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ORIGINAL ARTICLE

Applying stable isotope probing of phospholipid fatty acids and rRNA in a Chinese rice field to study activity and composition of the methanotrophic bacterial communities *in situ*

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Methanotrophs in the rhizosphere play an important role in global climate change since they attenuate methane emission from rice field ecosystems into the atmosphere. Most of the CH_4 is emitted via transport through the plant gas vascular system. We used this transport for stable isotope probing (SIP) of the methanotrophs in the rhizosphere under field conditions and pulse-labelled rice plants in a Chinese rice field with CH_4 (99% ^{13}C) for 7 days. The rate of $^{13}CH_4$ loss rate during ^{13}C application was comparable to the CH_4 oxidation rate measured by the difluoromethane inhibition technique. The methanotrophic communities on the roots and in the rhizospheric soil were analyzed by terminal-restriction fragment length polymorphism (T-RFLP), cloning and sequencing of the particulate methane monooxygenase (*pmoA*) gene. Populations of type I methanotrophs were larger than those of type II. Both methane oxidation rates and composition of methanotrophic communities suggested that there was little difference between urea-fertilized and unfertilized fields. SIP of phospholipid fatty acids (PLFA-SIP) and rRNA (RNA-SIP) were used to analyze the metabolically active methanotrophic community in rhizospheric soil. PLFA of type I compared with type II methanotrophs was labelled more strongly with ^{13}C, reaching a maximum of 6.8 atom-%. T-RFLP analysis and cloning/sequencing of 16S rRNA genes showed that methanotrophs, especially of type I, were slightly enriched in the 'heavy' fractions. Our results indicate that CH_4 oxidation in the rice rhizosphere under *in situ* conditions is mainly due to type I methanotrophs.
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Introduction

Flooded rice fields, an important source of atmospheric methane, are widely distributed in China (Cai, 1997; Li *et al.*, 2002; Cai *et al.*, 2003). The total emission of CH_4 from Chinese rice fields was estimated to be in the range of 8.05 ± 3.69 Tg CH_4 per year, depending on the type of rice paddy field (Cai, 1997). In these ecosystems, the main CH_4 emission to the atmosphere occurs through the aerenchyma system of the rice plants (Nouchi *et al.*, 1990; Schütz *et al.*, 1991). In turn, oxygen from the atmosphere is transported to the rice roots enabling aerobic and microaerophilic conditions in the rhizosphere (Liesack *et al.*, 2000). Aerobic methanotrophs in such rice rhizosphere environments play an important role in CH_4 emission, since they attenuate the CH_4 flux from the soil into the atmosphere covering a large range between 0 and 94% attenuation (Schütz *et al.*, 1989; Groot *et al.*, 2003). The activity of aerobic methanotrophs strongly depends on the environmental conditions, for example the *in situ* concentrations of not only CH_4 and O_2, but also of other compounds, such as ammonium, sulfide or ferrous iron. The latter compounds can also be microbially oxidized in the

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rhizosphere resulting in competition with methanotrophs for oxygen. Ammonia oxidizers may affect CH_4 oxidation due to the similarity between the key enzymes catalyzing the first step of CH_4 oxidation and ammonium oxidation (Mancinelli, 1995). In previous studies, inhibition (Bosse *et al.*, 1993; Nold *et al.*, 1999), stimulation (Bodelier *et al.*, 2000; Eller and Frenzel, 2001) or no effect (Lilkanen and Martikainen, 2003) on aerobic methanotrophs were observed in anoxic soils and sediments when fertilized with ammonium or urea (see review by Bodelier and Laanbroek, 2004). Another important factor for CH_4 emission is the composition of the methanotrophic communities, which seem to be different in bulk soil and rhizosphere and change dynamically over the season and upon fertilization with ammonium or urea (Bodelier *et al.*, 2000; Eller and Frenzel, 2001; Macalady *et al.*, 2002; Eller *et al.*, 2005; Shrestha *et al.*, 2008).

A further complication is the fact that not all members of the total methanotrophic community are equally active in attenuation of the CH_4 flux. Pulse labelling of rice plants with ^{13}C followed by stable isotope probing (SIP) of nucleic acid and membrane lipids has been demonstrated as a powerful approach to identify the metabolically active microbial communities in the rhizosphere of rice (Lu and Conrad, 2005; Lu *et al.*, 2006, 2007). SIP allows direct observations of substrate assimilation in minimally disturbed communities, and thus represents an exciting tool for linking microbial identity and function. As methanotrophs use CH_4 as carbon source, SIP of phospholipid fatty acids (PLFAs) (Boschker *et al.*, 1998; Boschker and Middelburg, 2002) or DNA/RNA (McDonald *et al.*, 2005) using ^{13}C-labelled CH_4 is a promising approach. Recently, rice microcosms were used to identify the methanotrophs actively assimilating CH_4-carbon that was introduced into the rice rhizosphere by means of permeable tubings (Shrestha *et al.*, 2008). However, a labelling approach under field conditions has not been conducted, and therefore the ability to compare laboratory-based results is still lacking.

Therefore, we applied SIP in a Chinese rice field by pulse labelling the rice plants with ^{13}C-labelled CH_4 through the aerenchyma systems of the rice plants into the rhizosphere. This enables an *in situ* labelling of methanotrophs located directly at the rice rhizosphere without disturbing the soil ecosystem. Furthermore, we tested if typical Chinese farm fertilization procedures had an effect on methanotrophic activity and on the global methane emission budget from this environment.

Materials and methods

Study site and treatments

The experimental field was located at the China National Rice Research Institute (CNRRI; Hangzhou, Zhejiang province, China; 30°25′N, 120°18′E). The fields cover an area of 494 ha, representing a typical rice growing area with annual single- and double-rice cropping systems. The climate is warm/cool humid subtropical with an annual rainfall of 1400 mm and a mean solar radiation of 12.8 MJ m^{-2} day^{-1}.

The field had been treated according to the common agricultural practice of the region. Fertilizers were applied directly to the rhizosphere of rice plants at the rates of 0.09 g P and 0.41 g K per plant and additional 0.6 g urea at 48 days after transplantation. For our study, urea treatment (PKN) was done in the same field, but separated with four rows of rice plants from those plots that were only fertilized with PK. The water level was kept constant at 5–10 cm depth. The field plots were shaded with transparent black fiber tissue to avoid excessively high solar radiation during our experiment to reduce the evaporation inside the gas chambers. The soil is a silty clay soil with a pH of 6.7, organic C of 24.2 g per kg soil, total N of 2.3 g per kg soil and cation exchange capacity of 144 mmol per kg soil.

Methane flux measurement

Methane flux was measured on 4 consecutive days starting 3 days after fertilization. The measurements were always done between 12:00 and 16:00 hours local time. The cylindrical flux chambers (diameter = 5 cm and height = 40 cm) were made of plexiglas. For flux measurements, rice plants were covered for a period of 4 h and gas samples were withdrawn every 30 min. Methane emission rates were determined from the slope of the linearly increasing CH_4 mixing ratio using a gas chromatograph with flame ionization detector.

Loss rates of $^{13}CH_4$ were determined after injection of 1 ml of ^{13}C-labelled CH_4 (99 atom-% $^{13}CH_4$; Sigma, Taufkirchen, Germany) into the flux chamber. The amount of $^{13}CH_4$ at time t (n_t) was calculated by:

$$n_t = m_t(a_t - a_p)$$

m_t = total amount of CH_4 at time t; a_t = ^{13}C atom-% of CH_4 at time t; a_p = natural ^{13}C atom-% of CH_4. The ^{13}C atom-% was analyzed using a gas chromatography–combustion–isotope ratio mass spectrometry as described previously (Conrad *et al.*, 2002).

The rate of CH_4 oxidation was determined by measuring the CH_4 flux in the presence and absence of difluoromethane (20 ml CH_2F_2), an inhibitor of CH_4 oxidation (Miller *et al.*, 1998), which was injected into the flux chamber. The measurements were done as described by Krüger *et al.* (2001). It was made sure that the concentration of CH_2F_2 did not decrease over the 4-h incubation time. Methane oxidation rates were calculated by

$$\text{Oxidation rate} = \text{Emission rate inhibited} - \text{Emission rate control}$$

The rates of the flux measurements are expressed in mmol CH_4 per m^2 per h giving the mean ± s.e. of $n = 4$ data obtained on 4 consecutive days.

^{13}C-labelling and sampling procedures
Labelling with $^{13}CH_4$ was started 36 h after fertilization. Plots were covered with closed chambers as described above. For both the N-fertilized (PKN) and the unfertilized (PK) plots the following experiments were performed: (1) rice plants were cut so that stubbles were 15 cm above the water surface and 15 ml of $^{13}CH_4$ (99 atom-% $^{13}CH_4$; Sigma) was added to the chamber twice a day ($^{13}CH_4$ cut). (2) Rice plants were left intact and treated as above ($^{13}CH_4$). (3) Rice plants were left intact and treated with 15 ml of unlabelled CH_4 ($^{12}CH_4$). (4) Rice plants were left intact and no CH_4 was injected into the chamber (air). At the end of 7-day labelling, the chambers were removed and the rice plants were pulled out. Four replicates were taken in each treatment. The bulk soil fell off the roots during plant tiller separation and with gentle shaking. The soil still adhering to the roots was defined as rhizospheric soil. After cutting off the shoots, the roots with the adhering soil were separated into three batches and immediately frozen in liquid nitrogen and stored at −80 °C. Transport to laboratories in Beijing (PLFA extraction) and Marburg (DNA/RNA extraction) was done within 20 h keeping the samples in a frozen state.

Phospholipid fatty acid extraction and ^{13}C profiling
After thawing one batch of the soil–roots samples for each replicate treatment, the soil was carefully washed from roots with deionized sterile water. The soil slurries were collected separately and centrifuged at 950 *g*. Total PLFA was extracted from 4 g fresh soil samples using the Bligh–Dyer method (Bligh and Dyer, 1969). The total amount of PLFA was determined by gas chromatography–mass spectrometry and the stable isotope ratio of individual fatty acid methyl esters by gas chromatography–combustion–isotope ratio mass spectrometry as described by Lu *et al*. (2007).

The relative amounts of individual fatty acid methyl esters were calculated on the basis of the amount of PLFA 16:0. This PLFA was chosen as it is ubiquitous in prokaryotes. The fractional abundance (F) of ^{13}C in the unlabelled (F_u) and labelled (F_l) PLFAs was used to calculate the concentration of ^{13}C incorporated into PLFAs ([PLFA-^{13}C]) from the total PLFA concentration ([PLFA]):

$$[\text{PLFA-}^{13}\text{C}] = (F_u - F_l)[\text{PLFA}]$$

The fractional abundance expresses the amount of ^{13}C as a proportion of the total amount of carbon in the PLFA:

$$F = {}^{13}\text{C}/({}^{12}\text{C} + {}^{13}\text{C})$$

DNA extraction and pmoA gene T-RFLP analysis
After thawing another batch of the soil-roots samples for each replicate treatment, the soil was carefully washed from roots with deionized sterile water. The soil slurries were collected separately and centrifuged at 950 *g* and used for nucleic acid extraction. DNA was extracted from rhizospheric soil according to Lueders *et al*. (2004) with minor modifications during the precipitation step. DNA was precipitated from the aqueous phase with 1 volume isopropanol and centrifugation at maximum speed (21 000 *g*) at 4 °C for 1 h. The precipitate was washed once with 500 µl of pre-cooled 70% ethanol, dried and re-suspended in 50 µl of PCR water (Sigma-Aldrich). Additional purification was done using a DNA clean-up system (Promega, Mannheim, Germany). Amplification of the particulate methane monooxygenase (*pmoA*) gene was performed using the primer sets 189f/682r and 189f-FAM/mb661r using nested PCR (Horz *et al*., 2005). Purified amplicons were digested by *Msp*I (*Hpa*II) (Fermentas, St Leon-Rot, Germany) at 37 °C for 3 h and inactivated at 65 °C. Aliquots of desalted digest (1–2 µl) were mixed with 12 µl of Hi-Di formamide (Applied Biosystems, Weiterstadt, Germany) and 0.25 µl of ROX-labelled MapMarker 1000 ladder (BioVentures, Murfreesboro, TN, USA), denaturated for 3 min at 95 °C, cooled on ice immediately and size separated on an ABI 310 genetic analyzer (Applied Biosystems). Electrophoresis was performed with POP-4 polymer in 47 cm by 50 µm capillaries (Applied Biosystems) with 15 kV injection voltage, 15 kV run voltage, 60 °C run temperature and 45 min analysis time. The peak heights were automatically quantified by the GeneScan software. The relative abundance of individual terminal-restriction fragments (T-RFs) were calculated based on the peak height of the individual T-RFs in relation to the total peak height of all T-RFs detected in the respective terminal-restriction fragment length polymorphism (T-RFLP) community pattern.

RNA extraction and gradient centrifugation
Total RNA was extracted from the rhizospheric soil samples $^{13}CH_4$ cut + PKN and $^{12}CH_4$ cut + PKN using a previously described protocol (Noll *et al*., 2005). RNA for reverse transcription (RT)-PCR was obtained by removal of co-extracted DNA using RNase-Free DNase (Promega) following the manufacturer's protocol. Additional RNasin (Promega) was added to inhibit RNases. The RNA pellets were re-suspended in 50 µl nuclease-free water (Promega). The absence of DNA in the resulting small-subunit ribosomal RNA (16S rRNA) preparation was checked by PCR of bacterial 16S rRNA genes and standard agarose gel electrophoresis on a 1% agarose followed by ethidium bromide staining. RiboGreen (500 ng; Invitrogen, Karlsruhe, Germany) was used to quantify RNA content in the extracts.

The RNA was density resolved by equilibrium density gradient centrifugation in CsTFA (cesium trifluoroacetate) (Amersham Biosciences, Freiburg, Germany) under the conditions reported previously (Lueders *et al.*, 2004). After centrifugation, the density of the collected gradient fractions was determined refractometrically, and RNA was precipitated and re-suspended in 30 µl nuclease-free water for subsequent community analyses. Control gradients were conducted with RNA from unlabelled soil ($^{12}CH_4$ cut−PKN).

RT-PCR of bacterial 16S rRNA gene and T-RFLP analysis

Terminal-restriction fragment length polymorphism analysis of density-resolved bacterial communities from gradient fractions was performed with primers Ba27f-FAM/Ba907r by RT-PCR using a one-step RT-PCR system (Access Quick1 RT-PCR-System; Promega). The reaction mixture (50 ml) contained 25 ml of 2 × reaction buffer, 0.5 mM of each primer (MWG Biotech, Ebersberg, Germany), 1.0 µl of template RNA and 1 µl (5 U) of AMV reverse transcriptase supplied by the manufacturer. The 2 × reaction buffer included 3 mM $MgSO_4$ (final concentration 1.5 mM), Tfl DNA polymerase (unknown amount) and the deoxynucleoside triphosphates (concentration was not provided by the manufacturer). The RT reaction was carried out at 48 °C for 45 min and followed by a denaturing step at 94 °C for 2 min to inactivate the AMV reverse transcriptase and to activate the Tfl DNA polymerase. The thermal profile of the PCR included 25–30 cycles of primer annealing at 52 °C for 45 s, primer extension at 68 °C for 1 min and denaturing at 94 °C for 45 s. The final elongation step was 5 min. Amplicons were digested by *Msp*I and separated as described above for the *pmoA* gene. To verify the assignments of T-RFs to our detected methanotrophs, we also tested individual methanotrophic clones indicated in sequence data by T-RFLP analysis.

Cloning, sequencing and phylogenetic analysis

Four clone libraries were created, one of *pmoA* genes from sample $^{13}CH_4$ cut + PKN (54 clones) and three of bacterial 16S rRNA genes from density-resolved 'heavy' (HM) (101 clones) and 'light' (LM) RNA fractions (74 clones) and control (CM) (78 clones) as indicated in Figure 5. Amplicons for sequence analyses were generated with the same primers and PCR conditions as described above and cloned into *Escherichia coli* using the pGEM-T Vector System (Promega). The randomly selected clones were sequenced by the ADIS DNA core facility (Max Planck Institute for Plant Breeding Research, Cologne, Germany) on an ABI Prism 3700 sequencer (Applied Biosystems) using BigDye terminator cycle sequencing chemistry. Raw sequence data were assembled and checked with DNAStar (Madison, WI, USA). All sequences were compared with those in the public databases by using NCBI megaBlast and the highest matched sequences were obtained from GenBank database (http://www.ncbi.nih.gov). Chimeras were checked for with the Bellerophon server (http://foo.maths.uq.edu.au/~huber/doc/doc.html) (Huber *et al.*, 2004) for all sequences and chimera sequences were excluded for further analysis. Phylogenetic analysis were conducted using the ARB software package (Ludwig *et al.*, 2004). A neighbor-joining tree was calculated for bacterial 16S rRNA gene sequences including a 50% base frequency filter. Neighbour-joining, maximum likelihood and tree-puzzle programs were used for calculating *pmoA*-based phylogenetic trees in which all showed the same topology.

Sequence data have been submitted to EMBL under accession numbers AM909841 to AM910093 for 16S rRNA gene and AM910094 to AM910147 for *pmoA* gene.

Results

Methane oxidation activity

We fertilized two plots of the same rice field with either phosphorous and potassium (PK treatment) or with phosphorous, potassium and nitrogen (PKN treatment) using the fertilization procedure typical for Chinese agriculture. Nine days after fertilization, ammonia concentrations in bulk soil were two times higher in the PKN treatment (27.6 ± 0.7 mg N per kg dry soil) compared with the PK treatment (11.5 ± 0.4 mg N per kg dry soil), while nitrate concentrations were low in both treatments (0.6 ± 0.1 and 1.1 ± 0.1 mg N per kg dry soil, respectively). Three to seven days after fertilization, CH_4 emission flux from the rice field plots was measured using the closed chamber technique. The flux was measured in the presence and absence of difluoromethane (CH_2F_2) as specific inhibitor of methanotrophs (Miller *et al.*, 1998), the difference being the rate of CH_4 oxidation (Table 1). Both CH_4 oxidation activity and the loss of $^{13}CH_4$ in the chambers to which ^{13}C-labelled CH_4 was added indicate that about 2–18% of the produced CH_4 was oxidized (Table 1). The rates of CH_4 oxidation or $^{13}CH_4$ loss were on the same order of magnitude irrespectively of whether the fields were fertilized with urea or not.

Table 1 Methane fluxes in rice field without (PK) and with (PKN) nitrogen fertilization; mean ± s.e., $n = 3$–4

CH_4 flux (mmol per m^2 per h)	*PK*	*PKN*
CH_4 emission rate	8.7 ± 1.4	12.1 ± 4.0
CH_4 oxidation rate	1.1 ± 0.8	0.3 ± 1.2
$^{13}CH_4$ loss rate	2.0 ± 0.3	1.6 ± 0.4

Isotopic enrichment of PLFAs

Rhizospheric soil samples were retrieved from rice field plots after different treatments, that is, fertilization with (PKN) and without (PK) urea, cutting plants above the water surface (cut), and addition of $^{13}CH_4$, $^{12}CH_4$ or no CH_4 (air) to the headspace of the flux chambers. Cutting plants above the water surface does not impede the CH_4 flux into the atmosphere (Seiler *et al*., 1984; Conrad and Schütz, 1988). The relative abundance of total PLFA was very similar in all the soil samples irrespectively of the treatment. The PLFA included Gram-positive bacterial biomarkers (15:0i, 15:0a, 17:0i, cy17:0d7,8 and 10Me-16:0) and Gram-negative biomarkers (16:1ω7, 18:1ω7 and cy19:0d8,9) (Figure 1).

Pulsed addition of $^{13}CH_4$ into the gas headspace led to strong labelling of methanotrophic PLFAs, reaching up to 6.8 ^{13}C atom-% in 16:1ω7 PLFA of the $^{13}CH_4$ cut + PKN treatment. Mainly, the saturated and monounsaturated PLFAs with 16 and 18-carbon atoms exhibited a significant isotopic enrichment (Figure 1). Notably, the monounsaturated 16:1ω7 and 16:1ω6 were highly ^{13}C-labelled in the PLFA profiles, which have been often affiliated to type I methanotrophs (Nold *et al*., 1999; Mohanty *et al*., 2007). Saturated C_{14} fatty acid, which occurs in type I *Methylomonas* species (Hanson and Hanson, 1996; Evershed *et al*., 2006), was also labelled to a less extent. The PLFAs 18:1ω7 and 18:1ω9, occurring in type II methanotrophs (Nold *et al*., 1999; Mohanty *et al*., 2007), were relatively strongly ^{13}C-labelled, but with less intensity than the PLFAs with 16-carbon fatty acids. There was no significant difference between the plots with and without ammonium fertilization (analysis of variance, $P > 0.05$) (Figure 1). Likewise, the treatments with and without cutting of the plants had similar isotope incorporation PLFA profiles (Figure 1). In PLFAs extracted from the unlabelled methane control soil, there was no enrichment of ^{13}C in the PLFAs.

Characterization of methanotroph communities

To characterize the functional gene diversity of the methanotrophic community, we applied *pmoA* gene-based T-RFLP fingerprint analysis of the different treatments after 7 days of incubation (Figure 2). Neither urea fertilization, nor addition of unlabelled or labelled CH_4, nor cutting of the plants had a significant (analysis of variance, $P > 0.05$) effect on the community structure of the T-RFLP patterns.

To assess the *pmoA* gene-based diversity, we cloned and sequenced in total 54 clones from pooled PCR products from all replicates of $^{13}CH_4$ cut + PKN. This treatment was chosen for cloning, as it was also used for SIP of rRNA (RNA-SIP) (see below). Out of 54 clones, 17 represented type II methanotrophs, which clustered within the *Methylocystis* group (Figure 3). These type II methanotrophs represented the 244-bp T-RF. All the other T-RFs represented type I methanotrophs. Type I *pmoA* made up approximately 80% of the total methanotrophic community. Some (15 clones) of the *pmoA* clones with a T-RF = 79 bp, represented *pmoA* from a group of type I methanotrophs that was distinct from *pmoA* from extant methanotrophs. Other clones clustered with *pmoA* from extant *Methylococcus/Methylocaldum*, *Methylomonas* and *Methylomicrobium/Methylobacter* species (Figure 3). The T-RF of 456 bp length occurred only with a low relative abundance (<1%) in the T-RFLP analyses and was affiliated to members of the genus *Methylomicrobium* (Figure 3).

Structure of bacterial community in rhizospheric soil

Ribosomal RNA was extracted from the rhizospheric soil of samples $^{13}CH_4$ cut−PKN and $^{12}CH_4$ cut−PKN, respectively. The RNA extracts were centrifuged in a density gradient and density fractions were amplified via RT-PCR with bacterial primers for subsequent T-RFLP analyses (Figure 4). Three different fractions were used to construct clone libraries of bacterial 16S rRNA, one from the control incubated with $^{12}CH_4$ (CM), one from the 'heavy' RNA (HM) and one from the 'light' RNA (LM) fraction as indicated in Figure 4. In total, 101 clones were sequenced from the HM library; 65% were affiliated to *Proteobacteria* and the remaining 35% belonged to diverse phylogenetic taxa including *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Gemmatimonadales*, *Thermomicrobia*, *Cyanobacteria*, *Nitrospirae*, *Verrucomicrobia*, *Bacteroidetes* and *Planctomycetes* (Figure 5, Table 2) (note the recent discovery of thermoacidophilic methanotrophs belonging to the *Verrucomicrobia*; Dunfield *et al*., 2007; Pol *et al*., 2007; Islam *et al*., 2008). Of the 74 clones sequenced from the LM library and the 78 clones sequenced from the CM library, 62 and 45%, respectively, were affiliated to *Proteobacteria*. *Deltaproteobacteria*-like clone sequences dominated the retrieved sequences affiliated to *Proteobacteria* (34, 28 and 33% in HM, LM and CM clone library, respectively). Methanotrophic bacteria within the *Alphaproteobacteria* and *Gammaproteobacteria* were relatively more frequent in the HM library than in the LM and CM clone libraries (Table 2). *In silico* prediction of T-RF sizes from sequence data (Table 2) allowed the assignment of the major T-RFs observed in the bacterial fingerprints (Figure 5).

Metabolically active methanotrophic community

The comparison between 16S rRNA T-RFLP fingerprinting and clone libraries indicated a relative increase of the 150-bp, 455-bp and 487-bp T-RFs in the 'heavy' compared with the other RNA fractions (Figure 4). The T-RFLP patterns are in agreement with the higher abundance of methanotrophs in the HM versus the LM and CM clone libraries (Table 2). Thus, it seems that methanotrophs were enriched in

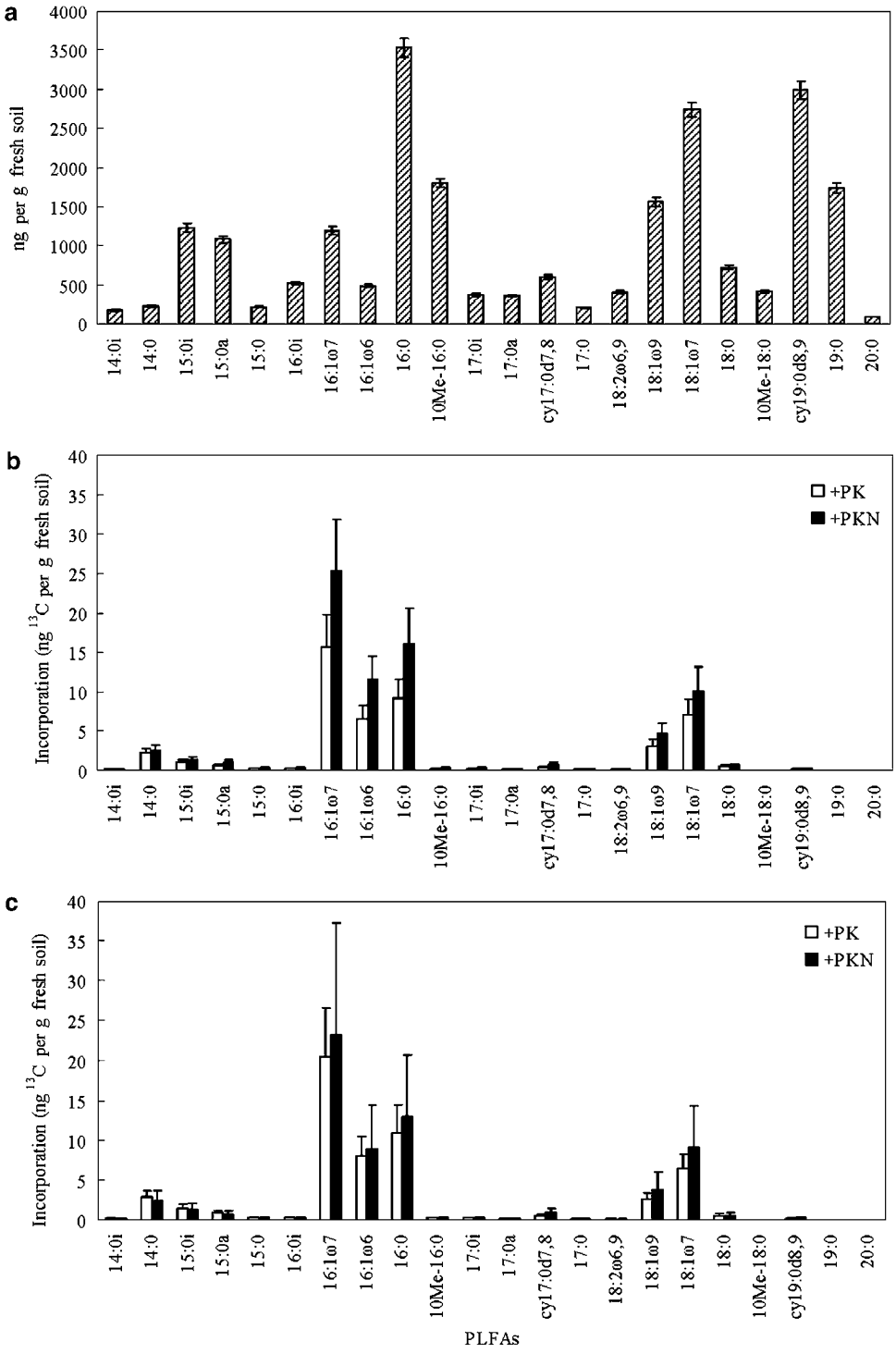

Figure 1 (**a**) Concentrations of individual phospholipid fatty acids (PLFAs) at the end of the field experiment using samples from all the different treatments including N fertilization and cutting of plants; mean ± s.e., $n = 32$. The small error bars show that concentrations of PLFAs were similar in the different treatments. (**b** and **c**) Concentrations of ^{13}C-labelled PLFA in the soil at the end of the field experiment with (PKN) and without (PK) addition of N fertilizer on plots with (**b**) intact plants and (**c**) plants cut above the water surface, both labelled with $^{13}CH_4$; mean ± s.e.; $n = 4$.

the 'heavy' fraction. The T-RFs of 455 and 487 bp represented type I methanotrophs. Only *Methylosarcina* and *Methylomicrobium* had a T-RF = 487 bp when considering all the bacterial 16S rRNA sequences retrieved (Table 2). Some of type I methanotrophs had 444-bp and 479-bp T-RFs as indicted by the HM clone library (Table 2), but these T-RFs were not seen in the T-RFLP patterns

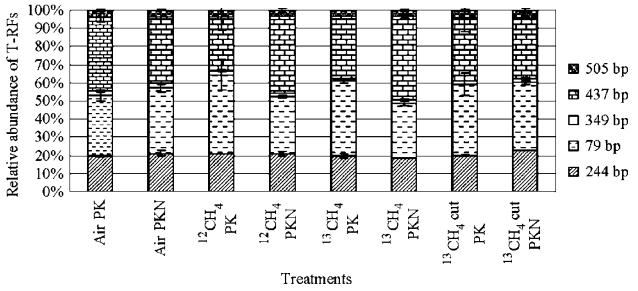

Figure 2 Relative abundance of terminal-restriction fragments (T-RFs) of particulate methane monooxygenase (*pmoA*) amplicons obtained from the various treatments at the end of the field experiment. 244-bp T-RF is representative of type II methanotrophs; the others (79, 349, 437 and 505 bp) are representative of type I methanotrophs. PK and PKN indicates the fertilizers applied; air, $^{12}CH_4$ and $^{13}CH_4$, the gas treatment; cut indicates that plants were cut above the water surface; mean ± s.e., $n = 2$.

(Figure 4). The T-RFs of 150 bp length were mainly found in clones belonging to *Methylocystis* species and exhibited a significant increase in the 'heavy' fractions (Figure 4). No other type II methanotrophs were found in the 'heavy' fractions. Hence, type I methanotrophs, including *Methylobacteria*, *Methylosarcina*, *Methylomicrobium* and *Methylomonas*, seemed to be more diverse than type II methanotrophs in the 'heavy' fraction (Figure 4).

Discussion

By applying $^{13}CH_4$ to the phyllosphere of rice, we were able to identify for the first time active methanotrophs under field conditions. Both PLFA-SIP (SIP of phospholipid fatty acids) and RNA-SIP

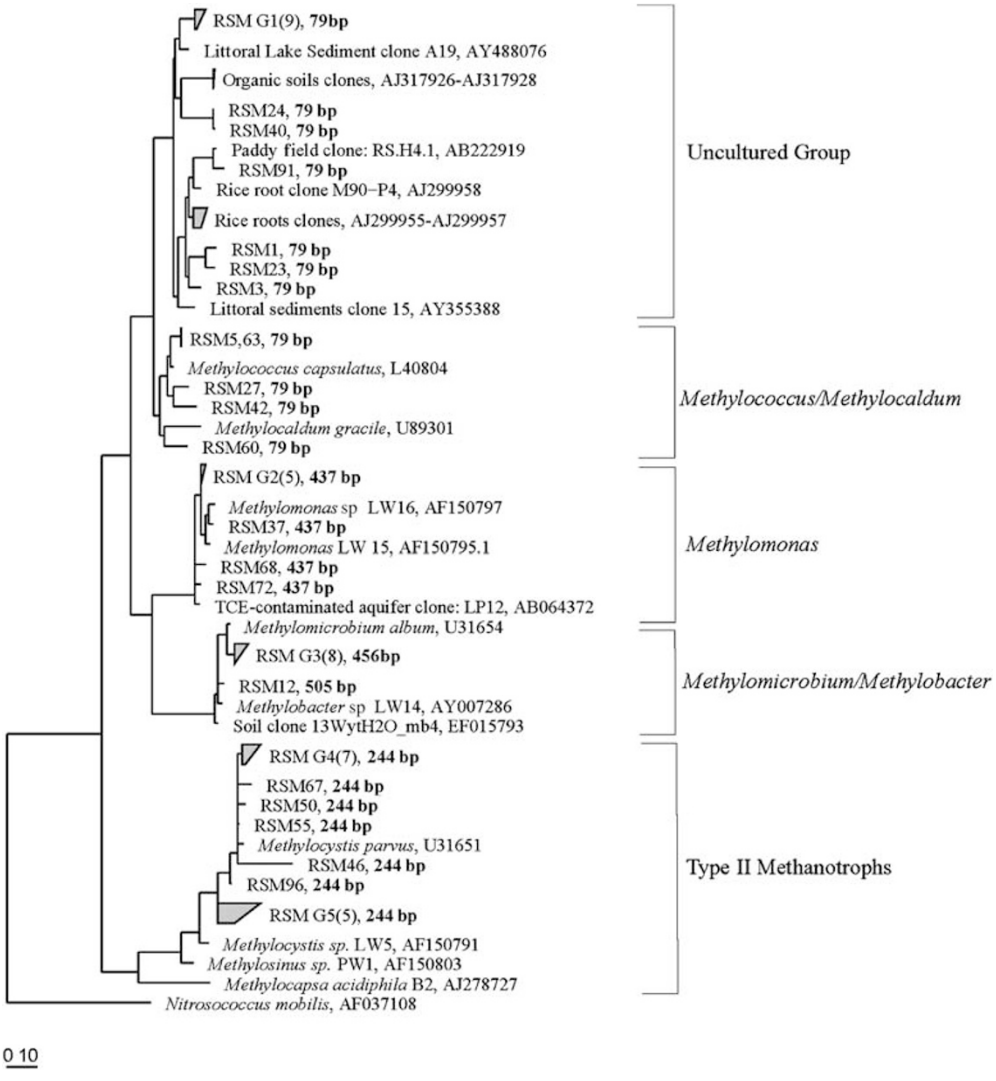

Figure 3 Phylogenetic relationship (neighbor-joining) of amino-acid sequences derived from particulate methane monooxygenase (*pmoA*) sequences cloned from rice soil methanotrophs (RSMs) retrieved from soil samples of the cut + PKN treatment. The terminal-restriction fragment (T-RF) sizes of clone sequences digested *in silico* with *Msp*I are shown in bold. Values in parentheses give the number of clones in the indicated group. The scale bar represents 10% sequence divergence; outgroup = *amoA*. GeneBank accession numbers of the reference sequences are indicated.

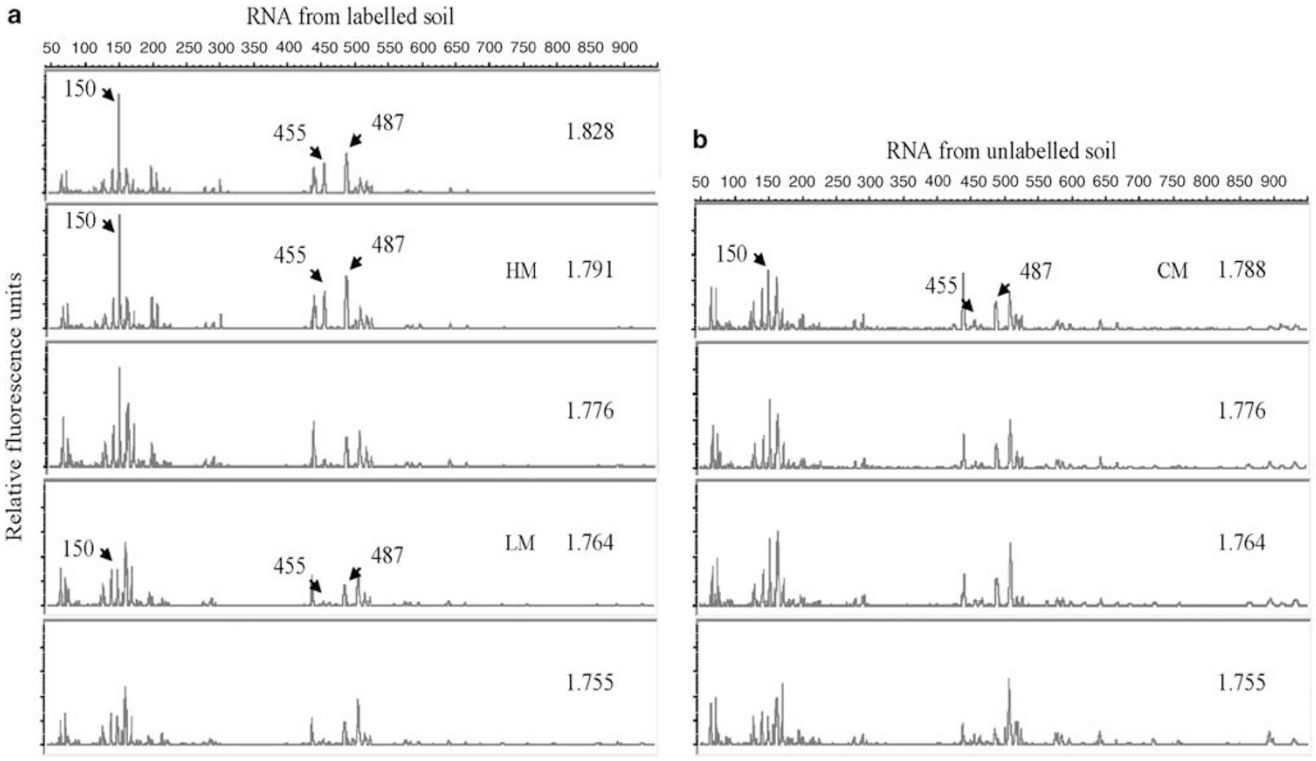

Figure 4 Bacterial terminal-restriction fragment length polymorphism (T-RFLP) fingerprints of 16S rRNA retrieved from density-resolved gradient fractions of (**a**) ^{13}C-labelled rhizospheric soil and (**b**) unlabelled control soil. CsTFA (cesium trifluoroacetate) buoyant densities (g ml^{-1}) of fractions are given. The specific fragment lengths (bp) of methanotrophic T-RFs (as mentioned in the text) are indicated by arrows. HM, LM and CM indicate the fractions from which clone libraries were generated.

indicated that mainly type I methanotrophs and to a less extent type II methanotrophs assimilated CH_4 in the rhizosphere.

In previous studies, CH_4 was supplied to the soil–plant system by the water from the bottom of rice microcosms (Groot *et al.*, 2003) or through permeable tubes buried into the soil (Shrestha *et al.*, 2008). Such technical approach is suitable for microcosm experiments, but can hardly be used for field studies. Since rice plants allow the transport of O_2 from the atmosphere into the soil and of CH_4 from the soil into atmosphere via their gas vascular system and aerenchyma tissue in roots and shoots by a diffusional process (Nouchi *et al.*, 1990; Schütz *et al.*, 1991), we inferred that transport of ^{13}C-labelled CH_4 from the atmosphere into the rhizosphere is also possible and that rhizospheric methanotrophs can be labelled in this way. The transport of ^{13}C-labelled CH_4 from the atmosphere into the rhizosphere was indeed operating as shown by the incorporation of ^{13}C into microbial PLFAs. Cutting plants above the water surface does not impede the CH_4 flux into the atmosphere (Seiler *et al.*, 1984; Conrad and Schütz, 1988). It apparently did also not impede the transport of $^{13}CH_4$ into the rhizosphere as microbial PLFAs were comparably labelled as with intact plants.

After labelling for 7 days, the ^{13}C content of some PLFAs (for example, 16:1ω7 and 16:1ω6) reached a value of almost 7 atom-%. Labelling experiments with $^{13}CH_4$ diffusing from permeable tubes buried into the soil achieved only about 3 atom-% incorporation (Shrestha *et al.*, 2008), indicating that the supply of CH_4 through the plant vascular system allows a more efficient labelling of the rhizosphere microbiota. Manefield *et al.* (2002) determined in a pure culture that the labelling of RNA should be >10 atom-% to allow separation by density centrifugation. However, recent studies of plants pulse labelled with $^{13}CO_2$ showed that root-inhabiting microbes incorporated within only 5 h sufficient amounts of ^{13}C to allow separation of heavy RNA by density gradient centrifugation (Vandenkoornhuyse *et al.*, 2007). This was possible although the δ^{13}C content of the bulk root biomass was rather low (equivalent to 1.4 atom-%). Although the labelled PLFAs detected in our experiments are biomarkers for a limited group of bacteria, they nevertheless represent not only methanotrophs but also other microorganisms, so that the methanotrophs might be ^{13}C-labelled by >7 atom-%. Therefore, we attempted RNA-SIP, which indeed was successful. The obtained results were consistent with the results of PLFA-SIP and indicated that type I methanotrophs were slightly more active in assimilation of $^{13}CH_4$ than type II methanotrophs. Hence, the application of $^{13}CH_4$ to the rice canopy seems to be a suitable technique for identification of rhizospheric methanotrophs active in CH_4 oxidation.

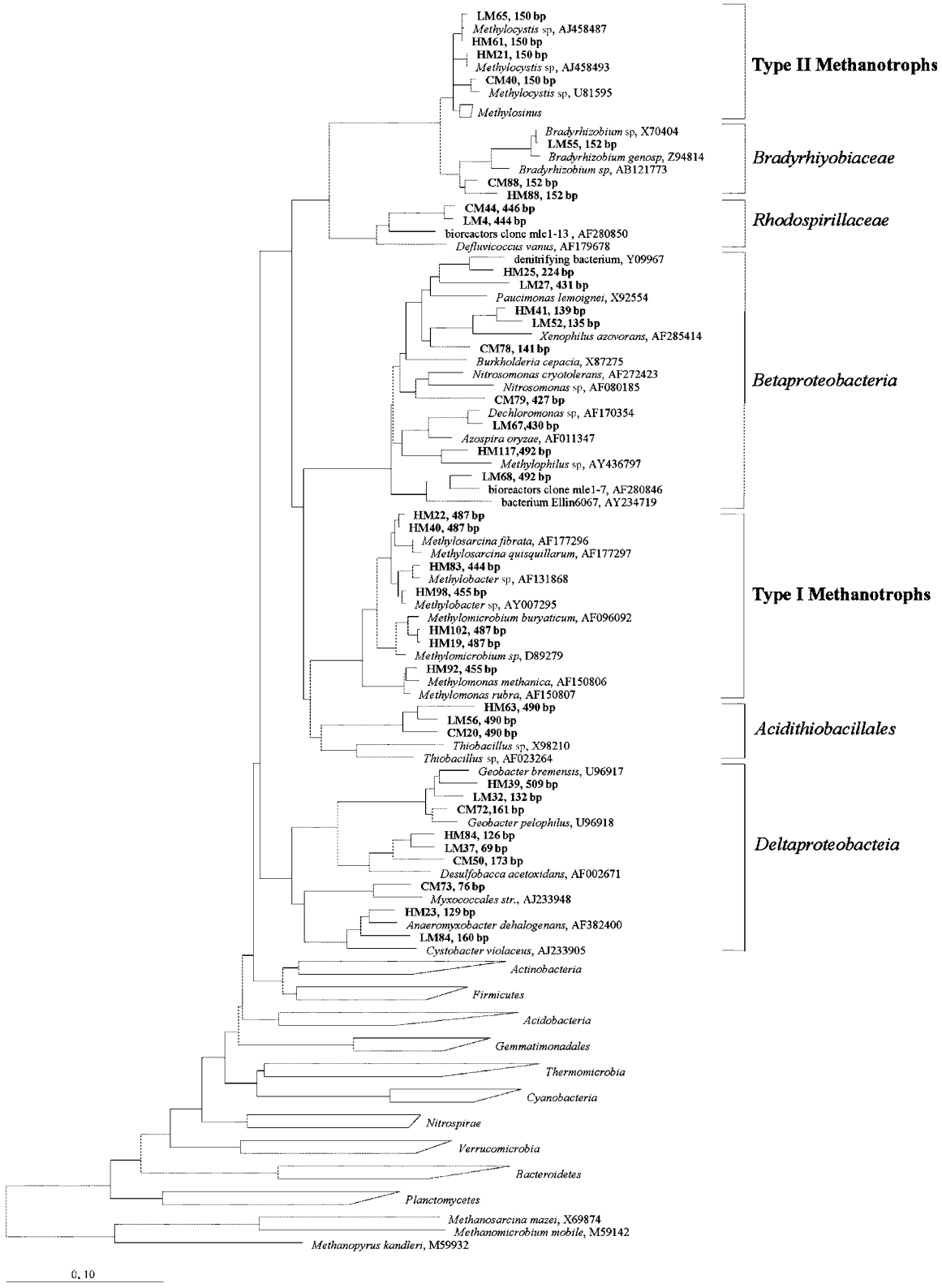

Figure 5 Phylogenetic relationship (neighbor-joining) of cloned bacterial 16S rRNA gene sequences retrieved from amplicons of the density-resolved 'heavy' (HM) and 'light' (LM) RNA fractions and from the control (CM) RNA. The new clone sequences and their terminal-restriction fragment (T-RF) sizes digested *in silico* with *Msp*I are shown in bold. The scale bar represents 10% sequence divergence. GenBank accession numbers of the reference sequences are indicated.

Table 2 Phylogenetic affiliations and numbers of 16S rRNA sequences retrieved in clone libraries generated from density-resolved RNA

RNA fractions	*Heavy (HM)*[a]	*%*	*T-RF (bp)*[b]	*Light (LM)*[a]	*%*	*T-RF (bp)*[b]	*^{12}C (CM)*[a]	*%*	*T-RF (bp)*[b]
Alphaproteobacteria									
Methylocystaceae	3	3		2	2.7		2	2.6	
Methylocystis	3	3	150*	2	2.7	150, 151	2	2.6	150*
Bradyrhizobiaceae	1	1	152	4	5.4	152*	1	1.3	152
Rhodospirillaceae				4	5.4	443, 64, 444, 113	2	2.6	446, 13
Caulobacterales				1	1.4	152			
Hyphomicrobiaceae	1	1	150						
Betaproteobacteria									
Burkholderiales	6	5.9	488, 116*, 139*	3	4.1	488, 135, 491	1	1.3	141
Rhodocyclaceae	7	6.9	224, 490*, 430, 432, 475, 427	5	6.7	431, 490, 430, 82, 434	1	1.3	492
Nitrosomonadales				3	4.1	492*, 490	1	1.3	427
Methylophilaceae	1	1	492						
Gammaproteobacteria									
Acidithiobacillales	1	1	490	3	4.1	490*,227	1	1.3	490
Methylococcaceae	12	12							
Methylobacter	2	2	444, 455						
Methylosarcina	4	4	487*						
Methylomicrobium	3	3	487*						
Methylomonas	3	3	455, 479*						
Unknown				1	1.4	141	1	1.3	461
Deltaproteobacteria									
Desulfobacterales	4	4	126*, 509*	4	5.4	173, 125, 69*	7	9	82, 68*, 69, 173*
Desulfuromonadales	12	12	509, 160, 159, 162*, 69, 499*, 497, 164, 127*	9	12	132, 160, 509, 164, 162*, 166, 499	5	6.4	161, 129, 212, 130, 133
Syntrophobacterales	2	2	508, 509				2	2.6	164*
Bdellovibrionales							2	2.6	67*
Myxococcales	13	13	444, 158, 129, 155, 78*, 76*, 216*, 183	7	9.5	121, 133, 160, 78, 216, 195, 498	8	10	158, 444, 121*, 76, 67*
Unknown	3	3	162*, 124				1	1.3	162
Verrucomicrobia	1	1	169				2	2.6	169, 209
Acidobacteria	9	8.9	264, 177*, 283, 295, 292*, 125, 204	6	8.1	201, 198, 141, 295*, 124	4	5.1	201*, 296*
Firmicutes	1	1	530	3	4.1	133, 519, 128			
Actinobacteria	6	5.9	84, 67, 132*, 163*				5	6.4	140, 132*, 142*
Nitrospirae				2	2.7	292, 127	3	3.8	157*
Cyanobacteria	7	6.9	159, 154*	1	1.4	159			
Thermomicrobia	9	8.9	644, 642, 592, 650*, 521, 515, 516, 518	13	18	172, 127, 461*, 643*, 167, 521, 515, 517*	16	21	179*, 170, 519, 169, 647, 511*, 515, 519, 163, 75, 139*
Gemmatimonadales				1	1.4	141	6	7.7	79*, 122
Planctomycetes	2	2	312*	2	2.7	97, 296	1	1.3	227
Sphingobacteria							6	7.7	89*
Total	101	100		74	100		78	100	

Abbreviations: rRNA, ribosomal RNA; T-RF, terminal-restriction fragment.
[a]'Heavy (HM)' and 'Light (LM)' are samples of ^{13}C enrichment of rRNA; ^{12}C (CM) is control rRNA.
[b]T-RF lengths are shown in base pairs (bp), T-RFs detected in more than one phylogenetic group are marked with an asterisk (*).

The fatty acid 16:1ω7 received most of the ^{13}C-label in our experiment (Figure 3). This fatty acid is common not only in type I methanotrophs but also in ammonia oxidizers, even in one type II methanotroph (Dedysh *et al.*, 2007). Although ammonia oxidizers can oxidize CH_4 and contain the PLFAs 16:0 and 16:1ω7 (Roslev and Iversen, 1999), they seem to play no role for CH_4 oxidation in rice field soil (Bodelier and Frenzel, 1999). Furthermore, ammonia oxidizers may not be able to assimilate CH_4 as a carbon source (Bedard and Knowles, 1989). Since the ^{13}C-labelling of PLFA was similar in samples from unfertilized and urea-fertilized plots, we assume that ammonia oxidizers indeed were not involved in CH_4 oxidation. A somewhat lower ^{13}C-labelling was found for 18:1ω7 PLFA, which is

present in all known type II methanotrophs (Bowmann *et al*., 1993). Labelling of 18:1ω9 PLFA, which also belongs to type II methanotroph, was still weaker. Hence, methanotophs characterized by C_{16}-PLFAs incorporated more labelled CH_4 than those characterized by C_{18}-PLFAs, suggesting that type I methanotrophs were more active in the rhizosphere of rice than type II methanotrophs.

To assess methanotroph diversity within environmental samples, functional genes, such as *pmoA*, have also been used extensively as targets for PCR amplification (McDonald and Murrell, 1997) in rice field ecosystems (Henckel *et al*., 1999; Hoffmann *et al*., 2002; Shrestha *et al*., 2008). This approach has been successful with methanotrophs as their 16S rRNA phylogeny is reflected in the phylogenetic relationships of *pmoA* (Holmes *et al*., 1995), with the exception of *Methylocella* spp, which do not contain *pmoA* (Dedysh *et al*., 2000). Thus, the analysis of *pmoA* in our study suggested a large diversity, including *Methylomonas*, *Methylococcus/Methylocaldum*, *Methylomicrobium*, *Methylobacter* and *Methylocystis* species. An uncultured group of type I methanotrophs with the same T-RF size as *Methylococcus/Methylocaldum* was detected in the clone libraries of *pmoA*. This uncultured group has formerly been detected on rice roots from Italian rice fields (Horz *et al*., 2001) and littoral lake sediment (Bussmann *et al*., 2004; Pester *et al*., 2004). Collectively, the *pmoA* analyses confirmed that type I methanotrophs formed the dominant methanotrophic populations in the rice rhizosphere. However, little difference was observed between plots that were fertilized or not fertilized with urea. This was consistent with methane oxidation measurement and PLFA analysis. Possibly, urea fertilization affects on methanotrophic populations only for a short period after the treatment (Dan *et al*., 2001; Krüger and Frenzel, 2003). Urea is the most important nitrogen fertilizer in rice agriculture (Dedatta and Buresh, 1989).

Among the type II methanotrophs, only *Methylocystis* spp was detected by analysis of both *pmoA* and 16S rRNA genes. RNA-SIP indicated that the T-RF = 150 bp increased in the 'heavy' fractions (Figure 4), which predominantly represented type II methanotrophs. Among the type I methanotrophs mainly the T-RFs of 455 and 487 bp increased with the density fraction and were only found in the clone library from the 'heavy' but not from the 'light' or 'control' rRNA clone libraries. The relative abundance of these different T-RFs in the different fractions indicate that both type I and type II methanotrophs incorporated ^{13}C from the labelled CH_4, but type I increased relatively more than type II methanotrophs (Table 3).

In conclusion, we found that SIP of methanotrophic bacteria is possible for rice fields under *in situ* conditions identifying individual groups of methanotrophs that are active in the rhizosphere and contribute to attenuate the emission of CH_4 into the atmosphere. It seems that type I compared with type II methanotrophs have a larger population (T-RFLP of *pmoA* gene) in the rhizosphere and are also more active in assimilating $^{13}CH_4$ into PLFA and RNA. A more precise determination, in particular by RNA-SIP, should be possible, if the production of unlabelled CH_4 by the methanogenic community in the soil would be relatively low so that the pool of $^{13}CH_4$ in the rhizosphere is not much diluted. This labelling approach may be adapted to other ecosystem studies to understand ecosystem functioning without laboratory-based limitations.

Table 3 Relative abundance of bacterial rRNA T-RFs assigned to methanotrophs

Buoyant density	*Relative abundance (%)*					
	Type II	*Type I*				
	150 bp	*444 bp*[a]	*455 bp*	*479 bp*[a]	*487 bp*	*Total*[b]
1.791 (HM)	14.2	3.0	4.3	ND	6.0	13.3
1.764 (LM)	4.3	ND	0.6	ND	2.3	2.9
1.788 (CM)	7.1	ND	1.0	ND	3.2	4.2

Abbreviations: CM, control rRNA; HM, heavy fraction rRNA; LM, light fraction rRNA, ND, not detected in T-RFLP; rRNA, ribosomal RNA; T-RF, terminal-restriction fragment; T-RFLP, terminal-restriction fragment length polymorphism.
[a]Not separated completely in T-RFLP.
[b]Total percentage of type I methanotroph T-RFs.

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