Molecular Analyses of the Methane-Oxidizing Microbial Community in Rice Field Soil by Targeting the Genes of the 16S rRNA, Particulate Methane Monooxygenase, and Methanol Dehydrogenase

THILO HENCKEL, MICHAEL FRIEDRICH, AND RALF CONRAD*

Max-Planck-Institut für terrestrische Mikrobiologie, D-35043 Marburg, Germany

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Rice field soil with a nonsaturated water content induced CH_4 consumption activity when it was supplemented with 5% CH_4 . After a lag phase of 3 days, CH_4 was consumed rapidly until the concentration was less than 1.8 parts per million by volume (ppmv). However, the soil was not able to maintain the oxidation activity at near-atmospheric CH_4 mixing ratios (i.e., 5 ppmv). The soil microbial community was monitored by performing denaturing gradient gel electrophoresis (DGGE) during the oxidation process with different PCR primer sets based on the 16S rRNA gene and on functional genes. A universal small-subunit (SSU) ribosomal DNA (rDNA) primer set and 16S rDNA primer sets specifically targeting type I methyloprophs (members of the γ subdivision of the class *Proteobacteria* [γ -*Proteobacteria*]) and type II methyloprophs (members of the α -*Proteobacteria*) were used. Functional PCR primers targeted the genes for particulate methane monooxygenase (*pmoA*) and methanol dehydrogenase (*mxhF*), which code for key enzymes in the catabolism of all methanotrophs. The yield of PCR products amplified from DNA in soil that oxidized CH_4 was the same as the yield of PCR products amplified from control soil when the universal SSU rDNA primer set was used but was significantly greater when primer sets specific for methanotrophs were used. The DGGE patterns and the sequences of major DGGE bands obtained with the universal SSU rDNA primer set showed that the community structure was dominated by nonmethanotrophic populations related to the genera *Flavobacterium* and *Bacillus* and was not influenced by CH_4 . The structure of the methyloproph community as determined with the specific primer sets was less complex; this community consisted of both type I and type II methanotrophs related to the genera *Methylobacter*, *Methylococcus*, and *Methylocystis*. DGGE profiles of PCR products amplified with functional gene primer sets that targeted the *mxhF* and *pmoA* genes revealed that there were pronounced community shifts when CH_4 oxidation began. High CH_4 concentrations stimulated both type I and II methanotrophs in rice field soil with a nonsaturated water content, as determined with both ribosomal and functional gene markers.

Methane oxidation by methanotrophic bacteria occurs in soil and aquatic environments; this process reduces CH_4 emission and may be a negative feedback mechanism for increases in atmospheric CH_4 levels (17, 50). The atmospheric CH_4 budget is a concern since CH_4 is one of the important greenhouse gases and affects the Earth's climate (15, 49). Methanotrophs are important regulators of CH_4 emission from rice fields. Only part of the CH_4 produced in rice field soil is released into the atmosphere; the remainder is oxidized by methanotrophic bacteria living in oxic niches in flooded fields (i.e., the surface soil layer and the rhizosphere) (7, 18, 25, 33, 55). Slurries of anoxic rice field soil change from production of CH_4 to consumption of CH_4 when they are aerated (29). In nonsaturated rice field soil CH_4 oxidation is induced when the soil is moistened and exposed to CH_4 concentrations higher than 1,000 parts per million by volume (ppmv) (3, 4). The most-probable-number counts of methanotrophic bacteria also increase under these conditions. Type II methanotrophs (organisms belonging to the α subdivision of the class *Proteobacteria* [α -*Proteobacteria*]) have been isolated from rice roots (27). However, the structure of the methanotrophic community and the possible

changes in this community that occur during induction of CH_4 oxidation in rice field soil are not known.

In general, little is known about the structure of the methanotrophic community in soil, but it seems that type II methanotrophs are found more frequently than type I methanotrophs are found (28). An immunofluorescent analysis of tundra soils revealed the presence of both type I and type II methanotrophs (62). A phospholipid analysis of soil microorganisms demonstrated that type II methanotrophs were dominant in boreal peatland soil (58). A hybridization analysis of 16S rRNA extracted from Alaskan soil also indicated that type II methanotrophs were dominant (11). Type II methanotrophs were also found to be the dominant methanotrophs in peat bogs (20), whereas type I methanotrophs seem to prevail in aquatic environments, such as lake water (28, 52) and lake sediments (6).

The introduction of denaturing gradient gel electrophoresis (DGGE) to microbial ecology provided a valuable molecular fingerprinting technique for studying microbial community structure (31, 44–46). DGGE facilitates separation of mixtures of PCR-amplified gene fragments based on sequence differences (47) and allows large numbers of samples to be analyzed simultaneously. Thus, this technique is ideally suited for monitoring the dynamics of microbial communities influenced by environmental changes.

Recently, DGGE was used to analyze oxic agricultural soils

* Corresponding author. Mailing address: Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch Strasse, D-35043 Marburg, Germany. Phone: 49-6421-178 801. Fax: 49-6421-178 809. E-mail: Conrad@mail.uni-marburg.de.

incubated with high CH_4 mixing ratios, and the results revealed that type I methanotrophs were present in soil extracts but type II methanotrophs were present in enrichment cultures prepared from the same soils (35, 48). In these studies 16S ribosomal DNA (rDNA) primer sets were used to detect a wide range of species belonging to the domain *Bacteria*. However, in another study methanotrophs could not be resolved by DGGE when universal 16S rDNA primers were used (61).

Methanotrophs are a phylogenetically heterogeneous group belonging to the α - or γ -*Proteobacteria* (28). Seven genera of type I (γ -*Proteobacteria*) and type II (α -*Proteobacteria*) methanotrophs have been proposed (8, 9). While the genera *Methylococcus* (also classified as type X [28]), *Methylomonas*, *Methylobacter*, *Methylomicrobium*, and *Methylosphaera* belong to the type I methanotroph group, the genera *Methylocystis* and *Methylosinus* make up the type II methanotroph group. Scientists have developed 16S rDNA probes that target methanotrophic bacteria belonging to either type I or type II (11, 40, 60). Hybridization of the total extracted environmental DNA in a blanket peat bog by using genus-specific probes suggested that members of the genera *Methylosinus* and *Methylococcus* were the dominant methanotrophs in this environment, while the genera *Methylomonas* and *Methylobacter* were not detected (20).

Methanotrophs have similar physiological characteristics. The key enzymes particulate methane monooxygenase (pMMO), soluble methane monooxygenase (sMMO), and methanol dehydrogenase (MDH) are highly conserved, so that enzyme-based gene markers may offer the possibility of detecting all known methanotrophs (28). Gene probes that target functional genes have been developed for the *pmoA* gene (41) coding for the α -subunit of the pMMO present in all known methanotrophs and the *mxrA* gene (40, 38) coding for the α -subunit of the MDH present in all methylobacteria (28, 39). The pMMO is closely related to the ammonium monooxygenase (AMO) of the ammonium oxidizers, and the degenerate *pmoA* primer set (primers A189f and A682r) also detects the homologous sequence of the *amoA* gene for the α -subunit of AMO (32). Scientists have also developed gene probes that target the *mmoB* gene coding for the sMMO (39) that is present in most type II methanotrophs and in members of the genus *Methylococcus* (type X) but not in most type I methanotrophs (28). A community analysis of environmental DNA from a blanket peat bog in which cloning and sequencing of these gene fragments were performed revealed a distinct phylogenetic cluster of type II methanotrophs that are probably new, uncultured acidophilic methanotrophs (38–41).

We studied changes in microbial community structure during induction of CH_4 oxidation in rice field soil by performing a DGGE analysis of small-subunit (SSU) rRNA-based and functional gene markers. A universal SSU rRNA-based primer set and two primer sets that target methylobacterial members of the γ - and α -*Proteobacteria* were used. In addition, we employed primer sets that target functional genes, such as the *pmoA*, *mmoB*, and *mxrA* genes (39–41). This approach facilitated detection of changes in microbial community structure during induction of CH_4 oxidation in a nonsaturated rice field soil.

MATERIALS AND METHODS

Soil. The rice field soil used in this study has been described previously in detail (29). This soil had a maximum water-holding capacity (WHC) corresponding to a gravimetric water content of 44% (wt/wt). In the experiments described below, the rice field soil was moistened with demineralized water until the gravimetric water content was 19% (wt/wt), which corresponded to 43% of the WHC. The moist soil was sieved with a 2-mm sieve in order to ensure homogeneity and to minimize the number of anoxic microsites.

CH_4 oxidation. Soil (4.1 ± 0.1 g [fresh weight]) was placed in 120-ml serum bottles, and the bottles were closed with butyl rubber stoppers. Fourteen bottles were flushed with moistened synthetic air (20.5% O_2 in N_2), and subsequently 50,000 ppmv of CH_4 was added. CH_4 was not added to a second set of eight bottles. All of the bottles were incubated at 25°C in the dark. Three bottles were used only for gas analysis, while the remaining bottles were used for DNA extraction. Four bottles were replenished three times with 5 ppmv of CH_4 after the soil had consumed enough of the CH_4 so that the mixing ratio was <1.8 ppmv. Bottles to which CH_4 was not added were used as controls for DNA extraction to provide community data for rice soil not supplemented with CH_4 . Rice soil samples used for DNA extraction (one bottle per sample) were removed at different times (see Fig. 1) and stored at -20°C .

Gas analysis. Methane and O_2 contents were periodically measured by gas chromatography as described previously (29). Oxygen was repeatedly added to the bottles to keep the O_2 concentration constant at about 17 to 20% (vol/vol).

Bacterial strains. For molecular analyses nine methanotrophic reference strains obtained from the collection at our institute were used as previously described by Gilbert and Frenzel (25). The bacteria were cultured in nitrate mineral salts medium (pH 6.8) with 15% CH_4 in the headspace (25). The cultures were harvested after 2 to 3 days by centrifugation, and the cell pellets were stored at -20°C until DNA was extracted.

DNA extraction. The method used to extract DNA from rice field soil and from pure cultures of methanotrophs was a modification of the method of Moré et al. (43). Cell pellets or 0.6-g (fresh weight) portions of soil were placed in 2-ml screw-cap tubes. Approximately 1 g of sterilized (170°C, 4 h) zirconia-silica beads (diameter, 0.1 mm; Biospec Products Inc., Bartlesville, Okla.), 800 μl of sodium phosphate buffer (120 mM; pH 8), and 260 μl of a sodium dodecyl sulfate solution (10% sodium dodecyl sulfate, 0.5 M Tris-HCl [pH 8.0], 0.1 M NaCl) were added to the soil, which was resuspended by vortexing. The cells were lysed by shaking the preparations with a cell disruptor (model FP120 FastPrep; Savant Instruments Inc., Farmingdale, N.Y.) for 45 s at a setting of 6.5 m s^{-1} . After centrifugation (3 min, $12,000 \times g$) the supernatant was collected, and the soil-bead mixture was extracted a second time by resuspension in 700 μl of phosphate buffer. Proteins and debris were precipitated from the supernatant by adding 0.4 volume of 7.5 M ammonium acetate and then incubating the preparation on ice for 5 min. After centrifugation at $12,000 \times g$ for 3 min, the nucleic acids were precipitated by adding 0.7 volume of isopropanol and then centrifuging the preparation at $12,000 \times g$ and 4°C for 45 min. Subsequently, the DNA pellet was washed with 70% ethanol at 4°C and dried under a vacuum. Finally, the DNA was resuspended in 200 μl of Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA; pH 8). Soil DNA was purified further by using a Prep-A-Gene kit (Bio-Rad, Munich, Germany) as specified by the manufacturer.

PCR amplification. For PCR amplification we used the following three SSU rRNA-based primer sets: a universal primer set that targeted all life, which was modified from the primers described by Weisburg et al. (63) by using primers 533f (GTGCCAGCAGCGCGGTAA) and 907r (AATTCCTTTGAGTTT) (*Escherichia coli* positions 515 to 533 and 907 to 922 [10]) as forward and reverse primers, respectively; and the MB10 γ and MB9 α primer sets. The latter two primer sets were formulated by utilizing hybridization probes 10 γ and 9 α , which target methylobacterial γ - and α -*Proteobacteria* (60), respectively, as forward primers and primer 533 as the reverse primer. In addition, three functional primer sets, the *pmoA*, *mmoB*, and *mxrA* primer sets, originally designated A189f/A682r, 77f/369r, and f1003/r1561, respectively, were utilized (38, 39, 41).

The primers and the PCR conditions used for each primer pair are summarized in Table 1. PCR buffer (20 mM Tris-HCl [pH 8.3], 50 mM KCl), 1 U of *AmpliTaq* DNA polymerase (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany), each primer at a concentration of 0.5 μM , each deoxynucleoside triphosphate (Amersham Life Science, Braunschweig, Germany) at a concentration of 100 μM , and 1 μl of template DNA were added to each 50- μl (total volume) reaction mixture at 4°C . Alternatively, as indicated in Table 1, a MasterAmp 2 $\times$ PCR premixture (Epicentre Technologies, Madison, Wis.) containing 100 mM Tris-HCl (pH 8.3), 5 to 7 mM MgCl_2 , each deoxynucleoside triphosphate at a concentration of 400 μM , and the PCR enhancer betaine was added to the reaction solutions. Amplifications were started by placing cooled (4°C) PCR tubes immediately into the preheated (94°C) thermal block of a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The thermal cycle profile consisted of initial denaturation for 3 min at 94°C , followed by 28 to 32 cycles (depending on the primer set) consisting of 30 s at 94°C , 30 s at the annealing temperature (as indicated in Table 1), and 45 s at 72°C (elongation) (5 min at 72°C for the last cycle). For PCR amplification with the *pmoA* and universal primer sets, the PCR conditions were optimized by using a touchdown program.

Aliquots (5 μl) of PCR products were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide, and quantified densitometrically. The gels were destained in water for 30 min. The Smart-Ladder DNA mass and size ruler (Eurogentec, Seraing, Belgium) was used for calibration (the calibration coefficient of all analyses was >0.9). The gels were photographed with an imaging system (MWG Biotech, Ebersberg, Germany), and DNA bands were analyzed with the RFLP-scan software (CSP Inc., Billerica, Belgium).

Cloning. 16S rDNA PCR products of soil DNA amplified with the MB9 α primer set were cloned by using a pGEM-TEasy cloning kit (Promega, Madison, Wis.). Randomly selected clones were sequenced as described previously (53).

FIG. 2. PCR yields of amplification reactions performed with different primer sets and template DNA isolated from soil samples without CH₄ oxidation (i.e., dry soil, control soil, lag-phase soil collected on days 1 and 2) and with CH₄ oxidation (days 4 to 23). The data are means $\pm$ standard deviations ($n = 6$ or 7).

added. PCR products of the expected sizes were obtained after amplification with the functional pmoA and mxaF primer sets and the 16S rDNA MB9 α , MB10 γ , and universal primer sets when the template DNAs were isolated from nine different methanotrophic reference strains and from soil samples. PCR products of the expected sizes were also obtained with the mmoB primer set that targets the *mmoB* gene coding for the β -subunit of sMMO for the appropriate reference strains. However, only a very weak PCR product was obtained from soil samples that oxidized CH₄. PCR amplification with the mmoB primer set containing a GC clamp failed, and thus, DGGE analysis of *mmoB* PCR products was not possible.

PCR amplification with the functional pmoA and mxaF primer sets and the 16S rDNA MB9 α and MB10 γ primer sets yielded significantly lower PCR product concentrations when we used template DNA extracted from control soil or lag-phase soil (at zero time and 2 days after CH₄ was added) than when we used DNA extracted from soil during the vigorous CH₄ consumption phase (4 to 23 days) (Fig. 2). In contrast, PCR amplification with the universal SSU rDNA primer set resulted in similar PCR product yields irrespective of the soil sample (Fig. 2). The uniform PCR amplification yields for all soil samples obtained with the universal primer set suggested that PCR-inhibiting substances in DNA templates, such as humic acids (59), did not bias amplification.

SSU rDNA DGGE and sequence analysis of members of the domain Bacteria. The DGGE analysis of PCR products amplified with the universal primer set revealed complex band patterns, which were similar for control soil without CH₄ and for soil supplemented with CH₄ (Fig. 3). The DGGE profiles obtained for dry rice field soil and control soil for the first 8 days of incubation contained fewer DGGE bands than the profiles obtained at later sampling times. The numbers and intensities of the bands increased after the soil was moistened. CH₄-supplemented soil obtained at zero time also produced fewer DGGE bands than soil obtained later. The sequences of the major DGGE bands (Fig. 3, bands I to V) grouped either with the gram-positive branch close to *Bacillus* species or with the *Cytophaga-Flavobacterium-Bacteroides* group (Fig. 3). However, sequences of methanotrophic or methylotrophic bacteria were not detected. Slight temporal changes in band patterns (Fig. 3) probably reflected CH₄-independent activation of bacteria by increases in the soil water content. The observation that even gram-positive, spore-forming *Bacillus* species known to be notoriously recalcitrant to lysis were detected by DGGE indicated that the lysis and DNA extraction protocols were

effective (42, 43). The sequences of DGGE bands with the same mobility were identical when they were obtained at different times (Fig. 3). This was also the case when we used primers other than the universal primers (see below).

16S rDNA DGGE and sequence analysis of type I methanotrophs. The DGGE profiles of the PCR products amplified with the MB10 γ primer set contained three major bands, which demonstrated that type I methanotrophs were less diverse than the total bacterial community (Fig. 4). The PCR product yields obtained from control and lag-phase soil samples (samples obtained at zero time and 2 days after CH₄ was added) were consistently lower than the PCR product yields obtained from soil samples consuming CH₄ (Fig. 4), which confirmed the PCR product yields shown in Fig. 2. Although twice the volume of the PCR product was loaded onto DGGE gels, the band intensities obtained with control soil were still lower than the band intensities obtained with CH₄-oxidizing soil (Fig. 4, samples obtained after days 4 to 23). Three bands were retrieved from DGGE gels. The DNA sequences of these bands grouped closely with the DNA sequences of *Methylobacter* species within the radiation of the type I methanotrophs (γ -*Proteobacteria*) (Fig. 4).

16S rDNA DGGE and sequence analysis of type II methanotrophs. The DGGE band patterns of PCR products obtained with the MB9 α primer set contained about 12 major bands after incubation with CH₄. Control soil samples produced about seven major bands, all of which were also present in the soil oxidizing CH₄ (Fig. 5, samples obtained after 18 and 23 days). The intensities of six bands increased after the soil started to consume CH₄ (Fig. 5), like the results obtained for PCR products obtained with the MB10 γ primer set. Pure excised and reamplified MB9 α DGGE bands could not be retrieved from DGGE gels, as indicated by the appearance of several DGGE bands when they were electrophoresed again on a DGGE gel. Therefore, the MB9 α PCR products from control soil (after 13 days) and soil consuming CH₄ (after 14 days) were cloned to obtain sequence data for type II methanotrophs ($n = 30$). The electrophoretic mobilities of the PCR products of the MB9 α clones were similar to those of the original soil DGGE bands. However, several DGGE bands of clones did not correspond to bands in the original DGGE band pattern, indicating that community analysis by cloning and community analysis by DGGE were subject to different biases (results not shown).

All of the clone sequences grouped with the α -*Proteobacteria*, but only one-third of the sequences grouped with the type II methanotrophs (i.e., *Methylosinus* and *Methylocystis* species) (Fig. 5). The other clones grouped with the genera *Caulobacter* and *Sphingomonas* (Fig. 5) (not all clones are shown). The sequences of two clones (clones c4 and c5) grouped closely with *Beijerinckia indica*. An acidophilic methanotroph (strain S6) that was recently isolated from a peat bog in Russia and probably represented a novel methanotrophic group (19) was also closely related to the genus *Beijerinckia* and exhibited 97% similarity to clones c4 and c5 from rice field soil.

DGGE and sequence analysis of the pMMO gene. Several methanotrophs contain at least two copies of the pMMO gene (56). This could explain the appearance of multiple strong DGGE bands for the pmoA PCR product of *Methylococcus capsulatus*, whereas *Methylosinus trichosporium* produced a very faint second band that was visible only on silver-stained DGGE gels (Fig. 6). It should be noted that the pmoA primer set is degenerate and also amplifies the *amoA* gene from ammonium oxidizers (32). The DGGE profiles of pmoA PCR products were markedly different for control soil and soil supplemented with CH₄ (Fig. 6). Control soil produced only one

FIG. 3. (A) DGGE band pattern obtained with the universal SSU rDNA primer set targeting all life. PCR product (20 μ l; $\sim 160 \pm 41$ ng) was loaded into each lane for all soil samples. The marked bands were sequenced. *Mcs*, *Methylocystis*; *Ms*, *Methylosinus*. (B) Phylogenetic tree constructed with partial 16S rDNA sequences, showing the relationship of the labeled DGGE bands to closely related members of the *Bacteria*. The sequences of the individual bands were obtained from the following lanes: band I, lanes 9d control, 4d, and 14d; band II, lanes 9d control and 14d; band III, lane 9d control; band IV, lane 4d; and band V, lane 9d control. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

major band, whereas CH_4 -oxidizing soil produced at least six additional bands, which appeared after 4 to 7 days of incubation and increased in intensity. At this time, the soil was oxidizing CH_4 at the maximal rate. Simultaneously, the intensities of the control soil bands decreased.

The deduced *pmoA* amino acid sequence data for the major DGGE bands confirmed that there was a shift in the population structure. The sequence for control soil DGGE band I (Fig. 6) (dry soil sample obtained at zero time) revealed an affiliation with ammonium oxidizers closely related to *Nitrosospora* species (β -*Proteobacteria*) (Fig. 6). On the other hand, the sequences of five DGGE bands obtained with CH_4 -consuming soil (samples obtained after 4 to 23 days) were

affiliated with type I and type II methanotrophs. Three sequences grouped most closely with members of the genus *Methylocystis*, while two type I sequences grouped with *Methylococcus capsulatus*. Bands II and III (Fig. 6) appeared to be clearly separated on a silver-stained gel, and we confirmed that these bands were two different bands by reamplification and subsequent DGGE analysis.

DGGE and sequence analysis of the MDH gene. The *mdhA* PCR products amplified from DNA extracted from control soil and soil supplemented with CH_4 were very weak and sometimes even undetectable at the beginning of the incubation period. Control soil produced only one DGGE band, whereas CH_4 -supplemented soil produced three bands after the onset

FIG. 4. (A) DGGE band pattern obtained with the MB10 γ 16S rDNA primer set targeting type I methanotrophs. For soil without CH₄ oxidation (lanes dry soil to 2d) 40 μ l (~63 to 100 ng) of PCR product was loaded into each lane, and for soil with CH₄ oxidation (lanes 4d to 23d) 25 μ l (~397 $\pm$ 80 ng) of PCR product was loaded into each lane. *Mc*, *Methylococcus*; *Mm*, *Methylomonas*. (B) Phylogenetic tree based on 16S rDNA sequences showing the relationship of the labeled DGGE bands to the most closely related members of the γ -Proteobacteria. The sequences of the individual bands were obtained from lane 18d. The scale bar indicates the estimated number of base changes per nucleotide sequence position.

of CH₄ oxidation (i.e., 4 days after CH₄ was added) (Fig. 7). Interestingly, the control soil band apparently was absent in the soil consuming CH₄ (Fig. 7). The *mxoF* gene sequences of purified DGGE bands from control soil and soil consuming CH₄ grouped with the cluster containing the type II methanotrophs (Fig. 7).

DISCUSSION

Rice field soil moistened to 43% of WHC and initially supplemented with 5% CH₄ oxidized CH₄ after a lag phase. The soil then consumed CH₄ at concentrations below atmospheric levels but was not able to maintain this capacity for a prolonged period of time if it was only supplemented with low CH₄ mixing ratios (<6 ppmv). We assumed that the initial CH₄ oxidation activity ($V_{\max}$) was high enough to allow consumption of CH₄ at atmospheric trace gas concentrations but that later CH₄ oxidation activity ceased, probably because oxidation of low CH₄ concentrations did not result in generation of the maintenance energy necessary for enzyme synthesis (16). Sim-

ilar observations of decreasing CH₄ oxidation rates after induction of CH₄ oxidation at high CH₄ concentrations have been made with other soils (4, 51, 54).

The molecular analysis of the soil microbial community during incubation in the presence of CH₄ revealed the following: (i) PCR amplification with the specific 16S rDNA MB10 γ and MB9 α primer sets, as well as with the functional *pmoA* and *mxoF* primer sets, resulted in significantly higher PCR product concentrations with soil consuming CH₄ than with control soil; (ii) the intensities of the DGGE bands of PCR products obtained with the MB10 γ , MB9 α , *pmoA*, and *mxoF* primer sets increased when soil consumed CH₄; and (iii) DGGE analysis of PCR products obtained with the MB10 γ , MB9 α , *pmoA*, and *mxoF* primer sets revealed differences in band patterns between control soil and CH₄-consuming soil.

We hypothesize that the increased PCR product concentrations observed after amplification with the specific MB10 γ and MB9 α primer sets and with primers targeting *pmoA* and *mxoF* from soil consuming CH₄ indicate that there are more target sites in soil oxidizing CH₄ than in control soil due to stimula-

FIG. 5. (A) DGGE band pattern obtained with the MB9 α 16S rDNA primer set targeting type II methanotrophs. For soil without CH₄ oxidation (lanes dry soil to 2d) 40 μ l ($\sim 360 \pm 118$ ng) of PCR product was loaded into each lane, and for soil with CH₄ oxidation (lanes 4d to 23d) 40 μ l ($\sim 572 \pm 162$ ng) of PCR product was loaded into each lane. *Mcs*, *Methylocystis*; *Ms*, *Methylosinus*. (B) Phylogenetic tree based on 16S rDNA sequences, showing the relationship of the MB9 α clones to the most closely related members of the α -Proteobacteria. The scale bar indicates the estimated number of base changes per nucleotide sequence position. c, clones from control soil (clones obtained after 13 days); ox, clones from soil with CH₄ oxidation (clones obtained after 14 days).

tion by CH₄. It is unlikely that the observed differences in PCR product concentrations were due to different concentrations of PCR-inhibiting compounds, such as humic acids, in the template DNA extracted from the soil. Since all soil samples were treated in the same way for DNA extraction, the concentrations of potential PCR inhibitors should a priori have been the same in all soil samples. This presumption was supported by PCR amplification results obtained with the universal primer set. These amplification results showed that the yield of PCR products was the same regardless of the soil sample. However,

it was surprising that the concentration of the *mxoF* PCR product was less than the concentration of the *pmoA* PCR product, since methanotrophs should contain both genes. We speculate that either there were more copies of the *pmoA* gene than of the *mxoF* gene per cell, the degenerate *pmoA* primer set also amplified the *amoA* gene from ammonium oxidizers present in the soil (see below), or PCR amplification operated more efficiently with the *pmoA* primer set than with the *mxoF* primer set.

Determination of microbial abundance based solely on PCR product concentrations is impossible (13, 21). However, Ferris

FIG. 6. (A) DGGE band pattern obtained with the *pmoA* primer set targeting the gene of the α -subunit of the pMMO. For soil without CH_4 oxidation (lanes dry soil to 2d) 35 μl ($\sim 181 \pm 59$ ng) of product was loaded into each lane, and for soil with CH_4 oxidation (lanes 4d to 23d) 35 μl ($\sim 707 \pm 203$ ng) of PCR product was loaded into each lane. *Mc.*, *Methylococcus*; *Ms.*, *Methylosinus*; *Mcs.*, *Methylocystis*. (B) Phylogenetic tree based on derived amino acid sequences of *pmoA* fragments, showing the relationship of the labeled DGGE bands to *pmoA* sequences of other methanotrophs. The sequences of the individual bands were obtained from the following lanes: band I, lanes dry soil, 9d control, 13d control, 4d, and 7d; bands II and III, lanes 7d and 14d; and bands IV through VI, lane 7d. The scale bar indicates the estimated number of changes per amino acid sequence position. Bootstrap values are given for 1,000 replicate trees.

and Ward (23) concluded that a change in the intensity of a particular DGGE band in a temporal or spatial environmental gradient may be used to infer that there has been a change in the size of a population. However, the intensities of different bands are not comparable to each other. Therefore, we concluded that the observed increases in the intensities of certain DGGE bands with longer exposure of soil to CH_4 indicated that there was an increase in the size of the methanotrophic population. Since the intensities of DGGE bands amplified with primers that target genes specific for methanotrophs were higher in the presence of CH_4 than in the absence of CH_4 , our data suggest that the sizes of CH_4 -oxidizing populations increased in rice field soil after it received CH_4 at high mixing ratios. A similar increase in the size of a methanotrophic population has also been demonstrated by most-probable-number

counting by using incubation mixtures having initial CH_4 mixing ratios of $>7,000$ ppmv (4).

The DGGE band pattern for 16S rDNA templates amplified with the universal primer set was as complex as expected for a habitat such as soil. Changes in the DGGE band pattern occurred only at the beginning of the experiment when the soil water content changed from dry to moist. Addition of CH_4 , however, had no effect on the DGGE band pattern. We assumed that the soil microorganisms responded to the difference in the soil water content and that this resulted in population shifts. The DNA sequences of the major DGGE bands grouped closely with the genera *Flexibacter* and *Flavobacterium* in the *Cytophaga-Flavobacterium-Bacteroides* group and with the genus *Bacillus* in the gram-positive bacteria with low G+C contents. Isolation of abundant fermentative rice field soil bac-

FIG. 7. (A) DGGE band pattern obtained with the *mxoF* primer set targeting the gene of the α -subunit of the MDH. For soil without CH_4 oxidation (lanes dry soil to 2d) 35 μl ($\sim 22 \pm 5$ ng) of PCR product was loaded into each lane, and for soil with CH_4 oxidation (lanes 4d to 23d) 35 μl ($\sim 75 \pm 56$ ng) of PCR product was loaded into each lane. *Mc.*, *Methylococcus*; *Ms.*, *Methylosinus*; *Mb.*, *Methylobacter*. (B) Phylogenetic tree based on derived amino acid sequences of *mxoF* fragments, showing the relationship of the labeled DGGE bands to *mxoF* sequences of other methylobacteria. The sequences of *Hyphomicrobium* spp. were obtained from reference 24. The sequences of the individual bands were obtained from the following lanes: band I, lane 23d; band II, lanes 13d control and 35d control; and band III, lanes 14d and 18d. The scale bar indicates the estimated number of changes per amino acid sequence position. The bootstrap values shown are the bootstrap values for 1,000 replicate trees.

teria and a molecular survey of microbial diversity performed by cloning 16S rDNA genes showed that, indeed, bacteria belonging to these two groups represent a major portion of the bacterial community in rice field soil (14, 30).

The DGGE band patterns of PCR products obtained with the 16S rDNA MB10 γ and MB9 α primer sets that target methylobacterial bacteria, as well as the functional primer sets that target *pmoA* and *mxoF*, contained fewer bands than the DGGE band patterns obtained with the universal SSU rDNA primer set. This observation is consistent with the assumption that the community of specialized CH_4 oxidizers was smaller than the community of microorganisms in general. DNA sequences of MB10 γ and *pmoA* primer set-generated DGGE bands grouped closely with DNA sequences of *Methylobacter* sp. and *Methylococcus capsulatus* within the type I methanotrophs. However, *mxoF* primer set-generated DGGE bands, some *pmoA* primer set-generated DGGE bands, and clone sequences obtained with the MB9 α primer set grouped with the type II methanotrophs. The presence of type II meth-

anotrophs (or type X methanotrophs, including *Methylococcus capsulatus*) in soil consuming CH_4 was also revealed by the PCR that targeted *mmoB*, but only soil samples that oxidized CH_4 at high rates produced an *mmoB* PCR product.

Differences between the DGGE band patterns obtained with soil incubated in the presence of CH_4 and soil incubated in the absence of CH_4 were observed when the *pmoA* and *mxoF* PCR products were examined. The major DGGE band detected and sequenced in control soil grouped close to *Nitrosospora* species. The *pmoA* primer set targeting *pmoA* also amplifies the *amoA* gene of ammonium oxidizers coding for the α -subunit of the AMO (32). *pmoA* primer set-generated DGGE bands that were related to bands of methanotrophs, as determined by sequencing, intensified after the onset of CH_4 oxidation, while the *pmoA* primer set-generated DGGE band closely related to the *Nitrosospora* band became fainter. Apparently, ammonium-oxidizing populations were outnumbered or outcompeted by methanotrophs when CH_4 consumption started. Our observation is in agreement with recent results of Bodelier and Frenzel

(5), who demonstrated that ammonium oxidizers did not contribute to CH₄ oxidation in rice microcosms.

16S rDNA sequence analysis of clones revealed that the MB9 α primer set was not specific for type II methanotrophs and detected other α -*Proteobacteria* as well. Therefore, changes in the DGGE band pattern observed with MB9 α primer set-generated PCR products after the onset of CH₄ oxidation cannot be linked to methanotrophs exclusively. However, 16S rDNA sequences that clustered with sequences of methanotrophic bacteria were related to type II methanotrophs, which supported the data obtained in the *mxhF* and *pmoA* analyses. The number and diversity of sequence types obtained by cloning MB9 α primer set-generated PCR products indicated that the diversity was greater than the diversity observed in the DGGE analysis of the same PCR products. DGGE analysis revealed only the most abundant populations, while cloning probably also detected less abundant populations.

Two MB9 α primer set-generated clones grouped close to *B. indica* and novel, recently isolated, acidophilic methanotrophs (19). Detection of these closely related populations in neutral rice field soil suggests that this new group of methanotrophs may not be limited to acidic peat bogs. However, these novel methanotrophs must be isolated from rice field soil to support our molecular findings.

Our knowledge of the community structure of methanotrophs in rice field soil has been limited. Recently, two type II methanotrophs (strains Rp1 and Rp2) were isolated from high dilutions of most-probable-number count preparations obtained from the rhizoplane of rice roots (27). Generally, type II methanotrophs have been detected more frequently in soil environments than type I methanotrophs (see above). Several factors that influence competition between type I and type II methanotrophs, such as CH₄ and O₂ concentrations and nitrogen and copper availability, are currently being examined (28). Amaral and Knowles (1, 2) studied the distribution of soil and sediment methanotrophs in agarose diffusion columns with opposing gradients of CH₄ and O₂. These authors concluded that type I methanotrophs may be favored at low CH₄ concentrations and high O₂ concentrations, whereas type II methanotrophs may be favored at high CH₄ concentrations and low O₂ concentrations (2). The prevalence of type II methanotrophs in soils and the presence of type I methanotrophs in aquatic sediments support this hypothesis (28). With respect to O₂ and CH₄ availability, flooded rice field soil provides at least two different niches for methanotrophs: (i) the soil surface of flooded rice field soil (bulk soil) and (ii) the rhizosphere. The soil surface is comparable to aquatic sediments and is characterized by steep opposing O₂ and CH₄ gradients (26). At the interface of these gradients, the concentrations of O₂ and CH₄ are both very low, which could favor both types of methanotrophs. In the rhizosphere, O₂ and CH₄ concentrations can both be very low, but this region is characterized by spatial and temporal heterogeneity of O₂ and CH₄ concentrations due to the influence of the rice roots (26). The results of *in situ* probing with 16S rDNA-based probes suggested that type II methanotrophs were numerically dominant in the rhizosphere of aquatic macrophytes (37), and two numerically relevant type II methanotrophs were isolated from the rhizoplane of rice (27).

In this study, we used rice field soil originating from all of the habitats described above. Our results show that both type I and type II methanotrophs were present in the original rice field soil. When the soil was incubated under moist conditions with high mixing ratios of CH₄, we obtained DGGE bands of both type I and type II methanotrophs, which indicated that these bacteria were active. Apparently, factors other than O₂ and

CH₄ availability may determine the composition of the methanotrophic community in rice field soil.

The active methanotrophs were apparently not able to consume CH₄ at atmospheric CH₄ mixing ratios, since CH₄ oxidation activity eventually ceased when CH₄ was supplied at low concentrations (<5 ppmv). Nevertheless, the populations of methanotrophs seemed to persist, as indicated by the results of the DGGE analyses. Recent experiments showed that some CH₄ production takes place even in drained, nonsaturated rice field soil, probably because there are anoxic niches in which active methanogenesis occurs (34). Therefore, it is possible that the methanotrophs were supplied with additional CH₄ that was produced inside soil aggregates.

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