Microbial Ecology

Archaeal Community Structure and Pathway of Methane Formation on Rice Roots

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Received: 7 April 2003 / Accepted: 16 May 2003 / Online publication: 5 November 2003

Abstract

The community structure of methanogenic Archaea on anoxically incubated rice roots was investigated by amplification, sequencing, and phylogenetic analysis of 16S rRNA and methyl-coenzyme M reductase (*mcrA*) genes. Both genes demonstrated the presence of Methanomicrobiaceae, Methanobacteriaceae, Methanosarcinaceae, Methanosaetaceae, and Rice cluster I, an uncultured methanogenic lineage. The pathway of CH_4 formation was determined from the ^{13}C-isotopic signatures of the produced CH_4, CO_2 and acetate. Conditions and duration of incubation clearly affected the methanogenic community structure and the pathway of CH_4 formation. Methane was initially produced from reduction of CO_2 exclusively, resulting in accumulation of millimolar concentrations of acetate. Simultaneously, the relative abundance of the acetoclastic methanogens (Methanosarcinaceae, Methanosaetaceae), as determined by T-RFLP analysis of 16S rRNA genes, was low during the initial phase of CH_4 production. Later on, however, acetate was converted to CH_4 so that about 40% of the produced CH_4 originated from acetate. Most striking was the observed relative increase of a population of *Methanosarcina* spp. (but not of *Methanosaeta* spp.) briefly before acetate concentrations started to decrease. Both acetoclastic methanogenesis and *Methanosarcina* populations were suppressed by high phosphate concentrations, as observed under application of different buffer systems. Our results demonstrate the parallel change of microbial community structure and function in a complex environment, i.e., the increase of acetoclastic *Methanosarcina* spp. when high acetate concentrations become available.

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Introduction

Flooded rice fields are an important source of the greenhouse gas CH_4, contributing about 13% to the total CH_4 budget [24]. The microbiota on rice roots may contribute a significant portion, since up to 50% of the emitted CH_4 originates from plant photosynthates that are excreted from the roots [40], and about 3–6% of the photosynthetically fixed CO_2 is converted to CH_4 and emitted [9]. The root-associated methanogenesis possibly affects not only the amount but also the isotopic signature of the emitted CH_4 [8, 21].

Rice field soil and rice roots have also become a model system for the microbial ecology of wetlands [4]. The methanogenic archaeal biota on rice roots seems to be different from that in the anoxic soil [17, 18]. Likewise, the pathway of CH_4 production and its temporal dynamics was found to be different in root and soil [5, 8, 23]. Acetoclastic methanogenic activity, in particular, was found to increase with time in root incubations and to be subject to inhibition by phosphate [6, 7]. However, data on the methanogenic archaeal community structure on rice roots are scarce. In particular, the contribution of the different major methanogenic groups to CH_4 formation and their population dynamics upon onset of methanogenesis are unknown. Furthermore, analysis of the methanogenic community structure on rice roots has been restricted to analysis of 16S rRNA genes. Functional marker genes, such as the *mcrA* gene encoding the methyl-coenzyme M reductase, the key enzyme of methanogenesis [12], have not been studied. This specific functional marker has been applied for the selective investigation of methanogenic communities in different anoxic habitats [2, 11, 16, 19, 25, 26, 30, 31, 33].

In this study, clone libraries targeting both 16S rRNA and *mcrA* genes of methanogens on anoxically incubated excised rice roots were constructed to compare the populations to those detected previously in anoxic bulk soil [3, 10, 18]. Furthermore, we investigated temporal

changes in the methanogenic archaeal community in correlation to the predominant pathways of CH_4 formation. Such analyses are necessary to improve our current knowledge of factors controlling the presence and activity of methanogenic populations on the surface of rice roots.

Materials and Methods

Incubation Experiments and Chemical Analyses. The growth of rice plants and the preparation of rice roots have already been described in detail [5]. Briefly, rice plants (*Oryza sativa*, var. Roma, type japonica) were grown in the greenhouse. After 12–14 weeks, plants were removed from the soil, and the roots were washed first with tap water, then with sterile demineralized water that was bubbled with N_2. The roots were then cut off with a razor blade, and aliquots (10 g) were incubated in moist state at room temperature (about 25°C) under a N_2 atmosphere, using stoppered glass bottles (150 mL volume) [5]. Alternatively, the roots were incubated in 50 mL anoxic sterile carbonate or phosphate buffer. The carbonate buffer consisted of demineralized water plus 5 g marble granules (0.5–2.0 mm size, consisting of $CaCO_3$; Merck, Darmstadt, Germany), the phosphate buffer of 50 mM KH_2PO_4, 17 mM NaCl and 0.2 mM $MgCl_2$ (pH 7.0) [6].

Gas samples (0.25–1.0 mL) and liquid samples (0.5 mL) were analyzed by gas chromatography and HPLC as described [5]. Stable isotope analysis of $^{13}C/^{12}C$ in gas and liquid samples was performed using a gas chromatograph combustion isotope ratio mass spectrometry (GCC-IRMS) system purchased from Finnigan (Thermoquest, Bremen, Germany) [8]. The fractions of CH_4 produced from reduction of CO_2 and acetate cleavage were calculated from the ^{13}C data of CH_4, CO_2 and acetate using the mass balance equations described by Conrad et al. [8], and assuming fractionation for the conversion of acetate to CH_4 and of CO_2 to CH_4 by $-21‰$ and $-70‰$, respectively.

Molecular Analyses. Root materials were taken before incubation as control and after incubation in presence of buffer or without additional buffer. The roots were cut in 1-mm pieces, and 0.5 g of roots was transferred to the 2-mL tubes containing DNA extraction buffer. The tubes were shaken vigorously to detach the microorganisms from rice roots before DNA extraction. DNA extraction from root material, PCR amplification of partial 16S rRNA and *mcrA* genes, cloning of amplicons, and sequence analysis were performed as published before [3, 26]. Phylogenetic trees were calculated from aligned 16S rRNA and deduced McrA amino acid sequences with the ARB software package using neighbor-joining, FITCH, maximum-parsimony, and maximum-likelihood methods as described earlier [15, 26]. Tree topologies reconstructed with different algorithms were compared and, in case of inconsistent branching orders, adjusted to a consensus tree by introducing multifurcations. T-RFLP analyses was done with 5′,6-carboxyfluorescein (FAM)-labeled 16S rDNA [3] and mcrA amplicons [26] on an ABI 373 DNA sequencer in GeneScan mode, and relative signal intensities of T-RFs were inferred by peak area integration.

Accession Numbers. The sequences generated in this study were deposited in the GenBank database under the following accession numbers: *mcrA* clones, MRR-mcr1 to MRR-mcr75 under AY125601 to AY125675; 16S rDNA clones, MRR1 to MRR49 under AY125676 to AY125724.

Results

Methane Production in Rice Root Incubations. The production of CH_4 was highest in rice roots suspended in carbonate buffer, followed by rice roots incubated in moist state (without additional buffer), and was lowest in rice roots suspended in phosphate buffer (Fig. 1A). Acetate accumulated in the rice root incubations (only measured in the buffered suspensions) to millimolar concentrations (Fig. 1B). Eventually, acetate decreased again, but not in the phosphate-buffered root incubations (Fig. 1B) or only after a longer delay (data not shown). These data confirm earlier observations [5–7, 23].

The relative contribution of H_2/CO_2-dependent and acetate-dependent methanogenesis to CH_4 production was determined by following the $\delta^{13}C$ in CO_2, CH_4 and acetate. The experimental procedure and a detailed data set obtained from anoxic root incubations (carbonate buffer) has already been published [8]. Similar data sets were obtained in the present study, both for incubations in carbonate and phosphate buffer. These experiments showed that the contribution of acetate-dependent methanogenesis to CH_4 production was ~40% and was reduced to ~20% when the roots were incubated in phosphate buffer (Table 1).

Archaeal 16S rRNA and Methyl-Coenzyme M Reductase Genes Retrieved from Rice Roots. DNA was extracted from four different rice root incubations, and each extract was used to generate clone libraries of amplified partial 16S rRNA and *mcrA* genes. The rice roots were from (i) 1 day incubation without buffer, (ii) 1 day incubation in phosphate buffer, (iii) 20 days incubation in carbonate buffer, and (iv) 20 days incubation in phosphate buffer. The libraries were screened randomly by T-RFLP analysis to recover clones representing different T-RFs. A total of 70 and 80 clones were screened

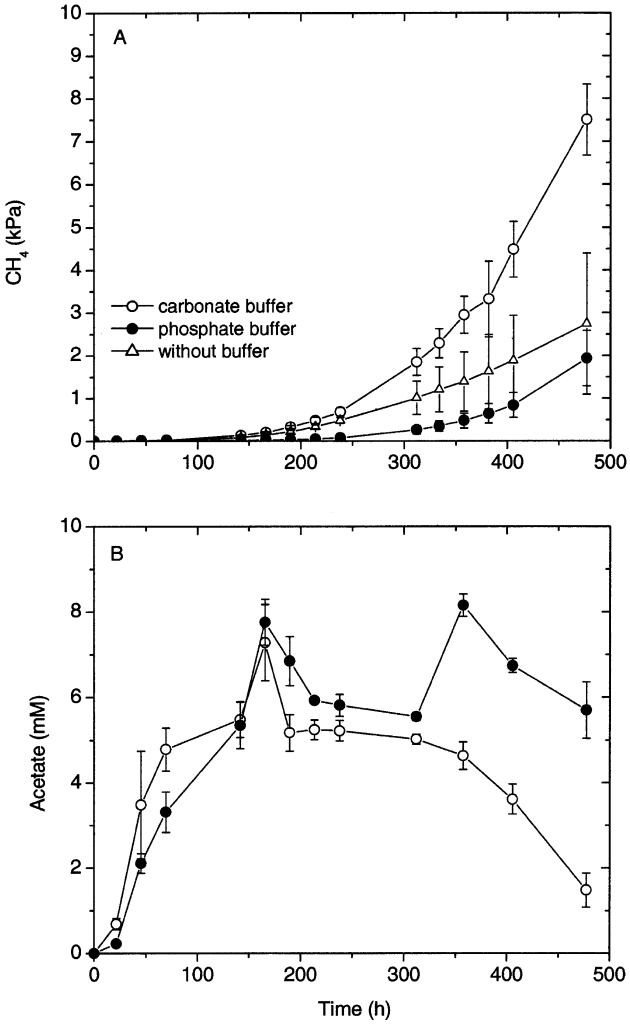

Figure 1. Time course of methane partial pressure (A) and acetate concentration (B) in anoxic incubations of excised rice roots (mean ± SD; $n = 3$).

from the 16S rRNA and *mcrA* gene clone libraries, respectively. Gene fragments from 49 and 75 of these clones, respectively, were sequenced and phylogenetic trees were calculated, both for the 16S rRNA genes (Fig. 2) and deduced McrA amino acid sequences (Fig. 3). The remaining 21 and 5 clones of the 16S rRNA and *mcrA* gene clone libraries represented highly redundant T-RF and were not sequenced, since these T-RF were already sufficiently covered by other clones.

The following methanogenic groups were detected in the clone libraries of both genes, i.e., methanogens within the Methanosarcinaceae, the Methanosaetaceae, the Methanomicrobiaceae, and the Methanobacteriaceae. The detection of methanomicrobial *mcrA* genes on the rice roots was a surprise, since we had failed to detect them in anoxic bulk soil earlier [26]. Furthermore, Rice cluster I [18] was detected by both 16S rRNA and *mcrA* genes, showing also the presence of this uncultured

Table 1. Effect of carbonate and phosphate buffer on the fractions of CH_4 produced from acetate, as determined from $\delta^{13}C$ of CH_4, CO_2, and acetate [8]

Experiment	*Carbonate buffer*	*Phosphate buffer*
1	0.47	0.26
2	0.35	0.12
3	0.45	0.25
4	0.41	0.27
Average ± SD	0.42 ± 0.05	0.22 ± 0.07

[a]Fractions were obtained by integration over the incubation period until depletion of acetate, using root material from different rice microcosms.

methanogenic lineage [26], which is assumed to produce CH_4 from H_2/CO_2 [13]. The as yet unidentified cluster of rice field soil *mcrA* genes (e.g., MRR-mcr24) related to the *Methanosarcinales* retrieved with low frequency from anoxic bulk soil [26] was also detected on rice roots in this study, which might prove valuable in future attempts to clarify the systematic affiliation of these sequences.

In addition to these different methanogenic lineages, the rice roots also contained 16S rRNA gene sequences related to uncultivated archaeal clusters that are probably not methanogenic (Fig. 2). These were the Thermoplasmales-related Rice cluster III (Group E3 Euryarchaeota [10]), Rice cluster V [18], and the Rice clusters IV and VI (Group C2 freshwater Crenarchaeota and Group C1b terrestrial Crenarchaeota, respectively [10]).

Archaeal Population Dynamics in Rice Root Incubations. Time-dependent shifts within the methanogen populations were followed by 16S rDNA-based T-RFLP analysis, a tool shown to give a reliable and quantitative measure of 16S rRNA gene abundance in archaeal communities [29]. The assignment of different characteristic 16S rDNA and *mcrA*-based T-RF to distinct lineages of Archaea and methanogens is shown in Tables 2 and 3. All peaks detected in T-RFLP analysis of 16S rRNA genes from rice roots incubated in moist state without additional buffer for 1 day (Fig. 4) were assignable to defined archaeal lineages (Table 2), notably those at 83 bp (Methanomicrobiaceae and Rice cluster IV), 91 bp (mainly Methanobacteriaceae), 185 bp (mainly Methanosarcinaceae), 284 bp (Methanosaetaceae), and 392 bp (Methanomicrobiaceae and Rice cluster I). The affiliation of T-RF on roots is thus similar to that in soil [3, 27, 34], with the exception that the 392-bp T-RF on roots, besides Rice cluster I, also represents members of the Methanomicrobiaceae. We did not find any 16S rDNA clones with terminal restriction sites that were not detected in direct T-RFLP analyses of root DNA.

Also the T-RF of *mcrA* genes retrieved from rice roots (Fig. 4) could all be assigned to distinct sequence clusters in the mcrA phylogeny (Table 3), notably those at 238 bp (Rice cluster I), 394 bp (Methanosarcinaceae), and 403, 470, and 503 bp (Methanobacteriaceae). Also,

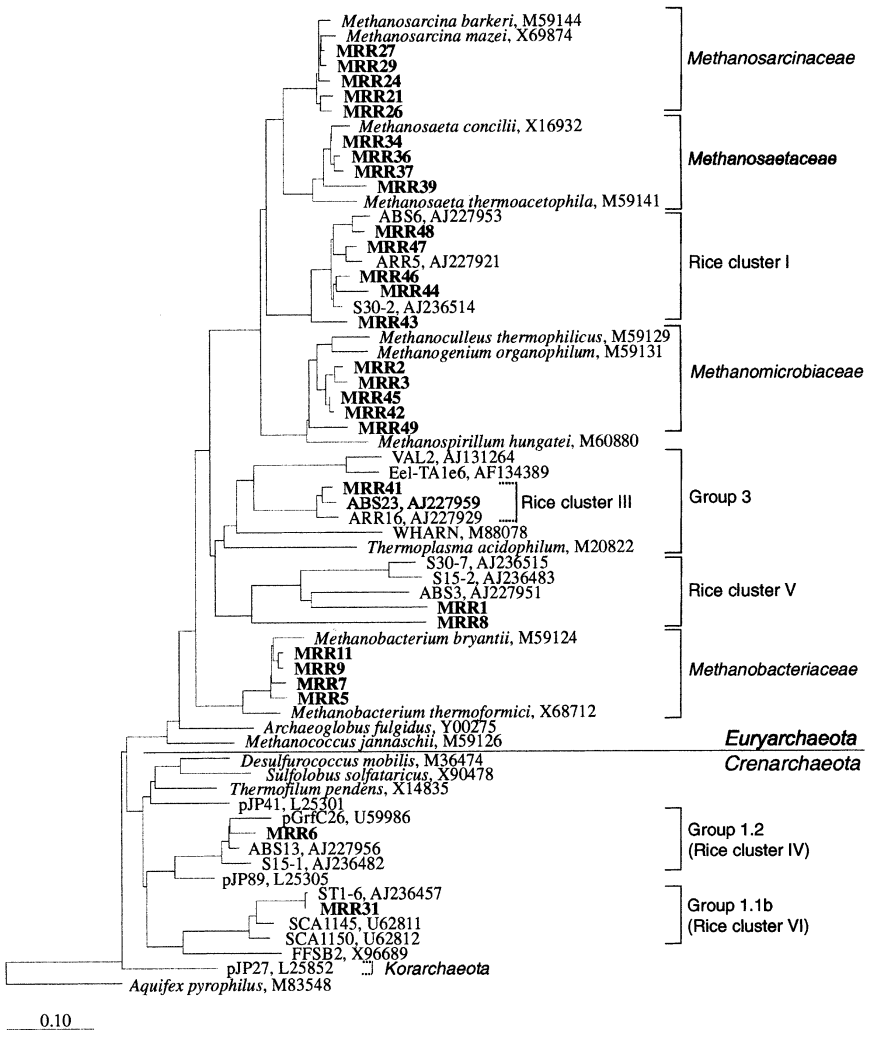

Figure 2. Phylogenetic dendrogram showing the relation of selected archaeal 16S rDNA sequences retrieved from anoxically incubated excised rice roots in this study (MRR clones, in bold) to those of cultivated Archaea and to other environmental clone sequences. The tree was reconstructed from partial (~800 bp) 16S rRNA genes using the maximum likelihood algorithm; the bar represents 10% sequence divergence. Nomenclature of uncultured archaeal lineages according to [3, 10, 18].

the 427 bp T-RF represented members of the Methanosarcinaceae. Although it was not observed for any *mcrA* clone in this study, it has previously been described for anoxic bulk soil [26]. The T-RFLP analysis of rice root DNA did not show any T-RF characteristic for the *mcrA* genes of the Methanomicrobiaceae (262 or 275 bp) and Methanosaetaceae (147 bp). Also, other *mcrA* T-RF predicted from the clone library were not detected in T-RFLP analysis of rice root DNA (e.g., 241, 347, 406, 409 bp, Table 3).

Root DNA was extracted from successive time points in parallel to the CH_4 production measurements shown in Fig. 1 and analyzed by 16S rDNA- and mcrA-targeted T-RFLP fingerprinting. Because of the highly degenerate *mcrA*-targeted primer set, this assay could not be utilized for quantitative community analyses [29]. The relative frequency of characteristic 16S rDNA T-RF of different groups of Archaea changed significantly with time and condition of the root incubations (Fig. 5). Initially, all incubations exhibited only a low frequency (up to 11%) of the T-RF characteristic for acetoclastic methanogens (Methanosarcinaceae and Methanosaetaceae), whereas the frequency of T-RF characteristic for H_2/CO_2-dependent methanogens (Methanomicrobiaceae and Rice cluster I, in particular) was high (up to 42 and 35%, respectively). With progress of incubation, the acetoclastic Methanosarcinaceae (but not Methanosaetaceae) became more frequent (up to 41% on day 6 in carbonate buffer). This increase was strongly delayed in phosphate-buffered incubations (Fig. 5); here the abundance of the methanosarcinal T-RF only increased strongly after 20 days. The relative increase in abundance of Methanosarcinaceae T-RF consistently occurred just before the acetate concentrations in the incubations started to decrease (Fig. 1), indicating increased acetate consumption by a growing population of acetoclastic methanogens. After 2 days, the T-RF of 392 bp (representing Rice cluster I and Methanomicrobiaceae) exhibited a relatively higher frequency in the carbonate-buffered than in the other root incubations, which in turn exhibited a relatively higher frequency of the T-RF at 83 bp (characteristic for Methanomicrobiaceae and crenarchaeotal Rice

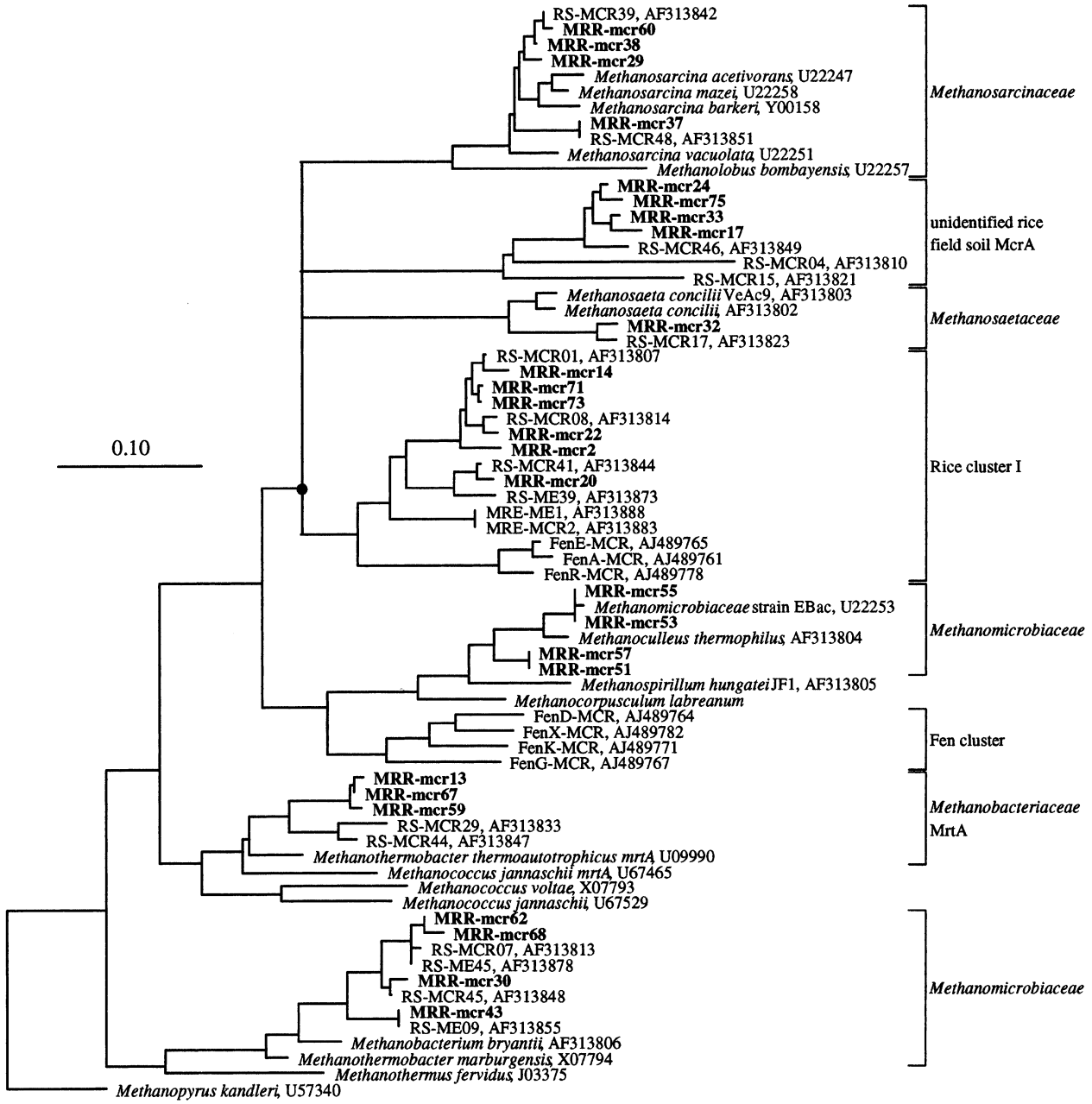

Figure 3. Phylogenetic dendrogram showing the relation of selected methanogen McrA and MrtA sequences retrieved from anoxically incubated excised rice roots in this study (MRR-mcr clones, in bold) to those of cultivated methanogens and to other environmental clone sequences. The tree was reconstructed from deduced amino acid (~150 positions) sequences using the FITCH algorithm. The full circle indicates a manually introduced multifurcation where inconsistent branching topologies for different sequence clusters were observed with different treeing algorithms; the bar represents 10% sequence divergence. Nomenclature of uncultured methanogenic lineages according to [16, 26].

cluster IV). The functional consequences of these population shifts, however, remain to be elucidated.

Discussion

Methanogenesis becomes the dominant pathway for anaerobic degradation of organic matter when environmental concentrations of inorganic electron acceptors such as nitrate, sulfate, or ferric iron are low. Otherwise, nitrate reducers, sulfate reducers, or iron reducers compete successfully for the methanogenic substrates H_2 and acetate and thus suppress methanogenesis. The dynamic interplay between inorganic electron acceptors and methanogenic pathways has been shown in several veg-

Table 2. Affiliation of T-RFs of the different archaeal 16S rDNA clones obtained from methanogenic rice roots to distinct phylogenetic lineages

T-RF (bp)	*Phylogenetic lineage*	*No. of clones*
74	Rice cluster V[a]	1
83	Methanomicrobiaceae	2
83	Rice cluster IV[a] (Group C2[b])	1
91	Methanobacteriaceae	8
91	Rice cluster V[a]	1
185	Methanosarcinaceae	19
185	Rice cluster VI[c] (Group C1b[b])	1
284	Methanosaetaceae	7
380	Rice cluster III (Group E3[b])	1
392	Methanomicrobiaceae	3
392	Rice cluster I[a]	5

[a]According to [18];
[b]According to [10];
[c]According to [3].

Table 3. Affiliation of T-RFs of the different methanogen *mcrA* and *mrtA* clones obtained from methanogenic rice roots to distinct phylogenetic lineages

T-RF (bp)	*Phylogenetic lineage*	*No. of clones*
147	Methanosaetaceae *mcrA*	1
238	Ricy cluster I *mcrA*	10
241	unidentified rice field soil *mcrA*	2
262	Methanomicrobiaceae *mcrA*	3
275	Methanomicrobiaceae *mcrA*	5
347	Methanosarcinaceae *mcrA*	1
394	Methanosarcinaceae *mcrA*	4
403	Methanobacteriaceae *mcrA*	2
406	Methanobacteriaceae *mcrA*	14
409	Unidentified rice field soil *mcrA*	3
470	Methanobacteriaceae *mrtA*	6
502	unidentified rice field soil *mcrA*	1
503	Methanobacteriaceae *mcrA*	23

etated methanogenic environments, e.g., rice fields [22, 37] or peatlands [1, 36]. Recently, it was shown that nitrate and sulfate affect not only the activity but also the community structure of methanogenic archaea using rice roots as model system [35]. However, little is known about the factors that influence the structure within the methanogenic community, i.e., the hydrogenotrophic and acetoclastic methanogenic archaea.

Our results establish a link between functionality and structure of a methanogenic population, i.e, the accumulation of acetate in rice root incubations leading to an increased relative abundance of the acetoclastic Methanosarcinaceae and a subsequently enhanced consumption of acetate. The pathway of CH_4 formation on the roots consequently shifted to a higher contribution of acetate as methanogenic precursor. The establishment of an abundant *Methanosarcina* population was obviously crucial for this pathway of CH_4 formation. High phosphate concentrations, which delayed the development of methanosarcinal populations, also delayed acetoclastic methanogenesis. Inhibition of acetoclastic methanogenesis by high phosphate has been observed before [6, 7], but it was unknown whether the effect was only due to inhibition of enzyme activity or also to inhibition of

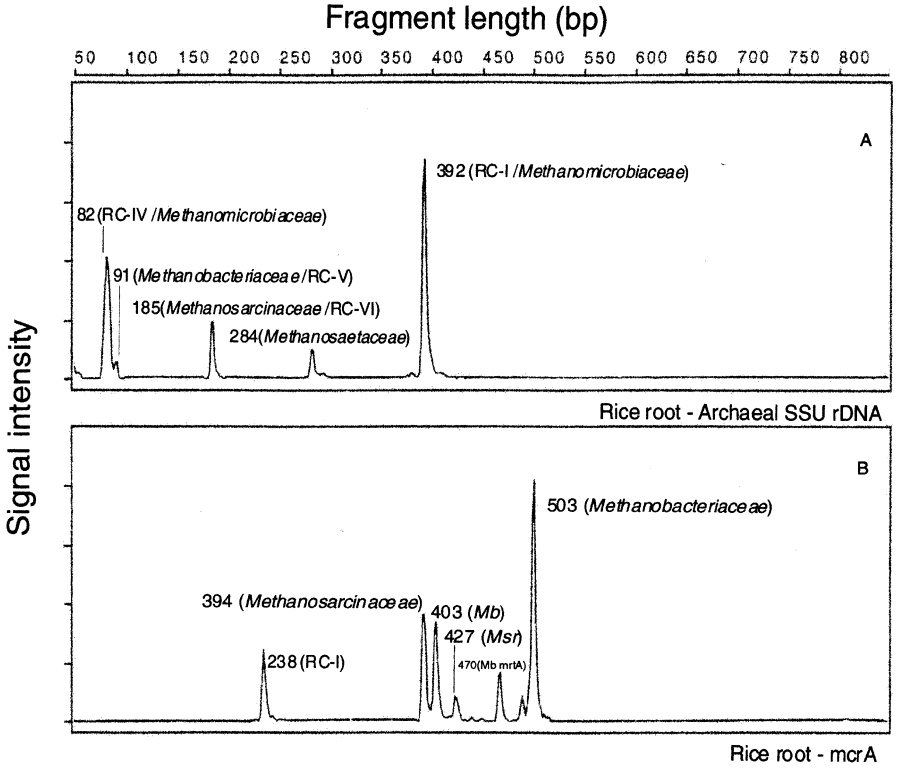

Figure 4. T-RFLP analysis of the methanogenic archaeal community on anoxically incubated excised rice roots, which were incubated in moist state for one day (without additional buffer). Electropherograms were generated targeting archaeal 16S rRNA (A) and methanogen *mcrA/mrtA* (B) genes. Characteristic T-RFs of defined lineages are indicated according to their length (bp). Mb, Methanobacteriaceae; Msr, Methanosarcinaceae.

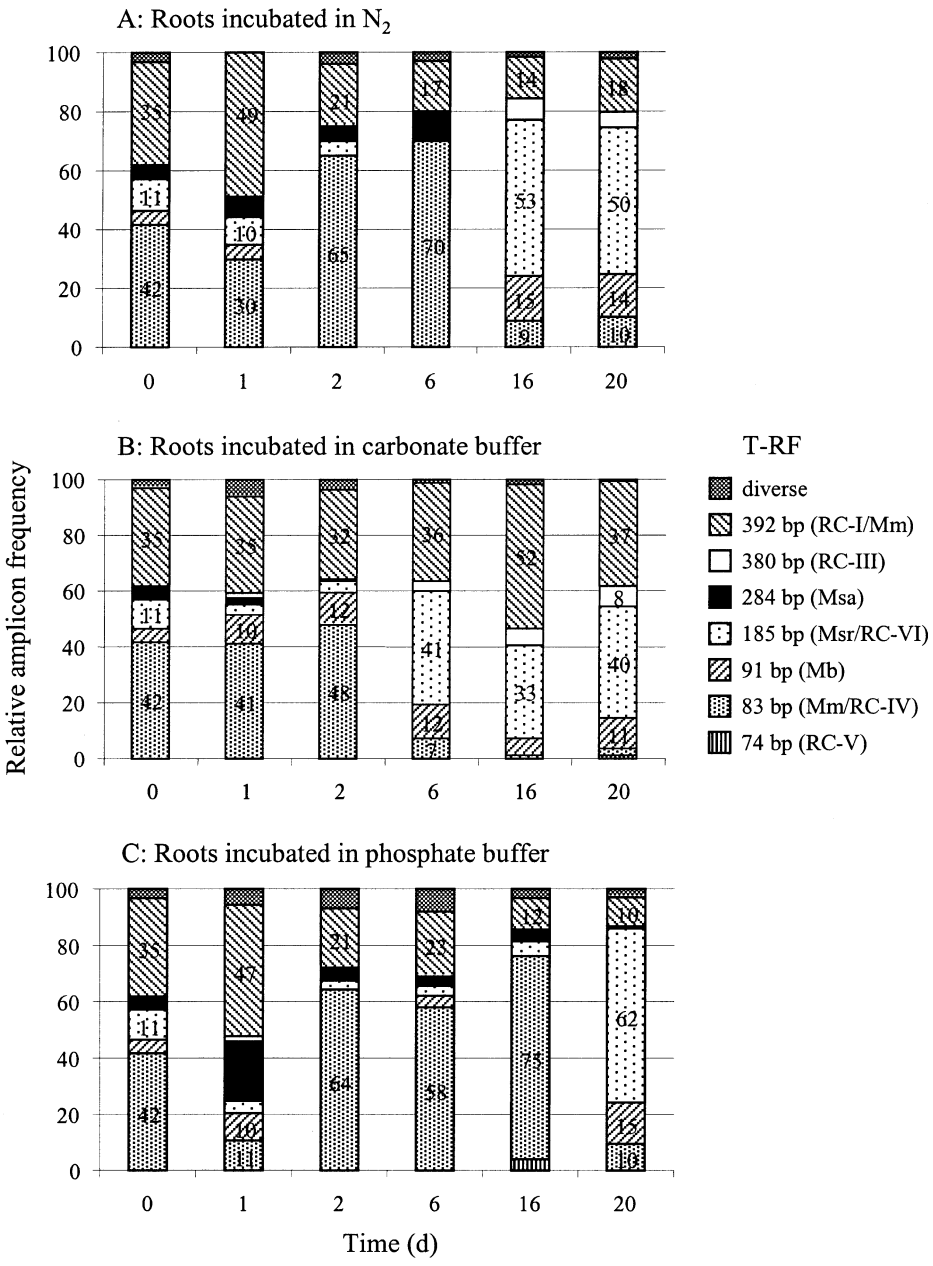

Figure 5. Time course of archaeal population dynamics on anoxically incubated excised rice roots as determined by 16S rDNA targeted T-RFLP analysis. Shown is the contribution percentage of individual T-RF to the total integrated fluorescence; percentages above 8% are given. Minor T-RF have been combined as "diverse". Mm, Methanomicrobiaceae; Msa, Methanosaetaceae; Msr, Methanosarcinaceae; Mb, Methanobacteriaceae; RC, Rice cluster.

growth. Obviously, proliferation of the acetoclastic *Methanosarcina* root population was negatively affected by high phosphate levels.

There are only few studies in which changes in methanogenic community function were successfully correlated to changes in community structure, and rarely were the observed community changes as pronounced as in the present study. In rice field soil incubations, for example, the relative abundance of the Methanosarcinaceae change only to small extent during the course of anoxic incubation [27], and an increased acetoclastic methanogenesis is mainly accompanied by increasing rRNA expression levels of existing *Methanosarcina* and *Methanosaeta* populations [28]. Brief exposure of methanogenic rice field soil to 50°C results in a pronounced inhibition of acetoclastic methanogenesis but only in a relatively small change in the acetoclastic methanogenic populations [41]. Prolonged incubation at 50°C, on the other hand, results in a drastic change in the methanogenic microbial community, resulting in the dominance of Rice cluster I and the production of CH_4 from H_2/CO_2 exclusively [13].

Interestingly, we observed a population shift for the acetoclastic Methanosarcinaceae, but not the Methanosaetaceae. In fact, Methanosaetaceae were hardly detectable at all on the rice roots, in accordance with earlier studies [18]. We assume that Methanosarcinaceae predominated, since they can grow faster than Methano-

saetaceae as long as acetate is available at relatively high concentrations. *Methanosarcina* spp. have a higher threshold and K_m value for acetate compared to *Methanosaeta* spp., due to the different mechanisms of acetate activation, i.e., by acetate kinase/phosphotransacetylase versus acetyl-CoA synthetase, respectively [20]. Hence, it is plausible to find *Methanosaeta* spp. mainly in anoxic bulk soil, especially at low acetate concentrations [14], but not on rice roots with high acetate concentrations. Acetoclastic methanogens on decomposing rice straw, which also shows relatively high acetate concentrations, also consist mainly of *Methanosarcina* spp. [40].

In addition to the acetoclastic methanogens, there may also have been a change in the hydrogenotrophic methanogenic populations. In particular, we observed a relatively higher frequency of the 392 bp T-RF (characteristic for Rice cluster I and Methanomicrobiaceae) in the carbonate-buffered root incubations, whereas the other root incubations exhibited a relatively higher frequency of the 83 bp T-RF (characteristic for Methanomicrobiaceae and the crenarchaeotal Rice cluster IV). Since both T-RF were characteristic for hydrogenotrophic methanogens, it is unclear what the functional meaning of these different community structures is.

The 16S rDNA sequences retrieved from rice roots were similar to those from rice field soil sampled in Vercelli (Italy) [3, 27], Gapan (The Philippines), and Shenyang (China) [34]. Root and soil populations also showed similar characteristic T-RF for distinct phylogenetic groups. The only exception was the 392 bp T-RF, which in soil almost exclusively represents Rice cluster I methanogens, but on roots equally represents members of the Methanomicrobiaceae. This is likely to be explained simply by a much higher frequency of these methanomicrobial methanogens in rice root incubations (~10% of both 16S rDNA and *mcrA* clones in this study) compared to anoxic bulk soil (between 1 and 5% of 16S rDNA clones [3, 27]). The same assignment of the 392 bp T-RF to members of the Methanomicrobiaceae has previously been described in the methanogenic sediments of Lake Kinneret, where rice cluster I methanogens apparently do not exist [32]. Hence, it is mandatory to create a 16S rDNA sequence library for each environment, in which methanogenic community structure is to be characterized by T-RFLP analysis, in order to correctly assign T-RF to phylogenetic groups.

Most of the *mcrA* sequences retrieved from rice roots were similar to those from rice field soil [26]. In contrast to soil, however, we also detected members of the Methanomicrobiaceae on the rice roots. Recently, *mcrA* genes related to the Methanomicrobiales were retrieved from a Finnish fen [16] and a landfill [30] with different primer systems. Because of the degenerate primer system, T-RFLP analysis of *mcrA* gene does not result in a quantitative recovery of the genes of different phylogenetic lineages [29]. This may explain why we detected methanomicrobial *mcrA* genes in the clone library in the present study, but no corresponding T-RFs in the *mcrA*-targeted T-RFLP analysis. The detection of methanomicrobial *mcrA* sequences in our present study proves that the primer system utilized here [38] is capable of detecting these microorganisms. The fact that we did not detect such genes in anoxic bulk soil earlier [26] therefore was probably due to a low abundance of methanomicrobial methanogens rather than to an insufficient utility of the *mcrA* primers designed by Springer et al. [38] to detect all orders of methanogens, as suggested by Luton and co-workers [30].

In conclusion, our data clearly show that the structure of the methanogenic community on rice roots changed with incubation time and conditions, which corresponded to a functional change in the dominance of acetoclastic versus hydrogenotrophic methanogenesis. The predominance of *Methanosarcina* spp. versus *Methanosaeta* spp. was consistent with the prevailing acetate concentrations. The community structures on the basis of 16S rRNA and *mcrA* genes were mostly consistent. In future, more detailed analyses of the Methanomicrobiaceae, Methanobacteriaceae, and Rice cluster I may provide valuable clues for structural and functional relationships among the hydrogenotrophic methanogenic community.

Acknowledgments

We thank Peter Claus for analysis of ^{13}C-isotopic data and the Fonds der Chemischen Industrie, Germany, for financial support.

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