APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1998, p. 960–969 Vol. 64, No. 3
0099-2240/98/$04.00+0

Diversity and Structure of the Methanogenic Community in Anoxic Rice Paddy Soil Microcosms as Examined by Cultivation and Direct 16S rRNA Gene Sequence Retrieval

REGINE GROßKOPF, PETER H. JANSSEN,† AND WERNER LIESACK*
Max-Planck-Institut für terrestrische Mikrobiologie, D-35043 Marburg, Germany

Received 19 August 1997/Accepted 17 December 1997

A dual approach consisting of cultivation and molecular retrieval of partial archaeal 16S rRNA genes was carried out to characterize the diversity and structure of the methanogenic community inhabiting the anoxic bulk soil of flooded rice microcosms. The molecular approach identified four groups of known methanogens. Three environmental sequences clustered with *Methanobacterium bryantii* and *Methanobacterium formicicum*, six were closely related but not identical to those of strains of *Methanosaeta concilii*, two grouped with members of the genus *Methanosarcina*, and two were related to the methanogenic endosymbiont of *Plagiopyla nasuta*. The cultivation approach via most-probable-number counts with a subsample of the same soil as an inoculum yielded cell numbers of up to 10^7 per g of dry soil for the H_2-CO_2-utilizing methanogens and of up to 10^6 for the acetate-utilizing methanogens. Strain VeH52, isolated from the terminal positive dilution on H_2-CO_2, grouped within the phylogenetic radiation characterized by *M. bryantii* and *M. formicicum* and the environmental sequences of the *Methanobacterium*-like group. A consortium of two distinct methanogens grew in the terminal positive culture on acetate. These two organisms showed absolute 16S rRNA gene identities with environmental sequences of the novel *Methanosaeta*-like group and the *Methanobacterium*-like group. *Methanosarcina* spp. were identified only in the less-dilute levels of the same dilution series on acetate. These data correlate well with acetate concentrations of about 11 μM in the pore water of this rice paddy soil. These concentrations are too low for the growth of known *Methanosarcina* spp. but are at the acetate utilization threshold of *Methanosaeta* spp. Thus, our data indicated *Methanosaeta* spp. and *Methanobacterium* spp. to be the dominant methanogenic groups in the anoxic rice soil, whereas *Methanosarcina* spp. appeared to be less abundant.

Rice paddy soils are estimated to contribute about 25% of the total budget of global methane emissions and therefore have a major impact on world climate due to their contribution to the greenhouse effect (39). Since these soils are flooded, they are largely anoxic and typically (but not exclusively) methanogenic. The major substrates for methanogenesis in submerged rice soil are H_2-CO_2 (and formate) and acetate (55). The fluxes of carbon and electrons through such a system are similar to those in other anaerobic habitats, such as sediments and anaerobic waste treatment systems (8). It has been possible to investigate the microbial community structure in some detail in various anaerobic waste treatment systems (7, 21, 40). Anoxic soils, with their large inorganic matrix components, gradients of oxygen and alternative electron acceptors, seasonal changes, high degree of heterogeneity, and influence of plants and mesofauna, are much more complex systems, and the study of their microbial community structure demands new conceptual and experimental approaches.

Current knowledge of the genera and species of methanogenic archaea occurring in flooded rice paddy soils comes mainly from enrichment studies. Enrichment cultures on a variety of substrates have led to the isolation and identification of a number of strains, including hydrogen- and/or formate-utilizing isolates assigned to the genera *Methanobacterium* (9, 16, 46) and *Methanobrevibacter* (4) and acetate-utilizing isolates assigned to the genus *Methanosarcina* (3, 16, 46). Recently, Kudo et al. (31) detected various methanogenic archaea in a number of Japanese rice paddy soils by analyzing 267-bp segments of the 3′ portions of 16S rRNA genes, amplified by PCR, in total community DNA extracted from the soils.

It is known that enrichment cultures select for fast-growing organisms with high growth yields and those best able to grow in the growth medium used for the cultivation (32). This means that the organisms isolated are not necessarily of numerical significance in the habitat under study. In addition, since these growth conditions favor organisms best adapted to the medium, there is also no guarantee that the organisms obtained have any significance in the biogeochemical processes within the environment under study. These problems, together with the general labor- and time-intensiveness of many cultivation studies, have meant that molecular approaches to studying microbial populations recently have gained popularity.

Generally, the 16S rRNA gene has been used as a molecular marker, enabling the identification or at least a phylogenetic assignment of the organism from which it originated without the necessity of cultivating and isolating the organism first (33; for detailed reviews, see references 2 and 32). Thus, selectivity imposed by cultivation conditions can be avoided. However, this approach is still in the phase of methodological evolution, especially for heterogeneous environments such as soils and sediments, so the correct interpretation of the data obtained is still difficult. The possibility that some bias may be introduced into the data is known from some investigations (32, 45, 48, 60). Nevertheless, the molecular approach enables the detec-

* Corresponding author. Mailing address: Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Straße, D-35043 Marburg, Germany. Phone: 49 (6421) 178 720. Fax: 49 (6421) 178 809. E-mail: liesack@mailer.uni-marburg.de.
† Present address: Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3052, Australia.

tion of as-yet-uncultured organisms and facilitates a more profound investigation of microbial diversity (32, 33).

The biogeochemical processes in flooded rice paddy soil microcosms have been investigated to some extent (8, 55), and the rates of methane production have been shown to be comparable to those occurring in the field (18, 51). We initiated a study using both culture and molecular ecology methods to investigate the methanogenic microflora occurring in anoxic flooded rice paddy soil microcosms. The aim was to obtain a more objective view of the methanogenic community in this habitat, since possible bias caused by each method could be assessed by comparing them. This biphasic approach enabled us to examine the relevance and utility of each of the methods. What we expected from the molecular approach was the detection of numerically important organisms that could play a role in the biogeochemical processes in the environment but might be difficult to cultivate. Cultivation studies, on the other hand, would give us phenotypes and allow the assignment of species to possible biogeochemical roles. Here we show that dominant methanogenic archaea detected by molecular methods correlated with those found by cultivation methods in the same habitat.

MATERIALS AND METHODS

Medium preparation. Two sulfide-reduced, bicarbonate-buffered mineral media, DM and FM (23), were used in this study. These media differed in the composition of the mineral salts solution which formed the basis of each medium. Trace element solution SL10 (70) was normally used, but trace element solution SL9 (61) was used as noted. Screw-cap bottles were filled, leaving a small gas bubble, while serum bottles or pressure tubes were partially filled with medium (with the headspace containing N_2-CO_2 gas [80:20, vol/vol]) and closed with butyl rubber stoppers.

Substrates were prepared as 200 mM to 2 M stock solutions, sterilized by autoclaving, and added to sterile media just before inoculation.

Microbial strains. *Methanosarcina barkeri* MS^T (DSM 800^T) and *Methanosaeta concilii* $GP6^T$ (DSM 3671^T) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *Methanosarcina* sp. strain VeA23 and *Methanobacterium* sp. strain VeH52 were isolated during the course of this study. *M. concilii* VeAc9 was isolated from a mixed culture that was growing on isopropanol and that was enriched from the same rice paddy soil described in the present study (24).

Methanosarcina spp. and *Methanosaeta* spp. were cultivated in 50-ml aliquots of FM in partially filled 125-ml serum bottles closed with butyl rubber stoppers under a headspace of N_2-CO_2 (80:20 [vol/vol]). The medium for *M. concilii* $GP6^T$ contained trace element solution SL9 instead of SL10. *Methanosarcina* spp. and *Methanosaeta* spp. were grown with 5 or 50 mM acetate, while *Methanobacterium* sp. strain VeH52 was grown with 60 kPa of H_2 added to the headspace and 5 mM acetate as a carbon source.

Enumeration and isolation of methanogens. Rice (*Oryza sativa* var. Roma, type japonica) was grown in the laboratory as described by Frenzel et al. (18) with plastic containers containing flooded soil obtained from wetland rice fields of the Italian Rice Research Institute in Vercelli, Italy. Three-tube most-probable-number (MPN) counts were made on soil cores from such laboratory rice cultures in which the plants were 90 days old. The cores were taken by pressing a plastic tube into the soil to a depth of about 15 cm. Only the lower 10-cm portions of the cores were used. The MPN counts were made as described previously (23) with 9 ml of DM plus 1 ml of sample in partially filled 23-ml pressure tubes with a headspace of N_2-CO_2 (80:20 [vol/vol]) and closed with butyl rubber stoppers. The counts were made with (i) 60 kPa H_2 added to the N_2-CO_2 headspace plus 1 mM acetate, (ii) 20 mM formate plus 1 mM acetate, (iii) 20 mM acetate, and (iv) 20 mM methanol plus 1 mM acetate. Incubations were done in the dark at 25°C. Tubes were considered positive if CH_4 was produced to levels of 10 Pa or higher. MPN counts were calculated from the dry weight of the soil (by drying to a constant weight at 105°C), the dilution factor, and tables for three parallel dilution series based on a statistical treatment of such counting methods (5).

Isolation and cultivation of pure cultures. The deep-agar method for isolating pure cultures was described by Pfennig (43). Sodium formate (20 mM) plus sodium acetate (5 mM) and sodium acetate (20 mM) alone were used as the growth substrates for strains VeH52 and VeA23, respectively. Purity was checked microscopically by growth on various growth substrates in FM, by growth in a complex medium consisting of FM with (per liter) 0.5 g of yeast extract, 2 mmol of glucose, 5 mmol of fumarate, 5 mmol of pyruvate, and 2 mmol of acetate, and by aerobic growth on nutrient agar (Difco Laboratories, Detroit, Mich.) plates supplemented with 4 mM glucose. Incubations were done at 25°C.

Characterization and analytical methods. Phase-contrast photomicrographs were made after immobilizing cells on an agar-coated microscope slide (44). Autofluorescence due to F_{420} was observed by use of an Axiophot (Oberkochen, Germany) microscope with filter set 05.

TABLE 1. Oligonucleotide primers used in this study for PCR amplification (Amp) and sequencing (Seq) of partial archaeal 16S rRNA genes

Oligonucleotide	Sequence (5′ to 3′)	Target site[a]	Use
A109f	ACKGCTCAGTAACACGT	109–125	Amp and Seq
A934b	GTGCTCCCCCGCCAATTCCT	915–934[b]	Amp and Seq
A112f	GCTCAGTAACACGTGG	112–127	Seq
A533b	TTACCGCGGCGGCTGGCA	516–533	Seq

[a] *E. coli* sequence numbering according to Brosius et al. (6).
[b] Stahl and Amann (58).

The threshold concentrations for acetate utilization by various methanogens were determined by growing the methanogens in serum bottles with 5 mM acetate as described above. Samples of 1 ml were taken at weekly intervals, and the residual acetate concentrations were determined by high-pressure liquid chromatography until no further decrease was detected over 4 weeks.

The concentration of acetate was measured by high-pressure liquid chromatography (30) with a detection limit of 5 μM. Pore water was extracted from soil by centrifugation of soil samples at 14,000 × *g* for 2 min, followed by membrane filtration (regenerated cellulose, 0.2-μm pore size) of the supernatant. Samples were frozen at −20°C until analyzed. The CH_4 concentration was measured by gas chromatography (17) with a limit of detection of about 0.005 Pa.

DNA extraction from bulk soil. The procedure used for DNA extraction was a modified version of previously described protocols (42, 56). Briefly, 1 g of fresh material from a 90-day-old rice microcosm was mixed with 1 ml of sodium phosphate buffer (0.12 M, pH 8.0). After three cycles of freezing and thawing (freezing at −70°C for 2 min, heating at 65°C for 2 min), 5 mg of lysozyme was added to the sample and the mixture was incubated at 37°C for 1 h. Subsequently, sodium dodecyl sulfate was added to a final concentration of 2%, followed by incubation for 10 min at 60°C. Then, an equal volume of glass beads (0.1-mm diameter) was added, and the suspension was shaken three times for 80 s each time at maximum speed in a bead beater (Mini-Bead-Beater; Biospec Products, Bartlesville, Okla.). After 10 min of centrifugation at 13,000 × *g*, the supernatant was extracted with phenol-chloroform (1:1 [vol/vol]) and chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was precipitated from the aqueous phase with 3 volumes of ethanol and, after being dried, was resuspended in 100 μl of deionized water. For further purification of the DNA, 0.1 g of cesium chloride was added and mixed well and the sample was incubated for 3 h at room temperature. After centrifugation at 13,000 × *g* for 20 min, the supernatant was transferred to a new tube and further purified by two rounds of precipitation with equal volumes of isopropanol. Finally, the DNA was resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). The amount of DNA extracted was estimated by electrophoresis of 5-μl aliquots on a 0.8% agarose gel and comparison to a *Hin*dIII digest of λ DNA. The gel was stained with ethidium bromide.

DNA extraction from cultures. Cells were pelleted by centrifugation and resuspended in 100 μl of deionized water. Lysis was achieved by boiling for 10 min. After centrifugation, the supernatant was transferred to a new tube and 1-μl aliquots were used directly for PCR amplification of the archaeal 16S rRNA genes.

PCR amplification of archaeal 16S rRNA genes. The oligonucleotide primer system was designed specifically to target archaeal 16S rRNA genes from positions 109 through 934 (*Escherichia coli* 16S rRNA numbering) (6) (Table 1). The reaction cocktail contained 1 μl of the template DNA, 10 μl of PCR buffer (0.2 M Tris-HCl [pH 8.3], 0.25 M KCl, 15 mM $MgCl_2$, 1 mg of bovine serum albumin, 0.5% Tween 20), 20 nmol of each deoxynucleotide (USB, Cleveland, Ohio), 30 pmol of each primer, and 2.5 U of *Taq* DNA polymerase (AmpliTaq DNA polymerase; Perkin-Elmer, Foster City, Calif.). The enzyme was added after the first denaturation step. The standard thermal profile for the amplification of the partial 16S rRNA gene sequences was as follows: 5 min at 94°C, after which the DNA polymerase was added (hotstart technique [38]); 38 cycles (DNA extracted from bulk soil) or 30 cycles (DNA extracted from cultures) consisting of primer annealing at 52°C for 60 s, DNA elongation at 72°C for 90 s, and denaturation at 94°C for 60 s; and a final cycle of 52°C for 60 s and 72°C for 6 min. Amplification was performed with a total volume of 100 μl in 0.2-ml reaction tubes and a DNA thermal cycler (model 2400; PE Applied Biosystems). Aliquots of the 16S rRNA gene amplicons (10 μl) were visualized by electrophoresis on a 1% agarose gel and staining with ethidium bromide.

Cloning and sequencing. The archaeal 16S rRNA gene PCR products obtained from bulk soil and from culture A5.1 (see Results) were cloned by use of a TA Cloning Kit (Invitrogen, San Diego, Calif.) following the manufacturer's instructions. Further analysis of randomly selected clones, i.e., extraction of phagemid DNA, PCR-mediated amplification of cloned inserts, and sequencing,

basically followed a previously described procedure (52). The primers used for the amplification of cloned inserts and for sequencing are listed in Table 1.

Phylogenetic placement. The phylogenetic analysis, i.e., data processing and reconstruction of the tree, was done by use of the ARB program package (59). The archaeal 16S rRNA gene sequences were added to a database of 123 complete or partial 16S rRNA sequences of archaea (35, 49, 64). The phylogenetic placement was done in comparison to reference sequences comprising the main lines of descent within the kingdom *Euryarchaeota*. The overall tree topology was evaluated by applying distance matrix methods (ARB and PHYLIP) (11). Evolutionary distances between pairs of sequences were calculated for a sequence stretch from positions 148 through 880 (*E. coli* 16S rRNA numbering) (6). To exclude highly variable regions prior to the phylogenetic analysis, only those nucleotide sequence positions which contained identical residues in at least 50% of the alignment positions within a data set of 104 euryarchaeotal sequences (614 nucleotide sequence positions) were used for the reconstruction of the tree. The branching patterns within defined lineages, i.e., within the *Methanosaeta* cluster, the *Methanosarcina* cluster, and the *Methanobacterium* cluster, were reanalyzed by use of the maximum-likelihood method (ARB and fastDNAml) (35) and all sequence information available for the cultured organisms and for the environmental sequences being compared.

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences obtained in this study for the environmental clones ABS1 to ABS23, culture A5.1, strains VeA23 and VeH52, and *Methanosaeta* sp. strain VeAc9 have been deposited in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers Y15385 through Y15402.

RESULTS

Sampling. Ninety days after transplantation of rice seedlings into flooded rice paddy soil microcosms, the plants began to flower. Several cores of the bulk soil were taken from the containers, the large plant pieces were removed under a stream of N_2 gas, and the samples were mixed well.

For the combined approach of cultivation and direct molecular retrieval of archaeal 16S rRNA gene sequences, a sample obtained from one flooded rice paddy soil microcosm was divided: one aliquot (from which strains VeH52 and VeA23 and culture A5.1 [see below] were obtained) was used for MPN cell counts, and the other was used for the extraction of total community DNA to generate a 16S rRNA gene library from the bulk soil. Pore water was extracted from other cores from the same microcosm, and the acetate concentration was measured as 10.6 ± 0.2 μM (mean ± standard deviation).

MPN counts were also made from two other microcosms, also 90 days after transplantation of the seedlings. These data are shown in Table 2.

Counting methanogenic archaea by the MPN method. Serial liquid dilution cultures in dilution steps of 10^{-1}, set up in triplicate, allowed an estimation of the population of methanogenic microorganisms that could be cultured by measurement of the production of methane and calculation of the number of viable organisms by a statistical treatment (5). The numbers obtained are minimal numbers, since only organisms which are able to metabolize in the medium used can be detected and since cells adhering to particles or forming multicellular aggregates or filaments will be counted as one cell. The development of methane production with time showed that incubation time has a profound effect on the number of organisms detected (Fig. 1). The counts on hydrogen-carbon dioxide, on formate, and on methanol were stable within 20 weeks or less. In contrast, the count on acetate increased over a 50-week incubation period for the MPN dilution series and may have continued to increase with even longer incubation periods. However, after 64 weeks of incubation, some of the tubes began to show signs of oxygen contamination, as evidenced by cloudiness due to precipitates such as sulfur, without bacterial growth, so the incubations were discontinued.

The numbers of methanogenic organisms determined in different microcosms by MPN counting revealed population sizes of up to 2.3×10^7 cells per g of dry soil able to grow with hydrogen (Table 2). The positive tubes were dominated by rod-shaped cells about 1.5 μm long and about 0.5 μm in diameter (Fig. 2a) which showed the strong blue fluorescence typical of methanogens due to F_{420}. Counts of formate-utilizing methanogens showed a population size of up to 2.7×10^6 cells per g of dry soil (Table 2), and the cultures were dominated by fluorescent rod-shaped cells very similar to those observed on hydrogen. Counts of methanogenic organisms able to use acetate revealed total population sizes of up to 1.3×10^6 cells per g of dry soil (Table 2). Cultures originating from less-dilute inocula, corresponding to population sizes of $<10^3$ cells per g of dry soil, were dominated by pseudosarcinae (Fig. 2b) which fluoresced strongly under UV illumination. Individual cells were about 3 μm in diameter. Cultures originating from more-dilute inocula, indicative of higher cell numbers in the soil, were dominated by nonfluorescent multicellular filaments with a diameter of about 0.5 μm (Fig. 2c). Counts of methanol-utilizing microorganisms showed populations of

TABLE 2. Counts of methanogenic populations in anoxic soil of different flooded rice paddy soil microcosms determined by the MPN dilution method

Substrate	Incubation time (wk)	Cell count (per g of dry soil)	95% Confidence interval
H_2-CO_2	64[a]	4.6×10^6	9.9×10^5–1.3×10^7
	57	2.0×10^6	4.6×10^5–6.1×10^6
	13	2.3×10^7	4.4×10^6–8.3×10^7
Formate	64[a]	2.7×10^6	5.2×10^5–9.9×10^6
	13	2.9×10^5	5.6×10^4–1.1×10^6
Acetate	64[a]	6.1×10^5	1.7×10^5–1.7×10^6
	57	1.3×10^6	2.5×10^5–4.6×10^6
	55	5.1×10^5	1.4×10^5–1.4×10^6
Methanol	64[a]	2.2×10^5	4.7×10^4–9.9×10^5
	13	7.5×10^5	1.3×10^5–3.7×10^6

[a] Total community DNA extracted from the same soil sample was used to generate the clone library analyzed in this study (see the text).

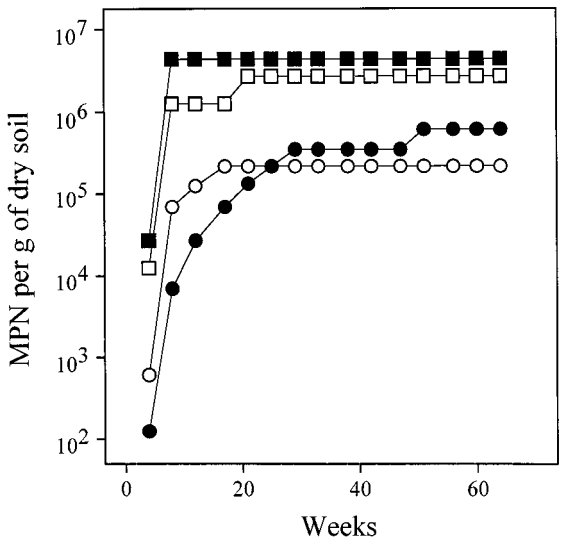

FIG. 1. Time course of the total cell counts determined with various substrates, showing the increase in the MPN index with increasing incubation time of the MPN dilution series. Symbols: ■, count on H_2-CO_2; □, count on formate; ●, count on acetate; ○, count on methanol.

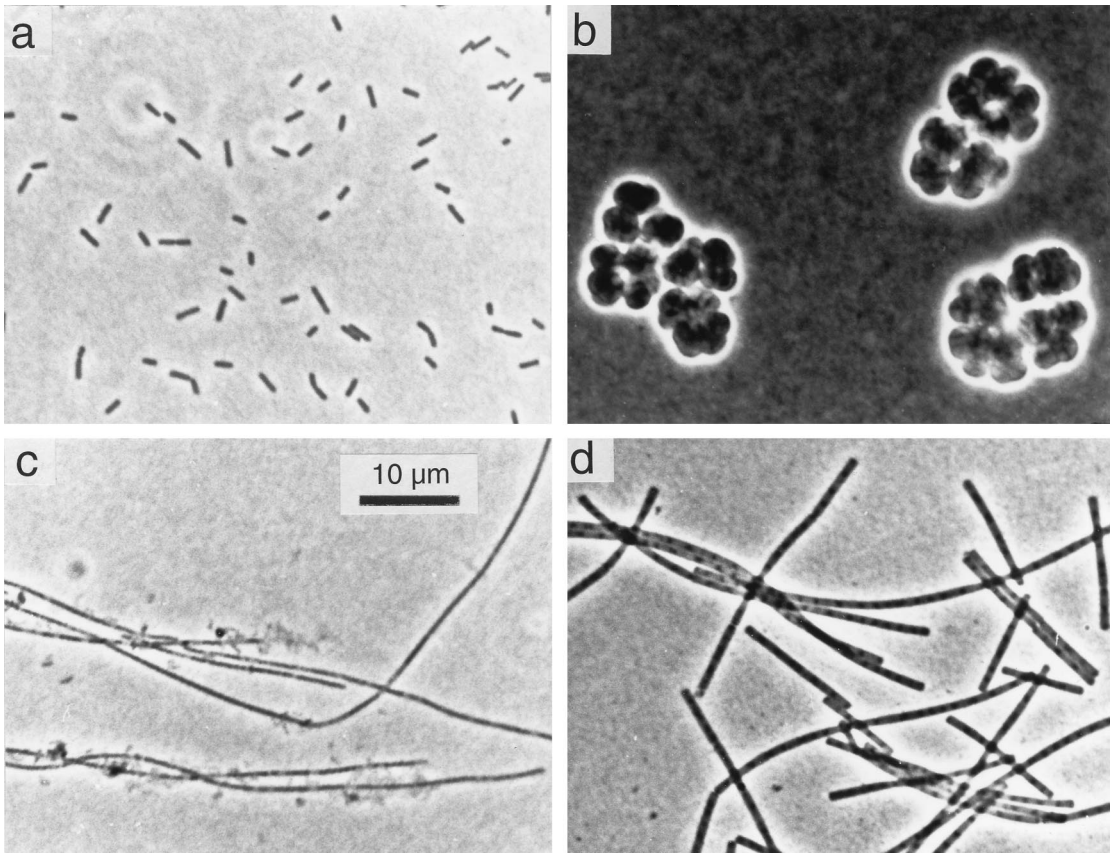

FIG. 2. Phase-contrast photomicrographs of dominant cell forms observed in liquid serial dilutions. (a) Mainly fluorescent rod-shaped cells in a culture on H_2-CO_2, from a dilution level corresponding to a population size of about 10^6 cells per g of dry soil. (b) Fluorescent *Methanosarcina*-like pseudosarcinae in a culture on acetate, from a dilution level corresponding to a population size of 10 to 100 cells or aggregates per g of dry soil. (c) Nonfluorescent *Methanosaeta*-like filaments in a culture on acetate, from a dilution level corresponding to a population size of 10^5 to 10^6 cells per g of dry soil. (d) *M. concilii* VeAc9 for comparison. Scale bar = 10 μm for all panels.

up to 7.5×10^5 cells per g of dry soil (Table 2). These cultures were dominated by mixed populations of nonfluorescent sporulating straight to curved rods and nonfluorescent multicellular filaments. The latter forms were very similar in appearance and size to those observed on acetate.

Isolation of methanogenic organisms. A pure culture of a hydrogen-utilizing methanogen was isolated from the terminal positive tube of the dilution series on hydrogen (corresponding to about 10^6 cells per g of dry soil). This isolate was able to grow with hydrogen and with formate (other substrates were not tested), produced methane, and displayed the blue fluorescence typical of methanogenic archaea. This strain was designated VeH52. A fluorescent methanogenic pseudosarcinal isolate able to grow on acetate, hydrogen, and methanol was also obtained in a pure culture from a lower dilution step (corresponding to about 10^2 cells per g of dry soil) of the dilution series on acetate. This strain was designated VeA23. Attempts to isolate in pure culture the multicellular filamentous microorganisms dominating the terminal positive dilutions of the MPN counts with acetate as a substrate have not been successful to date. One tube of the terminal positive dilution step corresponding to 10^5 to 10^6 cells per g of dry soil, designated culture A5.1, was investigated further by analysis of the 16S rRNA genes in a clone library (see below) derived from the organisms in this culture.

Subculturing of the methanol-utilizing bacteria from the terminal positive dilutions of the MPN counts with methanol resulted in a loss of methanogenic activity, although growth was maintained. The bacteria which grew in the subcultures were nonfluorescent, straight to curved rods, and some of them contained terminal endospores.

Identification of environmental sequences retrieved from bulk soil. In total, 23 environmental 16S rRNA gene sequences were obtained from the bulk soil of the flooded rice microcosm and further analyzed by determining almost the complete PCR-amplified primary structure (between 700 and 750 nucleotide sequence positions for each clone). Their phylogenetic placement confirmed the specificity of the primer system used in that only archaeal sequence types were found in the clone library. Fourteen of these environmental sequences could be assigned to known groups of archaea, all of them methanogenic (ABS clones) (Fig. 3). Seven of these 14 belonged to a coherent group of clone sequences that were closely affiliated with but distinct from *M. concilii*. Six of these *Methanosaeta*-like sequences formed a very tight cluster (environmental sequences ABS2, ABS8, ABS11, ABS14, ABS18, and ABS21). The ranges of overall sequence differences between these six environmental sequences were between 0.0% (identical sequences ABS2 and ABS8) and 1.5% (sequences ABS2 and ABS8 versus sequence ABS21), corresponding to 0 and 10 nucleotide substitutions, respectively. Some of these differences could be attributed to coordinated base-pair substitutions within the 16S rRNAs, e.g., between environmental sequences ABS2 and ABS8 and sequence ABS11 or ABS21 in

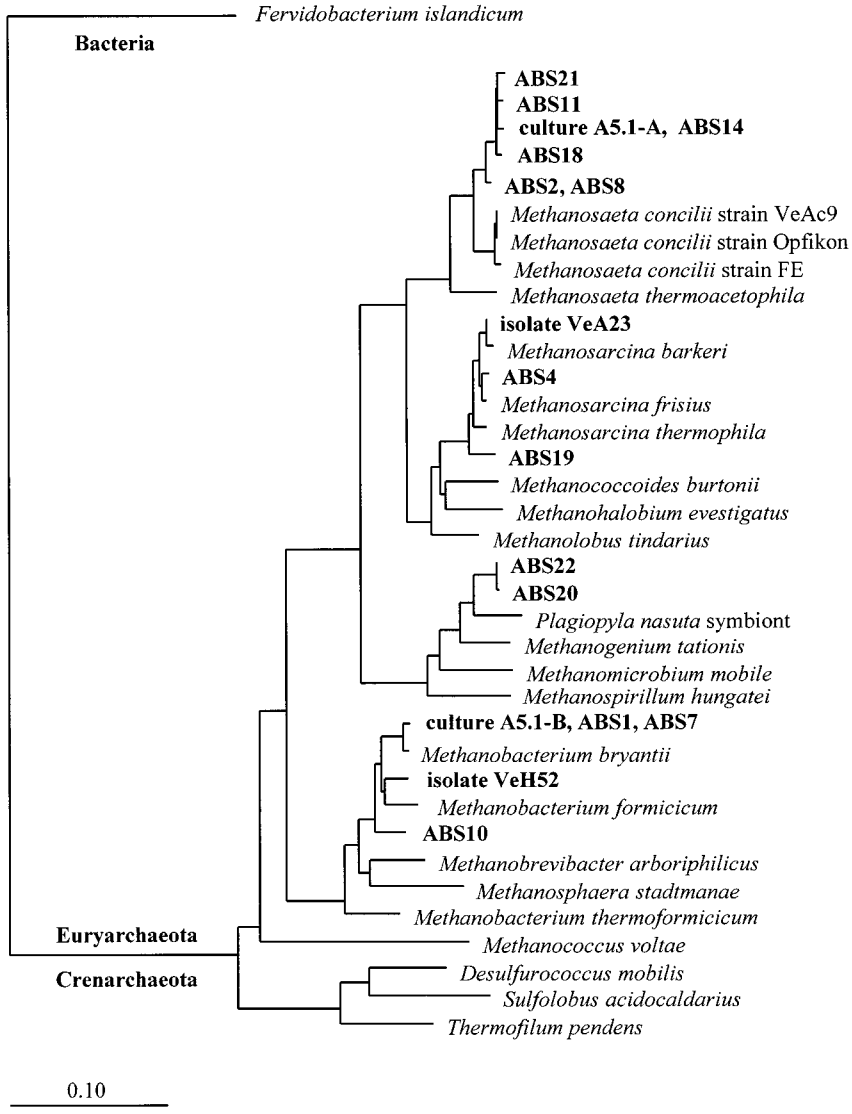

FIG. 3. 16S rRNA-based dendrogram showing the phylogenetic relationship between the cultured organisms (strains VeA23 and VeH52 as well as culture A5.1) and environmental sequences (ABS clones) obtained in this study and a representative selection of members of the *Euryarchaeota*. Members of the *Crenarchaeota* and *Fervidobacterium islandicum* were used as outgroup references. The tree topology was derived by performing a distance matrix analysis (calculation of the distance matrix was done with the Jukes-Cantor equation [27]; tree reconstruction was done with the neighbor-joining method [53]). The branching pattern within defined lineages, i.e., within the *Methanosaeta* cluster, the *Methanosarcina* cluster, and the *Methanobacterium* cluster, was corrected in accordance with maximum-likelihood analyses. The scale bar represents a 10% estimated difference in nucleotide sequences.

helix 13 or 24 (63), respectively; this finding indicated that at least the majority of the slight differences were real and were not introduced by methodological bias, such as amplification errors from the *Taq* polymerase used. The overall 16S rRNA gene sequence identity of this cluster to the corresponding stretch of *M. concilii* was approximately 94%, and that to *Methanosaeta thermoacetophila* was between 90.5 and 92%. The seventh clone sequence (ABS15) grouped slightly outside of the tight cluster characterized by the other six *Methanosaeta*-like sequences. A careful reanalysis of its primary structure showed *Methanosarcina*-like sequence motifs for the first 135 bp at the 5′ end, whereas the following 575 nucleotide sequence positions were indicative of a member of the novel *Methanosaeta*-like clone cluster. Consequently, this apparently chimeric (29, 34, 65) 16S rRNA gene clone sequence was excluded from the phylogenetic analysis.

Two environmental sequences were indicative of members of the genus *Methanosarcina* (ABS4 and ABS19). Three clones clustered within the genus *Methanobacterium* (ABS1, ABS7, and ABS10). Environmental sequences ABS1 and ABS7 were identical to each other and were most closely related to *Methanobacterium bryantii*, whereas sequence ABS10 grouped on a slightly separate branch. The almost identical clone sequences ABS20 and ABS22 (one mismatch) clustered next to the 16S rRNA sequence of the methanogenic endosymbiont of the anaerobic ciliate *Plagiopyla nasuta.*

The remaining nine environmental sequences formed several novel lineages within the domain *Archaea*. The overall similarity values of these nine phylotypes to the 16S rRNA gene sequences of the most closely related known methanogens were in all cases between 67 and 82% for the 700-bp stretch analyzed (data not shown). Thus, the assertion that

TABLE 3. Threshold acetate concentrations for methanogenesis by pure cultures of methanogenic archaea and steady-state concentrations of acetate in anoxic rice paddy soil systems

Strain or system	Acetate concn (μM)	Reference or source
Strains		
Methanosarcina barkeri Fusaro	244	25
Methanosarcina barkeri 227	1,180	69
Methanosarcina barkeri MS^T	850	This study
Methanosarcina mazei S-6	397	69
Methanosarcina thermophila CALS-1	190	37
Methanosarcina sp. strain VeA23	310	This study
Methanosaeta concilii Opfikon	7	25
Methanosaeta concilii VeAc9	<10	This study
Methanosaeta sp. Los Angeles River strain	69	69
Methanosaeta thermoacetophila CALS-1	12	37
Systems		
Pore water from Italian rice paddy soil	16–97	51
Pore water from rice paddy soil microcosms	10.6 ± 0.2[a]	This study
Methanogenic slurries of Italian rice paddy soil	20–40	1

[a] Mean ± SD.

these environmental sequences are derived from as-yet-uncultured methanogens is speculative as long as no cultures are available. One of these novel groups was the dominant phylotype in clone libraries generated from washed rice roots in a parallel study and will be discussed in more detail within this context (19).

Identification of cultivated methanogenic archaea strains. No pure culture could be isolated from the terminal positive dilutions of the MPN counts on acetate, in which the filamentous organisms dominated. Instead, a methanogenic culture (as confirmed by the active production of methane) of the terminal positive dilution step (designated culture A5.1) was investigated via the generation of a 16S rRNA gene clone library. Comparative sequencing analysis of 12 randomly selected clones detected only two different sequence types (A5.1-A and A5.1-B) (Fig. 3). The three A5.1-A sequences belonged to the *Methanosaeta*-like cluster of six environmental sequences that was directly retrieved from the bulk soil. Within this group, they showed a sequence identical to that of clone ABS14. The nine A5.1-B sequences of culture A5.1 were identical to each other and to environmental sequences ABS1 and ABS7, indicative of a hydrogenotrophic population closely related to *M. bryantii*. These data may indicate that the cultivation conditions favored the growth of a consortium of two methanogens.

The sequence of the fluorescent pseudosarcina dominating the less-dilute levels of the MPN counts on acetate, represented by the pure culture designated VeA23, was highly similar to sequences of *Methanosarcina* spp., with only seven nucleotide substitutions in comparison to the 16S rRNA sequence of *M. barkeri* (Fig. 3).

The phylogenetic placement of strain VeH52, isolated from the terminal positive dilution of the MPN counts on hydrogen, revealed a close relationship to *M. bryantii* and *Methanobacterium formicicum* and also an affiliation to environmental sequences ABS1, ABS7, and ABS10 (Fig. 3).

Threshold concentrations for acetate. The threshold concentrations for acetate determined in this study for strains VeAc9, VeA23, and MS^T in comparison with other aceticlastic methanogens are shown in Table 3.

DISCUSSION

One of the most fundamental tasks in studying microbial populations in natural environments is to make an objective survey of the structure of the microbial community. This task is also one of the most difficult, since methods used in microbial ecology are not yet able to answer many questions concerning microbial community structure in heterogeneous environments such as soils and sediments. State-of-the-art technologies, such as in situ hybridization with fluorescently labeled 16S rRNA-targeted oligonucleotide probes (FISH) and quantitative rRNA dot blot hybridization used to determine the relative abundances of natural populations in aquatic systems (2), have so far failed to be successfully applied to soils, mainly for method-inherent reasons. For example, FISH did not allow the detection of more than 1% of microorganisms that could be stained with 4′,6-diamidino-2-phenylindole (DAPI) (20), mainly due to autofluorescence of the soil and low specific signal intensities. Only very recently could the specific detection of microorganisms in soils by FISH be increased to 35 to 50% of cells that could be DAPI stained (71). We therefore used an alternative approach consisting of cultivation and direct retrieval of 16S rRNA gene sequence data from the same soil sample. One major goal was to evaluate the meaningfulness of this biphasic strategy for obtaining insights not only into the diversity but also into the structural composition of abundant microbial groups within heterogeneous environments such as soil. We chose the methanogenic community present in the anoxic bulk soil of flooded rice microcosms as a model system, since the biodiversity among this group is, to our recent knowledge, not as extensive as among most groups of the domain *Bacteria.*

Cultivation studies. The first approaches to investigation of the diversity of methanogenic archaea in flooded rice paddy soil systems were based on enrichment cultures (3, 4, 9, 16, 46). Enrichment cultures are selective in different ways due to the media used (14, 41, 54; for a review, see reference 32). Organisms of numerical significance but with low growth rates may be overgrown by those with high growth rates, even if the latter are of smaller numerical importance in the habitat under investigation. An alternative to enrichment cultures is cultivation by dilution series. The advantage of this technique is that, at higher dilution levels, numerically dominant organisms can be isolated, given that they are able to grow in the medium selected. This characteristic may explain why species of the generalist *Methanosarcina*, with their high growth rates (26), dominated the lower dilution levels of the MPN dilution series on acetate, whereas the more slowly growing *Methanosaeta* spp. dominated the highest dilution levels due to their numerical significance in the sample material.

Our results indicate that *Methanosaeta* spp. are an important part of the microbial community of the microcosms investigated, based on their dominance as determined by cultivation studies, their importance in the 16S rRNA gene clone library, and the correlation between their phenotype (low acetate threshold concentrations) and the acetate concentration in the soil. A comparison of 16S rRNA gene similarities versus DNA-DNA hybridization data for genomic DNA has revealed that microorganisms with overall 16S rRNA gene similarities below 97.5% never share a DNA reassociation value of 70% or more (57), the generally accepted threshold value for placing two closely related microbial strains into the same species (36, 67). In contrast, 16S rRNA gene similarities above 97.5% may or may not correlate with a DNA reassociation value above 70%. For the 700-bp stretch analyzed in this study, the overall similarity values of 16S rRNA gene sequences of the novel *Methanosaeta*-like cluster were approximately 94% to those of

strains of *M. concilii* and in the range of 90.5 to 92% to that of *M. thermoacetophila*. This finding permits the prediction that the full-length 16S rRNA gene sequences of this novel *Methanosaeta*-like cluster will not show 16S rRNA gene sequence similarities greater than 97.5% to 16S rRNA gene sequences of strains of *M. concilii*. Thus, it is feasible to postulate that a new species or group of species of the genus *Methanosaeta* is present in the anoxic rice paddy soil. Further support comes from differences in morphology (their filaments are about 0.5 μm in diameter [Fig. 2c]; the filament diameter is about 0.8 μm for *M. concilii* [Fig. 2d]). The tentative assignment of these organisms to the genus *Methanosaeta* is based on their apparent methanogenic growth on acetate, filamentous morphology, and lack of autofluorescence. Weak or no autofluorescence is a characteristic of most strains of *Methanosaeta* spp. (28).

Interestingly, based on morphology and the lack of autofluorescence, it can be assumed that mixed cultures of straight to curved spore-forming rod-shaped bacteria (probably homoacetogens) and *Methanosaeta* spp. dominated the terminal positive dilutions with methanol as a substrate, on which we had expected to see *Methanosarcina* spp. This result validates the low counts of *Methanosarcina* spp. (however, see below). What is apparently occurring in the MPN series on methanol is the enrichment of homoacetogenic bacteria and the subsequent enrichment of *Methanosaeta* spp. on the resulting acetate. Homoacetogenic bacteria have been found to occur in high numbers in similar rice paddy soil microcosms, and pure cultures of these are able to utilize methanol as a growth substrate, producing acetate (50).

Since the number of hydrogen-utilizing methanogens, as determined by the viable count (2.0×10^6 to 2.3×10^7 cells per g of dry soil), was higher than that of acetate-utilizing methanogens (5.1×10^5 to 1.3×10^6 cells per g of dry soil), hydrogen-utilizing methanogens could be expected to be present in cultures which yielded methane from acetate. The production of low levels of hydrogen could have enabled these to survive and perhaps even to grow, although fluorescent rods were not significant in these cultures. Their presence was confirmed by the recovery of 16S rRNA gene sequences indicative of *Methanobacterium* spp. in the clone library prepared from culture A5.1 (A5.1-B), the most dilute positive culture on acetate. The confirmation of hydrogen production by the new *Methanosaeta* spp. must await the isolation of a pure culture, but low levels of hydrogen production by a *Methanosaeta* sp. have been reported (72).

Although it was apparently a minor component of the archaeal population in culture A5.1, the *Methanobacterium*-like sequence A5.1-B dominated the clone library prepared from this culture. This result was perhaps indicative of methodological bias, which can result in a shift in the relative proportions of the different rRNA gene sequence types present, as discussed in more detail below (32, 45, 48, 60).

Cultivation-independent molecular investigations. The major portion of environmental sequences retrieved by the molecular approach could be assigned to known groups of methanogens. Six environmental sequences analyzed were related to obligately acetate-utilizing *Methanosaeta* spp., and three sequences grouped with hydrogen- and formate-utilizing *Methanobacterium* spp. The third group, containing two environmental sequences, was related to species of the generalist (utilizing both acetate and hydrogen) *Methanosarcina*. The 16S rRNA gene sequences obtained from strains VeH52 and VeA23 as well as from culture A5.1 were closely affiliated with one of these three sequence clusters.

For culture A5.1, obtained by the MPN dilution technique on acetate, the two sequence types identified (A5.1-A and A5.1-B) not only belonged to the clusters of *Methanosaeta*-like and *Methanobacterium*-like sequences, respectively, obtained by the molecular retrieval technique but also were in fact identical to some of the environmental sequences (*Methanosaeta*-like A5.1-A was identical to clone ABS14, and *Methanobacterium*-like A5.1-B was identical to clones ABS1 and ABS7). Complete 16S rRNA gene identities between microbial populations detected by molecular approaches and traditional cultivation (in our study, about 730 nucleotide sequence positions were analyzed, including the highly variable regions which roughly correspond to helices 9 to 11, 18, P23-1, 24, 28, and 29 [63, 64]) are by far exceptional findings and, to date, have been reported in only a few studies (14, 22, 41).

The successful demonstration of 16S rRNA gene identities between traditional cultivation and the molecular retrieval of archaeal 16S rRNA gene sequences by us might have resulted from (i) the use of the same soil sample for both approaches, (ii) the use of the serial dilution technique for cultivation, and (iii) the relatively low diversity of *Archaea* present in the anoxic rice paddy soil under study. However, an investigation is under way to evaluate the meaningfulness of this biphasic strategy in describing the more complex community structure of the abundant bacterial groups present in this anoxic rice paddy soil environment.

It is noteworthy that the three *Methanosaeta*-like A5.1-A sequences obtained from culture A5.1 were completely identical, suggesting that the cultivation conditions favored the growth of one defined aceticlastic population which corresponds to one defined 16S rRNA gene sequence type. However, some of the environmental 16S rRNA gene sequences belonging to the same cluster were slightly different, as verified by the detection of coordinated base-pair substitutions between them. Slight differences between molecularly retrieved 16S rRNA gene sequences were also detected in studies which analyzed hot-spring microbial mat communities (13, 66, 68). Ferris and Ward showed by using denaturing gradient gel electrophoresis that some of these differences were real and not methodological artifacts (15). Moreover, these differences could be assigned to closely related but clearly distinct populations which inhabited different ecological niches within the hot-spring microbial mat community. This result allows speculation that the slight differences between the environmental sequences belonging to the *Methanosaeta*-like cluster point to closely related but ecologically distinct populations, for example, in adaptation to varying concentrations of acetate or in response to physical parameters within the anoxic rice paddy soil.

The assignment of the environmental sequences ABS4 and ABS19 to members of the genus *Methanosarcina* suggested an abundance of such methanogens in the anoxic rice paddy soil under study. In contrast, the cultivation studies led to the detection of *Methanosarcina* spp. only in the less-dilute levels of the same serial dilution series on acetate that favored the growth of *Methanosaeta* spp. Even the use of methanol as a substrate, utilized by *Methanosarcina* spp. but not by *Methanosaeta* spp., did not result in higher viable counts of *Methanosarcina* spp. One explanation for this discrepancy between the molecular data and the cultivation data may be that, as previously shown, the relative contributions of different sequence types to a clone library of retrieved 16S rRNA gene sequences do not reflect the in vivo situation. The individual steps of the molecular approach, i.e., extraction of total community DNA (32), PCR amplification (10, 48, 60), and cloning (45), may be subject to methodological bias. A shift of the relative abundances of different sequence types may especially occur during the mixed PCR amplification of 16S rRNA genes because of

either preferential amplification (48) or differences in the kinetics of sequence type accumulation in the nonexponential phase of PCR, which may lead to an increasing frequency of originally less abundant sequence types in the final product (60).

Another explanation may be that *Methanosarcina* spp. often form multicellular pseudosarcinal packets, which will result in an underestimation of their abundance by serial dilution techniques. However, the same situation may occur with *Methanosaeta* spp., which form multicellular filaments. How these cells occur in the soil is not known. The molecular approach investigates at the level of the gene, and multicellular aggregates do not introduce bias, although multiple gene copies may (10). Finally, there may exist in the anoxic rice paddy soil abundant *Methanosarcina*-like populations which cannot grow in the medium used.

Two environmental sequences that grouped next to the 16S rRNA gene sequences from the endosymbiont of the anaerobic ciliate *P. nasuta* were identified. Since this endosymbiont had been characterized as an H_2-CO_2- and formate-utilizing methanogen, these molecular fingerprints might point to the presence of such anaerobic protozoa containing methanogenic endosymbionts in the anoxic rice paddy soil under study. Further support for this idea comes from the detection of polyunsaturated fatty acid biomarkers of eukaryotic microorganisms, which accounted for 13 to 16 mol% of the total phospholipids extracted from continuously cropped, irrigated rice paddies (47). It has been suggested that methanogenesis by such endosymbionts could be quantitatively important in some anaerobic systems (12, 62).

Biogeochemical aspects. The acetate concentration in the anoxic soil from the microcosms in this study was about 11 μM, in the lower range of concentrations detected in the same soil in Italy (51). This concentration is similar to the threshold concentration for acetate utilization by *Methanosaeta* spp. and is well below the concentration metabolized by *Methanosarcina* spp. (Table 3). The threshold concentration is that below which an organism is no longer able to degrade a particular substrate. These data suggest that *Methanosarcina* spp. are not able to utilize acetate in the rice paddy soil solution examined but that *Methanosaeta* spp. can utilize acetate at a concentration close to their threshold for acetate metabolism. Unfortunately, we were not able to isolate a pure culture of the new *Methanosaeta* species grown in culture A5.1, which was apparently numerically dominant in this system, and thus do not know the threshold concentration for acetate utilization by this organism.

Because of their different threshold concentrations for acetate, *Methanosaeta* spp. and *Methanosarcina* spp. could be indicative of different steady-state concentrations of acetate in this environment. There may be an acetate gradient from the rhizosphere to the bulk soil in which species of the generalist *Methanosarcina*, with their high acetate threshold concentrations, could be expected near the root system and species of the specialist *Methanosaeta*, with their low acetate threshold concentrations, could be expected in the bulk soil. We are currently investigating the methanogenic community structure of the rhizosphere soil to obtain more information.

By combining both cultivation and molecular retrieval techniques, we were able to identify aceticlastic *Methanosaeta*-like organisms and hydrogenotrophic *Methanobacterium*-like populations as dominant methanogens within the anoxic rice paddy soil microcosms under study. Due to method-inherent reasons, the cell numbers determined via MPN counts for both groups (up to 10^6 and 10^7, respectively, per g of dry soil) may still be an underestimation of the true abundances of these two methanogenic groups in the anoxic rice paddy soil examined. The overall pattern of sequence types retrieved by the cultivation and molecular approaches and their branching within the phylogenetic radiation of the methanogens confirmed earlier considerations that dominant populations of methanogenic archaea within rice paddies are well-known physiotypes characterized by cultured representatives. More important, our results agree with previous investigations that determined H_2-CO_2 (and formate) and acetate to be the main substrates for methanogenesis in flooded rice paddies from which the soil was taken for our microcosm studies (55).

An important question is if these findings can be generalized and transferred to other rice paddy systems or if they reflect the community structure in our microcosms only. Recently, Kudo et al. (31) published a comparative survey of the methanogenic flora present in Japanese rice paddies at different sites, based on the molecular retrieval of 267-bp 16S rRNA gene fragments via extraction of total community DNA followed by PCR amplification and cloning. The composition of the methanogenic community was deduced from ratios of the clones which were identified to the genus level based on signature nucleotides within the sequence stretches analyzed. The findings led to the conclusion that *Methanosarcina*-like or *Methanosaeta*-like organisms are the dominant aceticlastic methanogens in the different rice field sites under study. However, the identification of environmental sequences based on a very few signature nucleotides is rather problematic and, considering the possible pitfalls discussed above, the structural composition of microbial communities can by no means be deduced from the ratios of sequence types retrieved from environmental samples via cloning approaches. Nevertheless, these data may provide further evidence that *Methanosarcina*-like and *Methanosaeta*-like populations are ubiquitously distributed in rice paddy fields. Ultimately, FISH and quantitative rRNA dot blot hybridization will be of major importance in addressing questions related to the structural composition of methanogenic communities in anoxic rice paddy soils, once these approaches have been established as reliable tools for general use in soils.

ACKNOWLEDGMENTS

We thank Sonja Fleissner and Alexandra Schuhmann-Pidun for excellent technical assistance. We are grateful to Wolfgang Ludwig (Technische Universität Müchen, Munich, Germany) for providing the ARB package and for many helpful instructions. We thank two anonymous reviewers for constructive criticism of the manuscript.

This work was supported by grants from the Deutsche Forschungsgemeinschaft and from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (contract 0311121) awarded to W.L. and by a grant from the EC RTD Programme Biotechnology (contract BI04-CT96-0419).

REFERENCES

1. **Achtnich, C., A. Schuhmann, T. Wind, and R. Conrad.** 1995. Role of interspecies H_2 transfer to sulfate and ferric iron-reducing bacteria in acetate consumption in anoxic paddy soil. FEMS Microbiol. Ecol. **16:**61–70.
2. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Asakawa, S., M. Akagawa-Matsushita, H. Morii, Y. Koga, and K. Hayano.** 1995. Characterization of *Methanosarcina mazeii* TMA isolated from a paddy field soil. Curr. Microbiol. **31:**34–38.
4. **Asakawa, S., H. Morii, M. Akagawa-Matsushita, Y. Koga, and K. Hayano.** 1993. Characterization of *Methanobrevibacter arboriphilicus* SA isolated from a paddy field soil and DNA-DNA hybridization among *M. arboriphilicus* strains. Int. J. Syst. Bacteriol. **43:**683–686.
5. **Beliaeff, B., and J. Y. Mary.** 1993. The "most probable number" estimate and its confidence limits. Water Res. **27:**799–805.
6. **Brosius, J., M. L. Palmer, P. J. Kennedy, and H. R. Noller.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **75:**4801–4805.

7. **Chartrain, M., and J. G. Zeikus.** 1986. Microbial ecophysiology of whey biomethanation: characterization of bacterial trophic populations and prevalent species in continuous culture. Appl. Environ. Microbiol. **51:**188–196.
8. **Conrad, R.** 1993. Mechanisms controlling methane emission from wetland rice fields, p. 317–355. *In* R. S. Oremland (ed.), Biogeochemistry of global change: radiatively active trace gases. Chapman & Hall, New York, N.Y.
9. **Conrad, R., F. Bak, H. J. Seitz, B. Thebrath, H. P. Mayer, and H. Schütz.** 1989. Hydrogen turnover by psychrotrophic homoacetogenic and mesophilic methanogenic bacteria in anoxic paddy soil and lake sediment. FEMS Microbiol. Ecol. **62:**285–294.
10. **Farrelly, V., F. A. Rainey, and E. Stackebrandt.** 1995. Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl. Environ. Microbiol. **61:**2798–2801.
11. **Felsenstein, J.** 1989. PHYLIP-phylogeny inference package (version 3.2). Cladistics **5:**164–166.
12. **Fenchel, T.** 1993. Methanogenesis in marine shallow sediments: the quantitative role of anaerobic protozoa with endosymbiotic methanogenic bacteria. Ophelia **37:**67–82.
13. **Ferris, M. J., G. Muyzer, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. **62:**340–346.
14. **Ferris, M. J., A. L. Ruff-Roberts, E. D. Kopczynski, M. M. Bateson, and D. M. Ward.** 1996. Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring mat habitat. Appl. Environ. Microbiol. **62:**1045–1050.
15. **Ferris, M. J., and D. M. Ward.** 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. **63:**1375–1381.
16. **Fetzer, S., F. Bak, and R. Conrad.** 1993. Sensitivity of methanogenic bacteria from paddy soil to oxygen and desiccation. FEMS Microbiol. Ecol. **12:**107–115.
17. **Frenzel, P., and U. Bosse.** 1996. Methyl fluoride, an inhibitor of methane oxidation and methane production. FEMS Microbiol. Ecol. **21:**25–36.
18. **Frenzel, P., F. Rothfuss, and R. Conrad.** 1992. Oxygen profiles and methane turnover in a flooded rice microcosm. Biol. Fertil. Soils **14:**84–89.
19. **Großkopf, R., S. Stubner, and W. Liesack.** Unpublished data.
20. **Hahn, D., R. I. Amann, W. Ludwig, A. D. L. Akkermans, and K.-H. Schleifer.** 1992. Detection of microorganisms in soil after in situ hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J. Gen. Microbiol. **138:**879–887.
21. **Harmsen, H. J. M., H. M. P. Kengen, A. D. L. Akkermans, A. J. M. Stams, and W. M. de Vos.** 1996. Detection and localization of syntrophic propionate-oxidizing bacteria in granular sludge by in situ hybridization using 16S rRNA-based oligonucleotide probes. Appl. Environ. Microbiol. **62:**1656–1663.
22. **Huber, R., S. Burggraf, T. Mayer, S. M. Barns, and K. O. Stetter.** 1995. Isolation of a hyperthermophilic archaeum predicted by in situ analysis. Nature **376:**57–58.
23. **Janssen, P. H., A. Schuhmann, E. Mörschel, and F. A. Rainey.** 1997. Novel anaerobic ultramicrobacteria belonging to the *Verrucomicrobiales* lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. Appl. Environ. Microbiol. **63:**1382–1388.
24. **Janssen, P. H., and T. Wind.** Unpublished data.
25. **Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder.** 1990. Acetate threshold values and acetate activating enzymes in methanogenic bacteria. FEMS Microbiol. Ecol. **73:**339–344.
26. **Jetten, M. S. M., A. J. M. Stams, and A. J. B. Zehnder.** 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. FEMS Microbiol. Rev. **88:**181–198.
27. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York, N.Y.
28. **Kamagata, Y., and E. Mikami.** 1991. Isolation and characterization of a novel thermophilic *Methanosaeta* strain. Int. J. Syst. Bacteriol. **41:**191–196.
29. **Kopczynski, E. D., M. M. Bateson, and D. M. Ward.** 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. Appl. Environ. Microbiol. **60:**746–748.
30. **Krumböck, M., and R. Conrad.** 1991. Metabolism of position-labelled glucose in anoxic methanogenic paddy soil and lake sediment. FEMS Microbiol. Ecol. **85:**247–256.
31. **Kudo, Y., T. Nakajima, T. Miyaki, and H. Oyaizu.** 1997. Methanogen flora of paddy soils in Japan. FEMS Microbiol. Ecol. **22:**39–48.
32. **Liesack, W., P. H. Janssen, F. A. Rainey, N. L. Ward-Rainey, and E. Stackebrandt.** 1997. Microbial diversity in soil: the need for a combined approach using molecular and cultivation techniques, p. 375–439. *In* J. D. van Elsas, J. T. Trevors, and E. M. Wellington (ed.), Modern soil microbiology. Marcel Dekker, Inc., New York, N.Y.
33. **Liesack, W., and E. Stackebrandt.** 1992. Occurrence of novel groups of the domain *Bacteria* as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. **174:**5072–5078.
34. **Liesack, W., H. Weiland, and E. Stackebrandt.** 1991. Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. **21:**191–198.
35. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1996. The ribosomal database project (RDP). Nucleic Acids Res. **24:**82–85.
36. **Marteinsson, V. T., L. Watrin, D. Prieur, J. C. Caprais, G. Raguénès, and G. Erauso.** 1995. Phenotypic characterization, DNA similarities, and protein profiles of twenty sulfur-metabolizing hyperthermophilic anaerobic archaea isolated from hydrothermal vents in the southwestern Pacific Ocean. Int. J. Syst. Bacteriol. **4:**623–632.
37. **Min, H., and S. H. Zinder.** 1989. Kinetics of acetate utilization by two thermophilic acetotrophic methanogens: *Methanosarcina* sp. strain CALS-1 and *Methanothrix* sp. strain CALS-1. Appl. Environ. Microbiol. **55:**488–491.
38. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1992. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
39. **Neue, H.-U.** 1993. Methane emission from rice fields. BioScience **43:**466–473.
40. **Ney, U., A. J. L. Macario, E. Conway de Macario, A. Aivasidis, S. M. Schoberth, and H. Sahm.** 1990. Quantitative microbiological analysis of bacterial community shifts in a high-rate anaerobic bioreactor treating sulfite-evaporator condensate. Appl. Environ. Microbiol. **56:**2389–2398.
41. **Nold, S. C., E. D. Kopczynski, and D. M. Ward.** 1996. Cultivation of aerobic chemoorganotrophic proteobacteria and gram-positive bacteria from a hot spring microbial mat. Appl. Environ. Microbiol. **62:**3917–3921.
42. **Ogram, A., G. S. Sayler, and T. Barkay.** 1987. The extraction and purification of microbial DNA from sediments. J. Microbiol. Methods **7:**57–66.
43. **Pfennig, N.** 1978. *Rhodocyclus purpureus* gen. nov. and sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family Rhodospirillaceae. J. Syst. Bacteriol. **28:**283–288.
44. **Pfennig, N., and S. Wagener.** 1986. An improved method of preparing wet mounts for photomicrographs of microorganisms. J. Microbiol. Methods **4:**303–306.
45. **Rainey, F. A., N. Ward, L. I. Sly, and E. Stackebrandt.** 1994. Dependence of the taxon composition of clone libraries of PCR amplified, naturally occurring 16S rDNA on the primer pair and the cloning system used, p. 796–797. *In* U. B. Göbel and E. Stackebrandt (ed.), Methodological developments in nucleic acid diagnosis. Birkhäuser, Basel, Switzerland.
46. **Rajagopal, B. S., N. Belay, and L. Daniels.** 1988. Isolation and characterization of methanogenic bacteria from rice paddies. FEMS Microbiol. Ecol. **53:**153–158.
47. **Reichardt, W., G. Mascarina, B. Padre, and J. Doll.** 1997. Microbial communities of continuously cropped, irrigated rice fields. Appl. Environ. Microbiol. **63:**233–238.
48. **Reysenbach, A.-L., L. J. Giver, G. S. Wickham, and N. R. Pace.** 1992. Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. **58:**3417–3418.
49. **Rodriguez-Tomé, P., P. J. Stoehr, G. N. Cameron, and T. P. Flores.** 1996. The European Bioinformatics Institute (EBI) databases. Nucleic Acids Res. **24:**6–12.
50. **Rosencrantz, D., F. A. Rainey, and P. H. Janssen.** Unpublished data.
51. **Rothfuss, F., and R. Conrad.** 1993. Vertical profiles of CH_4 concentrations, dissolved substrates and processes involved in CH_4 production in a flooded Italian rice soil. Biogeochemistry **18:**137–152.
52. **Rotthauwe, J.-H., K.-P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704–4712.
53. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
54. **Santegoeds, C. M., S. C. Nold, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis used to monitor the enrichment culture of aerobic chemoorganotrophic bacteria from a hot spring cyanobacterial mat. Appl. Environ. Microbiol. **62:**3922–3928.
55. **Schütz, H., W. Seiler, and R. Conrad.** 1989. Processes involved in formation and emission of methane in rice paddies. Biogeochemistry **7:**33–53.
56. **Smalla, K., N. Cresswell, L. C. Mendonca-Hagler, A. Wolters, and J. D. van Elsas.** 1993. Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. J. Appl. Bacteriol. **74:**78–85.
57. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **4:**846–849.
58. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes in bacterial systematics, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd., Chichester, England.
59. **Strunk, O., and W. Ludwig.** 1996. ARB: a software environment for sequence data. Technische Universität München, Munich, Germany.
60. **Suzuki, M. T., and S. J. Giovannoni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
61. **Tschech, A., and N. Pfennig.** 1984. Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. Arch. Microbiol. **137:**163–167.

62. **Van Bruggen, J. J. A., C. K. Stumm, and G. D. Vogels.** 1983. Symbiosis of methanogenic bacteria and sapropelic protozoa. Arch. Microbiol. **136:**89–95.
63. **Van de Peer, Y., S. Chapelle, and R. De Wachter.** 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res. **24:** 3381–3391.
64. **Van de Peer, Y., S. Nicolaï, P. De Rijk, and R. De Wachter.** 1996. Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res. **24:** 86–91.
65. **Wang, G. C.-Y., and Y. Wang.** 1996. The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology **142:**1107–1114.
66. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature **345:**63–65.
67. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:**463–464.
68. **Weller, R., M. M. Bateson, B. K. Heimbuch, E. D. Kopczynski, and D. M. Ward.** 1992. Uncultivated cyanobacteria, *Chloroflexus*-like inhabitants, and spirochete-like inhabitants of a hot spring microbial mat. Appl. Environ. Microbiol. **58:**3964–3969.
69. **Westermann, P., B. K. Ahring, and R. A. Mah.** 1989. Threshold acetate concentrations for acetate catabolism by aceticlastic methanogenic bacteria. Appl. Environ. Microbiol. **55:**514–515.
70. **Widdel, F., G. W. Kohring, and F. Mayer.** 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. Arch. Microbiol. **134:**286–294.
71. **Zarda, B., D. Hahn, A. Chatzinotas, W. Schönhuber, A. Neef, R. I. Amann, and J. Zeyer.** 1997. Analysis of bacterial community structure in bulk soil by in situ hybridization. Arch. Microbiol. **168:**185–192.
72. **Zinder, S. H., and T. Anguish.** 1991. Carbon monoxide, hydrogen, and formate metabolism during methanogenesis from acetate by thermophilic *Methanosarcina* and *Methanothrix* strains. Appl. Environ. Microbiol. **58:**3323–3329.