Identification of Acetate-Assimilating Microorganisms under Methanogenic Conditions in Anoxic Rice Field Soil by Comparative Stable Isotope Probing of RNA[▽]

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Acetate is the most abundant intermediate of organic matter degradation in anoxic rice field soil and is converted to CH_4 and/or CO_2. Aceticlastic methanogens are the primary microorganisms dissimilating acetate in the absence of sulfate and reducible ferric iron. In contrast, very little is known about bacteria capable of assimilating acetate under methanogenic conditions. Here, we identified active acetate-assimilating microorganisms by using a combined approach of frequent label application at a low concentration and comparative RNA-stable isotope probing with ^{13}C-labeled and unlabeled acetate. Rice field soil was incubated anaerobically at 25°C for 12 days, during which ^{13}C-labeled acetate was added at a concentration of 500 μM every 3 days. ^{13}C-labeled CH_4 and CO_2 were produced from the beginning of the incubation and accounted for about 60% of the supplied acetate ^{13}C. RNA was extracted from the cells in each sample taken and separated by isopycnic centrifugation according to molecular weight. Bacterial and archaeal populations in each density fraction were screened by reverse transcription-PCR-mediated terminal restriction fragment polymorphism analysis. No differences in the bacterial populations were observed throughout the density fractions of the unlabeled treatment. However, in the heavy fractions of the ^{13}C treatment, terminal restriction fragments (T-RFs) of 161 bp and 129 bp in length predominated. These T-RFs were identified by cloning and sequencing of 16S rRNA as from a *Geobacter* sp. and an *Anaeromyxobacter* sp., respectively. Apparently these bacteria, which are known as dissimilatory iron reducers, were able to assimilate acetate under methanogenic conditions, i.e., when CO_2 was the predominant electron acceptor. We hypothesize that ferric iron minerals with low bioavailability might have served as electron acceptors for *Geobacter* spp. and *Anaeromyxobacter* spp. under these conditions.

In anoxic environments, a variety of microorganisms are involved in the degradation of organic matter by interacting with each other. The functions of the microorganisms consist of hydrolysis, fermentation, syntrophic oxidation, homoacetogenesis, methanogenesis, and oxidation coupled with the reduction of inorganic electron acceptors. Acetate is the most important intermediate under anaerobic conditions and is converted to CH_4 and/or CO_2 (21, 35, 48, 51). The degradation of acetate is strongly influenced by the availability of exogenous electron acceptors, such as sulfate or ferric iron. Thermodynamic theory predicts that in the presence of these electron acceptors (i.e., reduction phase), acetate is oxidized to CO_2 associated with the reduction, while it is converted to CH_4 and CO_2 in the absence of these electron acceptors (i.e., methanogenic phase).

The reduction phase and the methanogenic phase proceed sequentially in flooded rice field soils (1, 40). The pathway of acetate degradation during these phases was demonstrated by turnover experiments using [2-^{14}C]acetate. However, the carbon recovery of substrate and gas products was unexpectedly low in the methanogenic phase (4). This suggests that part of the acetate must have been assimilated by as-yet-unknown microorganisms. Whereas it is well known that aceticlastic methanogenic archaea (i.e., *Methanosarcina* spp. and *Methanosaeta* spp.) primarily degrade and assimilate acetate during the methanogenic phase (7, 17, 28, 29, 59), very little is known about the members of the domain *Bacteria* that are active under these conditions, probably because of the high diversity of the community composition and function.

Stable isotope probing (SIP) allows study of the ecophysiology of the microbial populations on the basis of the incorporation of a ^{13}C-labeled substrate into biomass under quasinatural conditions (9, 24, 34, 43). SIP involves the following experimental steps: (i) microbial assimilation of a defined ^{13}C-labeled substrate and ^{13}C labeling of the nucleic acids, (ii) extraction of the total nucleic acids, (iii) separation of the labeled nucleic acids by isopycnic centrifugation, (iv) genetic profiling, and (v) cloning, sequencing, and phylogenetic analysis. High substrate concentrations and long incubation times should be avoided because they may cause a discrepancy between the experimental and the actual environmental conditions, for which the carbon flux may be high although the substrate pool is low (11). In this study, we added several small dosages of substrate at a low concentration (500 μM) to prevent enrichment conditions. Comparative SIP with ^{13}C-labeled and unlabeled substrates was used to improve the detection of the ^{13}C labeling of rRNA (31). Even if the substrate concentration and incubation time are insufficient for complete ^{13}C

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labeling of nucleic acids, incorporation of substrate ^{13}C into nucleic acids can be detected by comparison with a control using unlabeled substrate.

Rice fields are a significant source of atmospheric methane and have attracted much attention because of the strong impact on global warming (2, 15, 42). In-depth knowledge of the microbial interspecies relationships in anoxic rice fields might be of particular importance for developing options for mitigation of methane emission. However, which bacteria compete for substrate with methanogens is still mostly unclear. The objective of this study was to identify the acetate-assimilating microorganisms during the methanogenic phase in rice field soil by using a combination of frequent label application and comparative RNA-SIP.

MATERIALS AND METHODS

Anaerobic incubation of rice field slurry. Rice field soil was collected from the rice fields at the Italian Rice Research Institute in Vercelli, Italy. Details of soil characteristics and the sampling site have been described in previous reports (52, 53). The soil was air dried, stored, and sieved as described previously (6). Prior to experimental use, a soil slurry was prepared by mixing the dry soil with distilled water at a ratio of 1:1. Aliquots (5 ml) of the homogenized soil slurry were placed in 25-ml serum vials, which were then sealed with butyl rubber septa. The soil slurry was preincubated anaerobically in the dark at 25°C for 30 days in order to allow for the activation of the soil slurry microorganisms and the reduction of available sulfate and ferric iron (28, 29, 30). After preincubation, the headspace of soil slurries was flushed with N_2. Three treatments were prepared: (i) ^{13}C treatment with [U-^{13}C]acetate (99 atom%; Sigma, Taufkirchen, Germany) as the substrate, (ii) unlabeled treatment with unlabeled acetate as the substrate, and (iii) control treatment with distilled water instead of substrate. Each soil slurry treatment was run in triplicate with static incubation for 12 days at 25°C. The appropriate substrate was added to each vial at a final concentration of 500 μM on days 0, 3, 6, and 9. Samples of headspace gas, slurry water, and soil were removed every third day from each vial of each set of different slurry treatments done in triplicate. Total CH_4 and CO_2 in the headspace samples were analyzed using a standard gas chromatography method (49). The ^{13}C atoms percent of CH_4 and CO_2 was analyzed by a gas chromatography-isotope ratio mass spectrometry method as described previously (8). The concentrations of volatile fatty acids from the slurry water sample were measured using a high-pressure liquid chromatography method (18). The soil samples were stored at −80°C for subsequent molecular analyses.

RNA extraction and density gradient centrifugation. RNA was extracted from 0.5 ml of each soil slurry sample from one set of the ^{13}C treatment and the unlabeled treatment after 12 days of incubation by a direct lysis protocol involving bead beating as described previously (39). Total RNA was quantified using the Ribogreen RNA quantification kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA extracts (500 ng of RNA) of the ^{13}C treatment and unlabeled treatment were mixed with cesium trifluoroacetate (CsTFA) (Amersham Biosciences, Freiburg, Germany) solution. The mixture was subjected to equilibrium density gradient centrifugation under the conditions reported previously (31). The centrifugation enabled the separation of RNA on the basis of density. Gradients of the density-separated RNA were fractionated, and the CsTFA buoyant density (BD) of each fraction was determined (31).

RT-PCR amplification and terminal restriction fragment length polymorphism analysis. Each density fraction of RNA from the ^{13}C treatment and unlabeled treatment was subjected to reverse transcription-PCR (RT-PCR) using a one-step RT-PCR system (Access Quick; Promega, Mannheim, Germany) for terminal restriction fragment length polymorphism (T-RFLP) fingerprinting. The PCR primer sets Ba27f-6-carboxyfluorescein (FAM)/Ba907r and Ar109f/ Ar912rt-FAM were used to amplify the transcripts of bacterial and archaeal 16S rRNAs, respectively (29, 32). Reverse transcription was carried out at 48°C for 45 min, and PCR was started with an initial denaturation step at 94°C for 3 min. The thermal profile of PCR amplification consisted of 20 cycles under stringent conditions, each including 30 s at 94°C, 45 s at 52°C, and 90 s at 72°C, which was followed by a final extension step of 5 min at 72°C. The products were checked by electrophoresis on 1% agarose gels. RT-PCR amplicons were digested using MspI and TaqI for *Bacteria* and *Archaea*, respectively. Digested amplicons were desalted using Auto Seq G-50 columns (Amersham Biosciences). Prior to electrophoresis, 1 μl of the digest was suspended in 12 μl of Hi-Di formamide

(Applied Biosystems, Weiterstadt, Germany) and 0.25 μl of carboxy-X-rhodamine (ROX)-labeled MapMarker 1000 ladder (Bio-Ventures, Murfreesboro, TN). The mixture was denatured at 95°C for 3 min and cooled immediately on ice. Size separation of terminal restriction fragments (T-RFs) was performed using an ABI 310 genetic analyzer (Applied Biosystems). The conditions of electrophoresis were the same as described previously (30).

Sequencing and phylogenetic analyses. Selected density fractions of bacterial RNA were amplified for cloning using the primer set Ba27f/Ba907r under the thermal conditions mentioned above. The RT-PCR products were ligated into the plasmid vector pGEM-T Easy (Promega), and the ligation mixture was used to transform *Escherichia coli* JM109 supercompetent cells (Promega) according to the manufacturer's instructions. We randomly selected 71 clones from the ^{13}C treatment and 67 clones from the unlabeled treatment. The 16S rRNA segments were sequenced at the automatic DNA isolation and sequencing facility of the Max-Planck-Institute for Plant Breeding Research, Cologne, Germany, using BigDye terminator cycle sequencing chemistry (Applied Biosystems) (25, 32). Chimeric structures were detected by separately analyzing the phylogenies of terminal stretches at the 5′ and 3′ ends ("fractional treeing") (26, 27). Phylogenetic analyses were conducted using the ARB software package (http://www .arb-home.de) (26) as described previously (33). Phylogenetic trees with reference 16S rRNA sequences (>1,400 nucleotides) were calculated using neighborjoining, maximum-parsimony, and maximum-likelihood methods. Bootstrap values were obtained from 1,000 replications. Partial 16S rRNA sequences (i.e., approximately 850 bp) obtained in this study were added to the trees by using the ARB parsimony tool. The similar topologies of the trees constructed with different algorithms were confirmed, and the trees best fitting the overall consensus were chosen for presentation.

Nucleotide sequence accession numbers. The nucleotide sequence data obtained in this study have been deposited in the DDBJ nucleotide sequence database under accession numbers AB265824 to AB265961.

RESULTS

Biogeochemical activities in rice field slurry incubation with ^{13}C-labeled and unlabeled acetate. Anoxic preincubated rice field soil was repeatedly supplemented with ^{13}C-labeled and unlabeled acetate. Before each new addition of substrate, the acetate concentration was monitored, and it was found to have decreased to less than 15 μM in all the treatments (i.e., ^{13}C treatment, unlabeled treatment, and control treatment). Apparently, the size of the acetate pool was low in the rice field slurry and the added acetate was degraded within 3 days. Other volatile fatty acids (e.g., propionate or butyrate) were not detectable during the incubation. In total, 10 μmol of acetate was added to the ^{13}C treatment and the unlabeled treatment. CH_4 and CO_2 were produced immediately after the beginning of the incubation in all three treatments (Fig. 1). The production of CH_4 and CO_2 in the control treatment (no acetate addition) was less than those in the ^{13}C treatment and unlabeled treatment (Fig. 1A, B, and C). Addition of acetate increased the production of CH_4 and CO_2. There was little difference in CH_4 and CO_2 production between the ^{13}C treatment and unlabeled treatment (Fig. 1A and B). CH_4 increased in both treatments linearly to a concentration of approximately 500 μM during 12 days of the incubation. On the other hand, the CO_2 concentration increased rapidly during the first 3 days of the incubation and thereafter increased gradually to 660 to 700 μmol liter^{-1} at day 12. The high CO_2 concentration in the initial stage of the incubation was probably caused by degassing and establishment of the CO_2-bicarbonate equilibrium. The similarity of biogeochemical activities in both the ^{13}C treatment and the unlabeled treatment indicates similar physiological actions of the indigenous microbial community.

To resolve the fate of the substrate ^{13}C, the ^{13}C atoms percent of the products CH_4 and CO_2 were followed over time
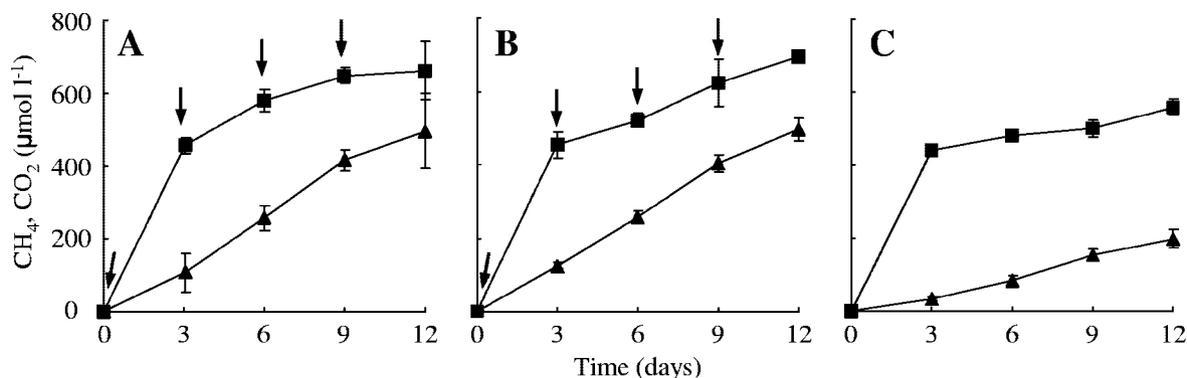
FIG. 1. Change in concentrations of CH_4 (▲) and CO_2 (■) during anaerobic incubation of a rice field slurry. (A) ^{13}C treatment; (B) unlabeled treatment; (C) control treatment. Arrows indicate when acetate was added to each vial at a final concentration of 500 μM. The error bars represent the standard deviations of three replications.

(Fig. 2). The ^{13}C atoms percent of CH_4 increased rapidly to more than 60% within the first 3 days of the incubation and afterwards remained at this level, while the ^{13}C atoms percent of CO_2 increased slowly to 23.4% at day 12 (Fig. 2A). The total amount of ^{13}C-labeled CH_4 and CO_2 was calculated using the concentrations in the gas and liquid phases (Fig. 1A) and the ^{13}C atoms percent (Fig. 2B). $^{13}CH_4$ and $^{13}CO_2$ were produced

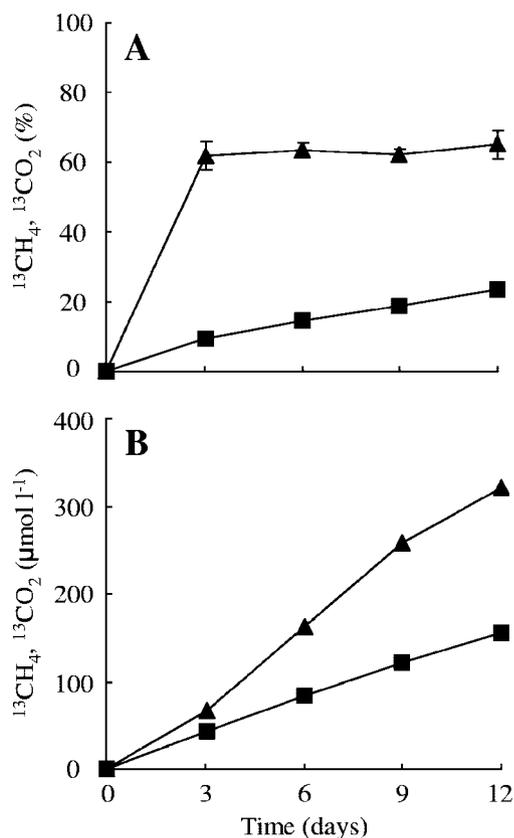FIG. 2. Conversion of ^{13}C-labeled acetate to $^{13}CH_4$ and $^{13}CO_2$ during incubation of the ^{13}C treatment. (A) Change in the ^{13}C atoms percent of CH_4 (▲) and CO_2 (■) in the headspace. Error bars indicates standard deviations of three replications. (B) Change in the concentrations of $^{13}CH_4$ (▲) and $^{13}CO_2$ (■).

immediately upon the start of incubation and increased linearly to concentrations of 320 μmol and 160 μmol per liter of headspace at day 12, respectively. The total amount of ^{13}C carbon from acetate added during the incubation was 20 μmol (2×10 μmol acetate). The amounts of $^{13}CH_4$ and $^{13}CO_2$ produced until day 12 were 6.4 and 3.1 μmol, respectively. Dissolved $^{13}CO_2$ components were estimated to be 2.6 μmol by using the gaseous $^{13}CO_2$ concentration and the pH of the rice field slurry (pH 7.7).

Comparative T-RFLP fingerprinting of bacterial and archaeal 16S rRNAs separated by density gradient centrifugation. Total RNA was extracted from the soil samples of the ^{13}C treatment and unlabeled treatment after 12 days of incubation, during which the added acetate (10 μmol) was mostly turned over. RNA was separated by isopycnic centrifugation according to molecular weight, and subsequently the gradients of the RNA were fractionated. Bacterial and archaeal rRNAs in each density fraction were used for RT-PCR-mediated T-RFLP analysis (Fig. 3). Incorporation of substrate ^{13}C into the microbial population was detected by comparing the T-RFLP fingerprints of rRNA derived from the ^{13}C treatment with those from the unlabeled treatment.

Bacterial rRNA from the ^{13}C treatment was successfully amplified in the fractions with BDs of 1.757 to 1.806 g ml^{-1}, while that from unlabeled treatment was amplified in the fractions with BDs of 1.760 to 1.791 g ml^{-1}. RNA obtained from the ^{13}C treatment became slightly heavier than that from the unlabeled treatment. The T-RFLP fingerprint patterns of the ^{13}C treatment and unlabeled treatment were similar in the low-density fractions with BDs of 1.757 to 1.769 g ml^{-1} (Fig. 3A and B). The fingerprints of the density fractions clearly showed a strong shift with increasing BD in the ^{13}C treatment, but there was little change in the unlabeled treatment. In particular, a T-RF of 161 bp increased in relative frequency in the fractions with BDs of ≥1.781 g ml^{-1} and predominated in the fractions with BDs of ≥1.793 g ml^{-1}. A T-RF of 129 bp could be detected as a second dominant peak in the fractions with BDs of ≥1.793 g ml^{-1}.

Archaeal rRNA from the ^{13}C treatment could be amplified only up to fractions with a BD of 1.757 to 1.793 g ml^{-1}, which is less heavy than that of the bacterial RNA. This may be attributed to the low abundance of the archaeal RNA in the
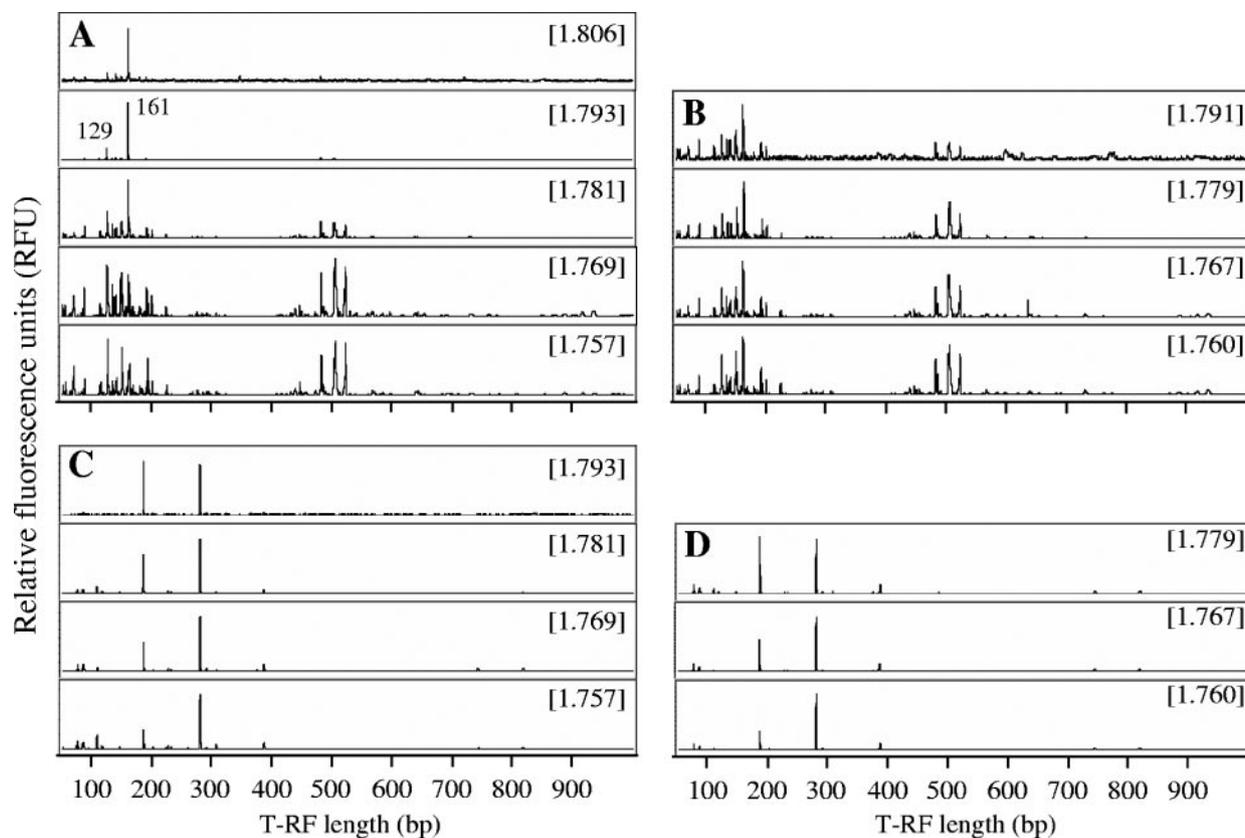
FIG. 3. T-RFLP fingerprints of the bacterial (A and B) and archaeal (C and D) 16S rRNAs separated by isopycnic centrifugation from the ^{13}C treatment (A and C) and the unlabeled treatment (B and D). The CsTFA BDs (g ml^{-1}) of the fractions are shown in brackets.

template, since the same cycle number for RT-PCR and the same amount of template were applied to the bacterial and archaeal fingerprintings. The archaeal population size was reported to be much lower than that of the bacterial population in rice field soil (30). A PCR product was obtained from a heavier fraction in the ^{13}C treatment (BD of 1.793 g ml^{-1}) compared to the unlabeled treatment (BD of 1.779 g ml^{-1}); however, the fingerprints were undistinguishable. Two predominated archaeal T-RFs of 188 bp and 286 bp were detected in the fractions with BDs of ≥1.779 g ml^{-1} in both the ^{13}C treatment and unlabeled treatment.

Comparative sequence analyses of bacterial 16S rRNAs from heavy gradient fractions. Two bacterial 16S rRNA clone libraries were constructed from the high ("H")-density fractions (Fig. 4): (i) the LH library ($n = 71$ clones) represented the ^{13}C treatment (BD of 1.793 g ml^{-1}), and (ii) the UH library ($n = 67$ clones) represented the unlabeled treatment (BD of 1.791 g ml^{-1}). The compositions of the LH and UH libraries were used to phylogenetically characterize the acetate-assimilating bacteria corresponding to the T-RFs of 161 bp and 129 bp found in the T-RFLP fingerprint analysis.

The composition of the LH library was less diverse than that of the UH library, as expected from the corresponding T-RFLP fingerprint patterns (Fig. 3A and B and 4A). The most remarkable difference between the LH and UH libraries concerned sequences within the class *Deltaproteobacteria*. In the LH library, the *Deltaproteobacteria* sequences were the primary

component, accounting for 46% of the total. In contrast, in the UH library, *Deltaproteobacteria* sequences were less than 13%. The *Deltaproteobacteria* sequences in the LH and UH libraries were identified at the genus level (Fig. 4B). Sequences related to the genus *Geobacter* were detected only in the LH library and represented the largest part of the clones (i.e., 48% of total *Deltaproteobacteria* sequences). These clones were affiliated with two novel clusters in the phylogenetic tree, which were closely related to *Geobacter psychrophilus* (accession no. AY653549; 98% and 96% sequence identity) (Fig. 5) (38). The expected T-RF size was 161 bp, which corresponded to the dominant peak in the T-RFLP fingerprint (Fig. 3A). The number of sequences for the second identified genus, *Anaeromyxobacter*, was much larger in the LH library ($n = 12$) than in the UH library ($n = 4$). These clones in the LH library formed a novel cluster within the genus *Anaeromyxobacter* (Fig. 5). The expected T-RF size was 129 bp, which corresponded to the second dominant peak in the T-RFLP fingerprint (Fig. 3A).

In addition to the class *Deltaproteobacteria*, the relative abundances of clones identified as *Acidobacteria* and *Planctomycetes* were higher in the LH library than in the UH library (Fig. 4A). However, these clones did not cluster into a specific branch of the phylogenetic trees (data not shown), and the expected T-RF sizes did not match with one of the dominant peaks in the T-RFLP fingerprint.
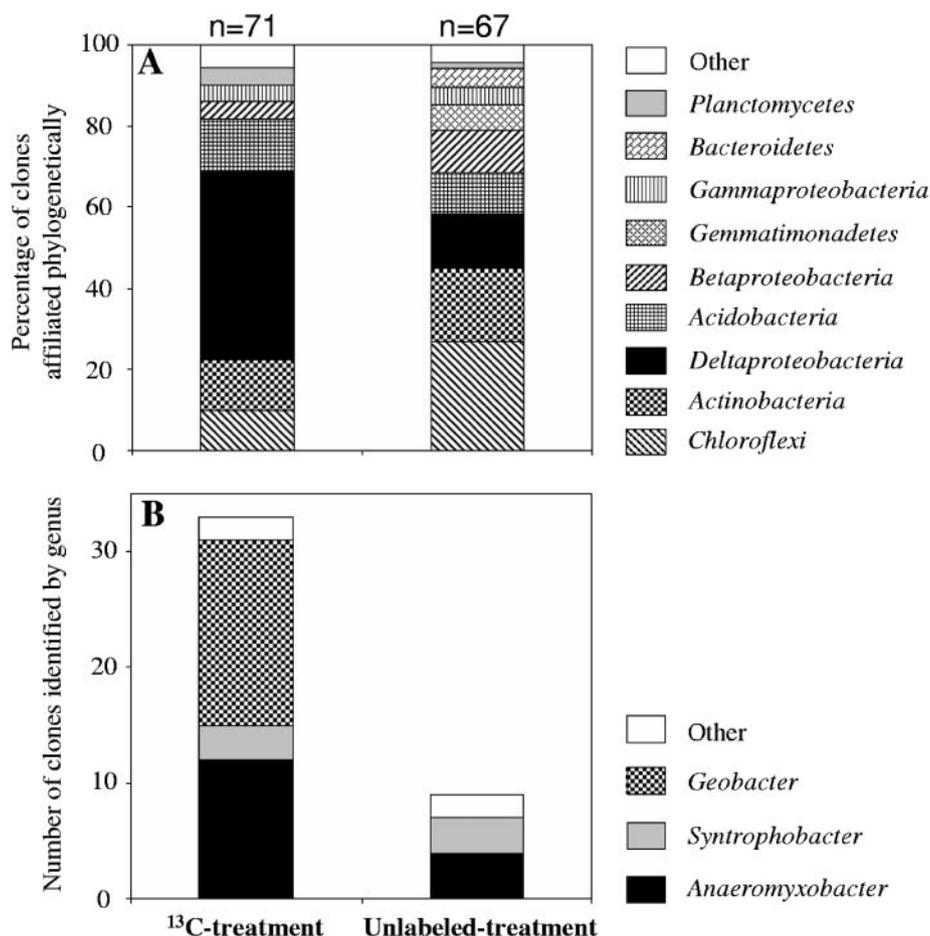
FIG. 4. Comparison of bacterial 16S rRNA gene sequences obtained from selected density fractions from the ^{13}C treatment and unlabeled treatment. (A) Relative proportions of 16S rRNA gene sequences affiliated phylogenetically. "Other" represents *Alphaproteobacteria*, *Cyanobacteria*, *Deinococcus-Thermus*, *Firmicutes*, or candidate division OP10 combined. *n* indicates the total numbers of clones obtained in this study. (B) Clone numbers of 16S rRNA gene sequences identified by genus within the *Deltaproteobacteria*. The phylogenetic relationships of the deltaproteobacterial clones are shown in Fig. 5.

DISCUSSION

We investigated the acetate-assimilating microorganisms in a methanogenic rice field soil, in which acetate was immediately converted to CH_4 and CO_2. Frequent substrate supplementation at low concentration enabled the indigenous microorganisms to incorporate ^{13}C substrate under conditions that are relatively close to those found in the natural environment (61). Comparative SIP with labeled and unlabeled substrates allowed the detection of RNA labeled with ^{13}C from the assimilated acetate. The combination of repeated label addition at low dosage and comparative RNA-SIP thus provided insight into the microbial assimilation of acetate under methanogenic conditions in a rice field soil.

The CH_4 produced in the control treatment was derived from endogenous organic matter present in the rice field soil (Fig. 1C). This organic matter was probably also degraded via acetate, as shown in numerous previous studies (21, 35, 48, 51) and supported by the observation of a low acetate concentration under steady-state conditions. The addition of acetate resulted in a 2.5-fold increase of the CH_4 production activity (Fig. 1A and B). The carbon balance of the substrate and gas

products showed that 20 μmol of acetate ^{13}C was converted to at least 12.1 μmol of ^{13}C-labeled gaseous products (CH_4 plus CO_2). The carbon recovery in the ^{13}C treatment was approximately 60%, which is nearly consistent with the data of the previous turnover experiment using $[2-^{14}C]$acetate (4). The carbon not recovered was probably assimilated into biomass including rRNA, which became labeled with ^{13}C.

The CsTFA BD of the heaviest RNA obtained in this study was 1.806 g ml^{-1}, which is not as high as that of the RNA fully labeled with ^{13}C (BD of 1.815 g ml^{-1}), as reported previously (31). Thus, the active microbial populations were not completely labeled but were heavily enriched with substrate ^{13}C. Incorporation of the substrate ^{13}C into RNA was clearly demonstrated by comparative T-RFLP analyses of the ^{13}C treatment and unlabeled treatment (Fig. 3). The ^{13}C treatment showed that the bacterial population detected clearly changed, because increasing BD of the density fraction and two T-RFs of 161 bp and 129 bp length predominated in the fingerprints of the high-density fraction (Fig. 3A and B). Clone library analyses revealed that the sequences of *Geobacter* spp. and *Anaeromyxobacter* spp. were detected preferentially in the LH
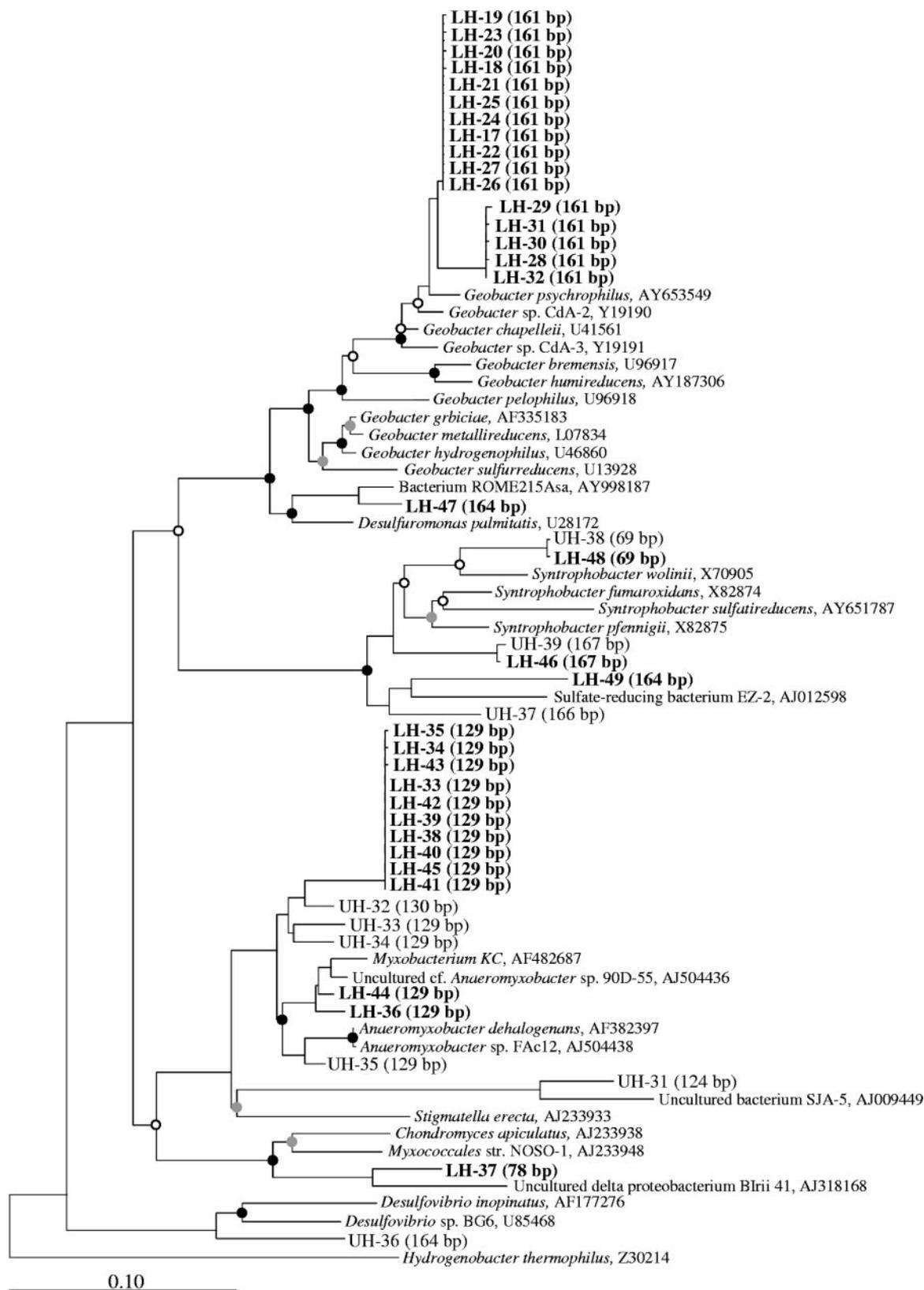
FIG. 5. Phylogenetic tree showing the relationships of 16S rRNA phylotypes affiliated with the *Deltaproteobacteria*. The basic tree of reference sequences was reconstructed using the maximum-parsimony method based on a comparison of more than 1,400 nucleotides. Bootstrap values were obtained from 1,000 replications, and >90%, 70 to 89%, and <69% are shown with black, gray, and open circles, respectively. Clones were designated LH (heavy fraction of the labeled ¹³C treatment; in boldface) and UH (heavy fraction of the unlabeled treatment). Numbers in parentheses represent the expected T-RF lengths of the clones. The scale bar represents 10% sequence divergence. GenBank accession numbers of reference sequences are given.

library (Fig. 4B and Fig. 5). The expected sizes of T-RFs for *Geobacter* spp. and *Anaeromyxobacter* spp. were in accordance with those of the dominant peaks in the T-RFLP fingerprint (Fig. 3A and 5). These results demonstrate that *Geobacter* spp. and *Anaeromyxobacter* spp. played a role in acetate assimilation in methanogenic rice field soil. We believe that labeling of these bacteria with ^{13}C was realistic for in situ conditions and was not caused by artificial enrichment due to the increased acetate concentrations, since the respective T-RFs were increased only upon addition of ^{13}C-labeled acetate and not upon addition of unlabeled acetate.

Geobacter and *Anaeromyxobacter* spp., both of which are known as dissimilatory iron reducers (13, 22), have been found in a wide variety of anoxic environments (41, 54, 55), possibly because of their ability to oxidize acetate, which is a central intermediate in the anaerobic degradation of organic matter. It has been reported that on rice roots, where oxygen released from the aerenchyma is probably available for iron(II) oxidation, the reduction of ferric iron is an important biogeochemical process (10). Furthermore, rice roots were found to be colonized by uncultured *Anaeromyxobacter* species (see, e.g., environmental clone 90D-55 in Fig. 5) (57). In flooded rice field soils, fine-scale profiling of iron(II) and iron(III) oxide distributions has revealed a spatial and temporal activity of iron(III) reduction (19). Although iron reducers have not been detected as a dominant population by using culture-independent approaches (14, 19, 39), *Anaeromyxobacter* sp. strain FAc12 (shown in Fig. 5) was isolated from the bulk soil of rice microcosms by using acetate and ferrihydrite as substrates (57).

Presently, it is not known how *Geobacter* spp. and *Anaeromyxobacter* spp. can be metabolically active during the methanogenic phase in rice field soil. Acetate most likely was the energy substrate used for dissimilation. However, it is unclear how it might have been used other than by acetoclastic methanogenesis, since our experimental setup excluded electron acceptors such as O_2, nitrate, or sulfate. Indeed, CH_4 was the predominant product of ^{13}C-labeled acetate, but $^{13}CO_2$ was also formed, and we cannot exclude the possibility that some of the CO_2 was formed from the methyl group of acetate, i.e., by acetate oxidation rather than methanogenic acetate cleavage. A fermentative type of metabolism has never been demonstrated so far for either *Geobacter* spp. (22) or *Anaeromyxobacter* spp. (13, 50, 57); also, it is unlikely that acetate alone could serve as a major carbon source for growth and energy metabolism, as this would require syntrophic acetate oxidation, i.e., that involving interspecies electron transfer (62), a process that has not been detected in rice field soil as yet (3). Oxidative acetate dissimilation may be achieved by using humic acids as primary electron acceptors (23). However, it is unclear whether humic acids in rice field soil have a large capacity for serving as ultimate oxidants for acetate. In lake sediments, for example, the electron uptake capacity of humic acids was found to be low in deeper layers, and humic acids were regarded as unlikely to be a significant oxidant (16). Rather than a final oxidant, humic acids seem to serve as an electron shuttle to ferric iron serving as terminal electron acceptor (16). In rice field soil, *Geobacter* spp. and *Anaeromyxobacter* spp. may oxidize acetate as an energy source by using such a shuttle mechanism. Alternatively, the *Geobacter* spp. and *Anaeromyxobacter*

spp. identified might directly utilize iron(III) oxides that are only poorly available for dissimilatory iron reduction, e.g., crystalline rather than amorphous iron oxide phases (45, 46). In this study, the rice field slurry was preincubated for a long time, and bioavailable iron(III) oxides were therefore considered to be largely depleted. In another study (60), the production of Fe(II) was hardly detectable in rice field soil. However, Fe(II) production never completely balances the amount of ferric iron minerals originally present in rice field soil. Therefore, it is assumed that much of the Fe(III) minerals are left unreduced in the methanogenic rice field soil because they are hardly available to microbial metabolism. We therefore hypothesize that *Geobacter* spp. and *Anaeromyxobacter* spp. can utilize some of the barely accessible iron(III) oxides as electron acceptors under methanogenic condition in rice field soil, albeit at a low rate. Various strategies that may be used by microorganisms to access insoluble Fe(III) oxides have been suggested to date [e.g., production of electron-shuttling components and/or Fe(III) chelators, chemotaxis to contact with Fe(III) oxides, and electron transfer via microbial nanowires] (5, 36, 37, 44).

Environments in which iron(III) reduction occurs are geochemically characterized by low acetate and H_2 concentrations (12, 56). Acetate-oxidizing iron reducers were reported to outcompete aceticlastic methanogens by maintaining the substrate concentration at levels too low for methanogenesis (20, 47). On the other hand, the reduction of Fe(III) is greatly influenced by the availability of ferric iron minerals. In this study, the steady-state acetate concentration of below 15 μM was lower than the acetate threshold concentration for aceticlastic methanogenesis, which ranges from 69 to 1,180 μM (58), indicating that a biogeochemical process that is more thermodynamically favorable than methanogenesis operated in the methanogenic rice field soil. Comparative SIP of RNA showed that while potential dissimilatory iron reducers of the genera *Geobacter* and *Anaeromyxobacter* were able to produce RNA from ^{13}C-labeled acetate, the archaeal population assimilated only a small amount of the acetate ^{13}C into their RNAs (Fig. 3). Energy obtained from iron(III) reduction is much greater than that from methanogenesis (1, 40). It is thought, therefore, that the iron(III) reducers can use acetate as a carbon source more readily than methanogens. The aceticlastic methanogens may have utilized acetate mainly for energy metabolism, with only a small amount being assimilated. Biomass production by methanogenic archaea probably was slight.

In summary, the results of this study showed that *Geobacter* spp. and *Anaeromyxobacter* spp. assimilated acetate during the methanogenic phase of a rice field soil. We assume that these genera were dissimilating acetate by reduction of crystalline ferric iron minerals. These bacteria would therefore compete for acetate with aceticlastic methanogens in situ but apparently did not outcompete them, since CH_4 nevertheless was the primary metabolic product of acetate degradation. It has been reported that *Geobacter* and *Anaeromyxobacter* coexist in acidic contaminated sediments, depending on the environmental pH (41). The ability of *Geobacter* spp. and *Anaeromyxobacter* spp. to access different forms of ferric iron minerals should be investigated to learn more about the ecophysiological niches of these iron reducers in anoxic rice field soil.

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