 2b). After substantial amounts of acetate were accumulated via propionate degradation, *Methanosarcinaceae* gained an advantage due to their ability to utilize both hydrogen and acetate (Fig. 2b).

To conclude, it should be noted that T-RFLP analysis of enrichment cultures is a convenient method for the study of the effects of various physicochemical factors on the structural changes of soil methanogenic communities. We have demonstrated that the structure of a hydrogenotrophic methanogenic community changes in the course of incubation and depends on the hydrogen concentration in the medium. High hydrogen concentrations favor *Methanosarcinaceae*, while under low hydrogen concentration, the diversity of the methanogenic community increases with time.

ACKNOWLEDGMENTS

The authors are grateful to the Fund for Chemical Industry (Germany) for financial support. They thank the researchers of the Department of Biochemistry, Max Planck Institute for Terrestrial Microbiology, especially Dr. Dana Kemnitz, Dr. Christoph Erkel, Dr. Matthias Noll, Dr. Steffen Kolb, Dr. Holger Penning, and Martina Metje for their help and interest in our work.

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