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ORIGINAL ARTICLE

Activity and diversity of methanotrophs in the soil–water interface and rhizospheric soil from a flooded temperate rice field

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Abstract

Aims: To combine molecular and cultivation techniques to characterize the methanotrophic community in the soil–water interface (SWI) and rhizospheric soil from flooded rice fields in Uruguay, a temperate region in South America.
Methods and Results: A novel type I, related to the genus *Methylococcus*, and three type II methanotrophs were isolated from the highest positive dilution steps from the most probable number (MPN) counts. Potential methane oxidation activities measured in slurried samples were higher in the rhizospheric soil compared to the SWI and were stimulated by N-fertilization. *PmoA* (particulate methane monooxygenase) clone libraries were constructed for both rice microsites. SWI clones clustered in six groups related to cultivated and uncultivated members from different ecosystems of the genera *Methylobacter*, *Methylomonas*, *Methylococcus* and a novel type I sublineage while cultivation and T-RFLP (terminal restriction fragment length polymorphism) analysis confirmed the presence of type II methanotrophs.
Conclusions: Cultivation techniques, cloning analysis and T-RFLP fingerprinting of the *pmoA* gene revealed a diverse methanotrophic community in the rice rhizospheric soil and SWI.
Significance and Impact of the Study: This study reports, for the first time, the analysis of the methanotrophic diversity in rice SWI and this diversity may be exploited in reducing methane emissions.

Introduction

Greenhouse gas methane (CH_4) emission from wetland rice can account for as much as 25% of the global CH_4 budget (Neue 1997). The increase in the flooded rice cultivated area is considered to be one of the factors responsible for the continuous increase in the atmospheric CH_4 concentration. Several factors have been reported to influence methane emission among them the input of fertilizer N, rice cultivars and flooding regime. A better understanding of the underlying processes is required to design strategies to mitigate and control CH_4 emission from wetland rice fields due to biological oxidation.

Methanotrophs or methane-oxidizing bacteria (MOB) are a unique group of bacteria that oxidize CH_4 with molecular O_2 and use it as a carbon and energy source (Bowman 2000). MOB have the potential to substantially decrease the emission rates of CH_4 from wetland rice agriculture into the atmosphere by oxidizing part of the CH_4 that is produced in these soils (Reeburgh 2003). CH_4 oxidation in MOB is catalysed by either a soluble or a membrane-bound, particulate methane monooxygenase (sMMO and pMMO, respectively). MOB mostly belong to the γ-*Proteobacteria* (Type I methanotrophs) and α-*Proteobacteria* (Type II methanotrophs) (Hanson and Hanson 1996). Recently, an extremely acidiphilic methanotrophic bacteria have been reported to belong to the phylum Verrucomicrobia, outside the established methanotrophs subphyla (Dunfield *et al.* 2007). All MOB described to date except

Methylocella spp. have a particulate methane monooxygenase (pMMO) as the key enzyme for CH_4 oxidation. These genes are sufficiently conserved between representative strains to be of use in constructing phylogenies that compare well with 16S rRNA phylogenies (Kolb *et al.* 2003). As a result, the *pmoA* has been used as a molecular marker of MOB diversity in numerous environmental studies (Murrell *et al.* 1998; Henckel *et al.* 1999; Rahalkar and Schink 2007).

Methane oxidation occurs at anaerobic–aerobic interfaces with available oxygen and methane: the soil–water interface (SWI) and the rice rhizospheric soil. The extent to which CH_4 oxidation takes place close to the rice roots compared with at the SWI remains controversial (Macalady *et al.* 2002). Although the activity and diversity of the methane-oxidizing community in the rice rhizospheric soil have been the subject of a series of studies in Italian (Vercelli) rice fields (Henckel *et al.* 2000; Eller and Frenzel 2001; Eller *et al.* 2005), there is a lack of information about the diversity associated with the SWI. Yet, expanding our knowledge of the MOB inhabiting this microsite is important because around 80% of the potentially emitted methane may become oxidized at the oxic surface of flooded rice soils (Conrad and Rothfuss 1991).

In an attempt to address this problem, we used both molecular and cultivation techniques in a combined approach to characterize and investigate the function of the MOB community inhabiting rhizospheric soil and SWI from a Uruguayan rice field. From a wider perspective, the renewed acknowledgment of the importance of cultivation methods in environmental studies (Leadbetter 2003; Oremland *et al.* 2005) is challenging the generally accepted paradigm (Amann *et al.* 1995; Fry 2000; Rappe and Giovannoni 2003) that only a small fraction of microbial species are culturable and that molecular approaches always cover a broader spectrum of microbial diversity than cultivation methods. For instance, Donachie *et al.* (2007) recently compared data from several different habitats where both 16S rDNA sequencing and cultivation techniques had been used and revealed that cultivation methods can detect many organisms undetected by molecular techniques. Therefore, a polyphasic approach is required to reveal the microbial diversity in any given habitat.

In addition, we assessed the effect of ammonium-based fertilization on the activity of MOB. The irrigated rice field chosen for the present study is rotated with pastures for livestock production in a low-intensity system way of using natural resources. This management is typical of the Uruguayan temperate rice production system which decisively contributes to the sustainability of the agroecosystem.

Materials and methods

Field site and sampling

The experimental site was located at the National Rice Research Institute (Instituto Nacional de Investigación Agropecuaria, Unidad Paso de la Laguna) in Treinta y Tres, southeast Uruguay (32°55′S and 54°50′W). Soil characteristics were: silty loam in texture, pH 5·2, $N\text{-}NH_4^+$ 0·3 mmol l^{-1}, 3·0–3·5% organic C and 5·0–5·5% organic matter. On 15 November 2003, the rice (*Oryza sativa*, type *Japonica*, variety INIA Tacuarí) was broadcast seeded into dry soil and flooded 30 days later. This variety has been obtained from local crossing and selection and has a good cold tolerance during the reproductive phase of the crop. Sampling was conducted after the last fertilization event when the rice was 70 days old. This sampling time was chosen accounting for previous reports that have shown that significant methane oxidation takes place after fertilization events when ammonium availability is greatest (Bodelier *et al.* 2000a,b). The experiment was laid down in a completely randomized block design having six plots (three fertilized and three unfertilized) each 3 m^2. Three nitrogen fertilizations, $(NH_4)_2SO_4$, were applied at the rate of 10 kgN ha^{-1} in three different occasions: at the time of seeding, at tillering and at panicle initiation. Samples (0–2 cm depth) of SWI were collected from five points distributed randomly in each plot between the rows of rice plants and then mixed separately for each plot. After careful visual inspection, any plant roots were removed by hand before incubations. For rhizospheric soil, 10 rice plants were dug out by hand from each plot, and were shaken to remove loose soil. The root-containing soil (rhizospheric soil) was firmly attached to the roots and therefore was removed by gently shaking the pooled plants for each plot in 100 ml of sterile distilled water. The resulting soil suspensions are referred to below as the rhizospheric soil. Dry weight was determined for SWI and rhizospheric soil suspensions by drying 10·0 g of each sample at 100°C until constant weight.

Potential CH_4 oxidation in soil slurries

Potential CH_4 oxidation rates of rhizospheric soil and SWI were determined for nine replicate field samples of three fertilized and three unfertilized plots each. All samples were measured in three parallel incubations. Soil slurries were prepared by mixing SWI or rhizospheric soil suspension with autoclaved distilled water to a final concentration of approx. 0·2 g of dry soil per milliliter. Each rhizophere and SWI sample was incubated in three replicates of 25-ml soil slurries in 120-ml sterile glass serum

bottles closed with butyl stoppers under an air atmosphere containing about 7% CH_4 (v/v). Slurries from unfertilized plots were supplemented with $(NH_4)_2SO_4$ (final concentration 5 mmol l^{-1} N) when indicated. The bottles were incubated in darkness at 28°C with agitation at 120 rev min^{-1}. Methane depletion was monitored by sampling the headspace and subsequent GC analysis on a SRI 8610, equipped with a thermal conductivity detector (column temperature 35°C, detector temperature 100°C and a Molecular Sieve 13× column (SRI, Torrance, CA, USA). Potential CH_4 oxidation rates were computed by linear regression through the linear portion of the plot of CH_4 concentration (five time points at 2-h intervals during 10 h) *vs* time. Soil slurries experiments containing half of the amount of soil (2·5 g dry weight) showed a 50% decrease in the CH_4 oxidation rate, indicating nonlimitation by gas phase transfer and nongrowing cell conditions (data not shown). Comparison of rates of treatments and samples means (analysis of variance, ANOVA) was performed using Tukey's test in case of equal variances and using Kruskall–Wallis test with inhomogeneous variances. All statistical tests were performed using SIGMA STAT software ver. 3.1 (Systat Software Inc., Point Richard, CA, USA).

Enumeration and isolation of MOB

The numbers of MOB in the fertilized plots of the rhizospheric soil and SWI were determined by the most probable number (MPN) method (De Man 1975). The soil slurries were further diluted in two-fold steps in triplicates down to 10^{-9} (g dry weight of soil)$^{-1}$ in 10-ml tubes with 4 ml of sterile nitrate mineral salts (NMS) medium (Whittenbury *et al.* 1970). Tubes were closed with butyl stoppers and methane was added to reach a 30% (v/v) concentration in air in the headspace. Tubes were incubated at 30°C with agitation for 4 months. Growth was monitored by measuring optical density, determined routinely photospectrometrically at 660 nm, and by checking the accompanying decrease in the methane concentration. Inoculated tubes without methane served as controls. MPN values with a nonoverlapping 95% confidence interval were considered significantly different, with $P < 0{\cdot}05$.

MOB were isolated from the highest positive dilutions of the MPN series. Pure cultures were obtained by repeated dilution in liquid NMS medium until extinction. Plates were prepared with the same medium solidified with purified agar. Purity was assured by phase-contrast microscopy and checking for nonmethanotrophic contaminants after streaking on nutrient agar plates incubated under an air atmosphere and re-inocultaing any colonies into NMS tubes with methane.

DNA extraction and PCR amplification

DNA from the rhizospheric soil and SWI from two replicate fertilized experimental plots were extracted using PowerMax Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) following the manufacturer's instructions. Cultures were centrifuged (10 min at 15 000 ***g***, 4°C) and the pellets were extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA).

Extracted DNA from soil samples, MPN tubes and isolates were used for the amplification of the partial *pmoA* (approx. 500 bp) using the *pmoA* primer pair A189f-mb661r (Costello and Lidstrom 1999). All PCRs were carried out in 30-μl (total volume) mixtures containing approx. 25 ng of DNA, 0·4 mmol l^{-1} of each primer, 1·5 mmol l^{-1} $MgCl_2$, Taq buffer, 0·17 mmol l^{-1} of each dNTP and 1·2 U of *Taq* DNA polymerase (Invitrogen, San Diego, CA, USA). The reactions were performed in a Perkin-Elmer, GeneAmpPCR System 2400 thermocycler using the following program: initial denaturation step at 94°C for 30 s, followed by 30 cycles at 92°C for 60 s, 60°C for 60 s and 72°C for 60 s, with a final extension step at 72°C for 5 min.

Amplification of the 16S rRNA gene was performed for the isolates obtained. All reactions were carried out in 25-μl (total volume) mixtures using primers 27F and 1492R and containing approx. 25 ng of DNA, 0·5 mmol l^{-1} of each primer, 0·2 mmol l^{-1} of each dNTP, 1·5 mmol l^{-1} $MgCl_2$, 0·2 mg ml^{-1} of bovine serum albumin, *Taq* buffer and 1·2 U of *Taq* DNA polymerase (Invitrogen). The program used including an initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 3 min, with a final extension step at 72°C for 7 min.

Clone libraries, restriction fragment length polymorphism (RFLP), sequencing and phylogenetic analysis

PCR products were cloned using a TOPO TA cloning kit (Invitrogen Corp.) following the protocol of the manufacturer. Clones were subjected to tooth pick PCR, using primers A189f-mb661r. The amplified products were digested with *Msp*I–*Hha*I, grouped into Operational Taxonomic Units (OTUs) based on their restriction patterns, and each clone was assigned to an OTU which represented a unique RFLP pattern. Rarefaction analysis was done for rhizosphere and SWI libraries using the free software Analytic Rarefaction 1.3 (http://www.uga.edu/~strata/software/), developed by Steven Holland. At least two clones representative from each OTU group were sequenced with vector specific T3 and T7 primers. All sequencing reactions were carried out at Macrogen

Sequencing Service, Korea using an ABI PRISM 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). 16S rRNA gene was sequenced for isolates obtained using the primer 27F described above.

All sequences were manually checked for chimeras. For *pmoA* phylogenetic analysis, sequences were translated to obtain deduced amino acid sequences, alignments and phylogenetic distance dendrograms were constructed using the neighbour-joining and maximum parsimony algorithms as implemented in MEGA software version 3.1 (Kumar *et al.* 2004). At least one representative clone for each OTU was used to construct the phylogeny. Clones are grouped into six groups based on at least 90% nucleotide sequence identity. Evolutionary distances between pairs of inferred amino acid sequences were calculated using the model of Dayhoff. Close relatives and phylogenetic affiliation were checked using the BLAST search program (Altschul *et al.* 1997) at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The 16S rRNA gene sequence of strain E10 was phylogentically analysed using Jukes–Cantor distance in a neighbour-joining tree as implemented in MEGA.

All sequences of *pmoA* fragments have been deposited in GenBank under accession numbers EU358959–EU358999 (sequences from clones) and EU359000–EU359003 (sequences from isolates) while16S rDNA sequences from isolates B8, H8 and E10 have been deposited under accession numbers EU672870, EU672872 and EU672873, respectively. Unfortunately, strain C9 was lost upon repeated transfers.

T-RFLP analysis

pmoA fragments were amplified as described above with the primer A189f (5′ end labeled with HEX fluorescent dye, IDT). Amplicons always resulted in the predicted size. After purification with a QIAquick gel extraction kit (Qiagen, MD, USA), approx. 100 ng of the amplicons were digested with 20 U of the restriction endonuclease *Msp*I (Invitrogen). The digestions were carried out overnight at 37°C according to the instructions of the manufacturer. Enzyme inactivation was done by incubation at 65°C for 20 min followed by desalting the samples with Microcon-50 columns (Amicon, Inc., Beverly, MA, USA). The subsequent T-RFLP analysis was performed at the Genomics Technology Support Facility (http://genomics.msu.edu/; Michigan State University, East Lansing, MI, USA). Briefly, the T-RFs were separated by capillary electrophoresis on an ABI Prism 3700 DNA analyzer. The DNA bands were automatically identified and sized using GeneScan software (Applied Biosystems, Foster City, CA, USA) and compared to internal lane standards. T-RFLP results were standardized between different samples as recommended by Dunbar *et al.* (2001) calculating the relative abundance of individual T-RFs in a given *pmoA* PCR product based on the peak height of the individual T-RFs and multiplying by a correction factor calculated as the quotient between the sum of all peak heights >100 fluorescence units in the given sample and the sum of all the peaks in the sample with the smallest total fluorescence. After adjustment of a profile, peak heights <100 fluorescence units were discarded again.

Results

Methane oxidation activity and ammonium effect

Potential CH_4 oxidation rates were measured in soil slurries from planted rhizospheric and top layer soil (Fig. 1). CH_4 oxidation started after a lag time that varied between 24 and 48 h. A linear increase of CH_4 oxidation rates was observed up to 5 g dry weight of soil, indicating that assay conditions were of nongas phase transfer limitation and of nongrowing cells (data not shown). The potential CH_4 activities, calculated from the first 8–10 h after the beginning of CH_4 oxidation, showed significant higher rates for the rhizospheric soil slurries compared to the SWI slurries, whether fertilized or unfertilized (Tukey's test, $P = 0{\cdot}004$ and $P < 0{\cdot}001$, respectively). The highest CH_4 oxidation rates were always found in the ammonium

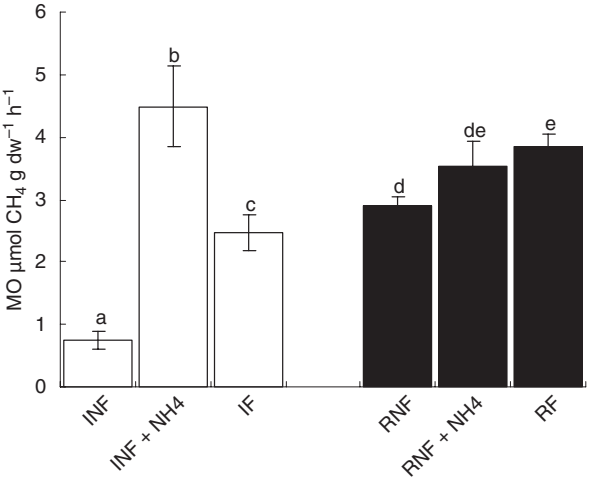


Figure 1 Potential CH_4-oxidizing (MO) activities in slurries from the soil–water interface (I, white bars) and from rhizospheric soil (R, black bars) which were either fertilized (IF and RF) or unfertilized (INF and RNF). The effect of ammonium supplement (final concentration 5 mmol l^{-1} N-NH_4^+) on unfertilized slurries (INF + NH_4^+; RNF + H_4^+) Error bars denote standard error for samples from three replicate fertilized and unfertilized plots, respectively. Different letters indicate significant differences ($P < 0{\cdot}05$) between treatments.

fertilized treatments, irrespective of the microsite. Thus, fertilization produced a nearly three-fold increase in the potential CH_4 oxidation rates of SWI slurries (Kruskall–Wallis test, $P < 0{\cdot}001$) while a smaller increase was detected for activities associated to the rhizospheric soil (Tukey's test, $P < 0{\cdot}001$). However, ammonium addition to unfertilized soils revealed a clear stimulation of methane oxidation activity for the SWI (Tukey's test, $P < 0{\cdot}001$), in contrast to a statistically insignificant increase for the rhizospheric soil (Tukey's test, $P = 0{\cdot}135$).

MPN counts and isolations

Results from MOB counts performed on two replicate fertilized rice plots from each microsite were as follows MPN MOB per g^{-1} dry soil (in brackets 95% confidence interval): SWI, $1{\cdot}7 \times 10^5$ ($4{\cdot}3 \times 10^4$–$7{\cdot}0 \times 10^5$); $7{\cdot}0 \times 10^5$ ($1{\cdot}6 \times 10^5$–$3{\cdot}1 \times 10^6$) and rhizospheric soil, $1{\cdot}7$–10^4 ($6{\cdot}4 \times 10^3$–$4{\cdot}3 \times 10^4$); $3{\cdot}3 \times 10^4$ ($1{\cdot}2$–$9{\cdot}1 \times 10^4$). Although the SWI population size of MOB was slightly higher in both samples, these differences cannot be considered significantly different.

Four strains of MOB from the rhizospheric soil and the SWI (B8 and C9; H8 and E10, respectively) were isolated from the highest positive dilutions of the MPN counts and comparative sequence analysis of their partial *pmoA* sequences of 500 bp performed. Three isolates clustered within the phylogenetic radiation of the type II MOB belonging to the *α-Proteobacteria* (Fig. 2). Two of these isolates were affiliated with *pmoA* sequences of the *Methylocystis* cluster (96–99% sequence identity). Specifically, isolate B8 was closely affiliated with *Methylocystis* sp. strain M (98–99% sequence identity) while isolate H8 was most closely related to *Methylocystis* sp. IMET 10484 (96% sequence identity) and to clones R13 and RB39. Another isolate, C9, grouped with members of the *Methylosinus* genus and was most closely related to *Methylosinus trichosporium* strain M23 (98% sequence identity). Finally, isolate E10 represented type I MOB. Isolation of E10 initially proved to be difficult. Despite repeated streaking on solid medium, microscopic examination of isolated colonies always revealed the presence of cells with morphological features typical of *Hyphomicrobium* spp. Hyphomicrobia are known to copurify with methanotrophs since they utilize intermediate metabolic products (e.g. methanol) produced by the latter. Therefore, repeated liquid subculturing as soon as methane consumption began was successfully assayed to favour the outcompetition of E10 with respect to these contaminants. Small (1–2 mm in diameter), white, semi-transparent colonies were observed on solid mineral medium within 1 week at 28°C.

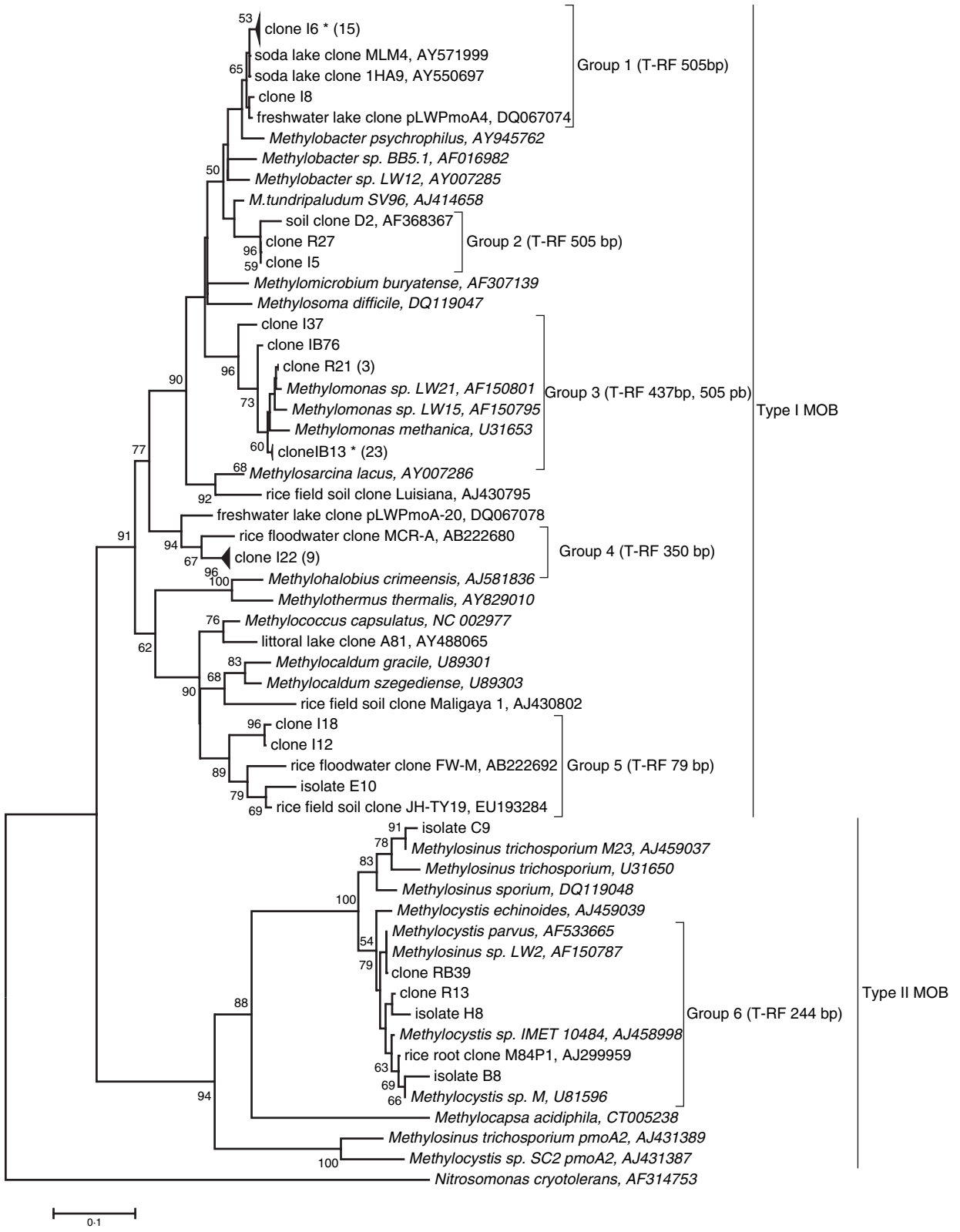
Database searches revealed that the closest related characterized bacterium to this strain was *Methylococcus capsulatus* but only at approx. 84% *pmoA* identity. The phylogenetic analysis indicated that isolate E10 clustered with clones I12 and I18 in a separate group supported by a high bootstrap value, close to the *Methylococcus pmoA* sequences (Fig. 2). Since this isolate appeared to be novel, a nearly full-length (1451 bp) 16S rDNA sequence was obtained. The closest cultured relatives of this strain were *Methylococcus capsulatus* and *Methylocaldum szegediense*, with 92% and 91% similarity, respectively (Fig. 3).

Clone libraries and phylogenetic analysis

Differential yields of *pmoA* PCR products were obtained when we used template DNA extracted from soils not supplemented with CH_4 or lag-phase soils, as has been reported for other wetland soils (Henckel *et al.* 1999; Hoffmann *et al.* 2002). Therefore, to compare the community structure of MOB in both rice field soil microsites, soil samples for DNA extraction were taken just before the onset of CH_4 oxidation. The *pmoA*-specific primers (primers A189f and mb661r) were used to construct in total four separate *pmoA* clone libraries of rhizospheric soil and SWI from two replicate fertilized experimental plots. These primers are known to cover a large diversity of MOB and exclude the homologous *amoA* gene of nitrifiers (Costello and Lidstrom 1999). A total of 174 randomly selected clones, 88 and 86 for the rhizospheric soil and SWI library, respectively, were exposed to RFLP analysis which identified 15 OTUs. Rarefaction calculations showed that species accumulation curves reached the plateau in SWI and rhizospheric soil libraries, respectively (data not shown), indicating that the analysis performed gave a good coverage of the number of OTUs and thereby of MOB diversity.

Representative clones from each OTU and from each soil were sequenced and phylogenetically analysed (Fig. 2). The *pmoA* sequences were clustered in six groups

Figure 2 Neighbour-joining phylogentic tree of the inferred amino acid sequences (153 amino acids) of *pmoA* from methanotrophs and representative rice soil clones retrieved in this study (bold). Clones are designated by R and I to denote the rice rhizospheric soil and soil–water interface, respectively, followed by the clone number. When more than one clone was observed, the number was included within parenthesis following the clone designations. The reference sequences are shown by species or clone name followed by the GenBank accession number. Numbers next to the clone groups represent the length of the respective T-RFs. Clones marked with an asterisk did not contain a restriction site. Bootstrap values ≥50% (100 data re-samplings) are given. The bar represents 10% divergence.



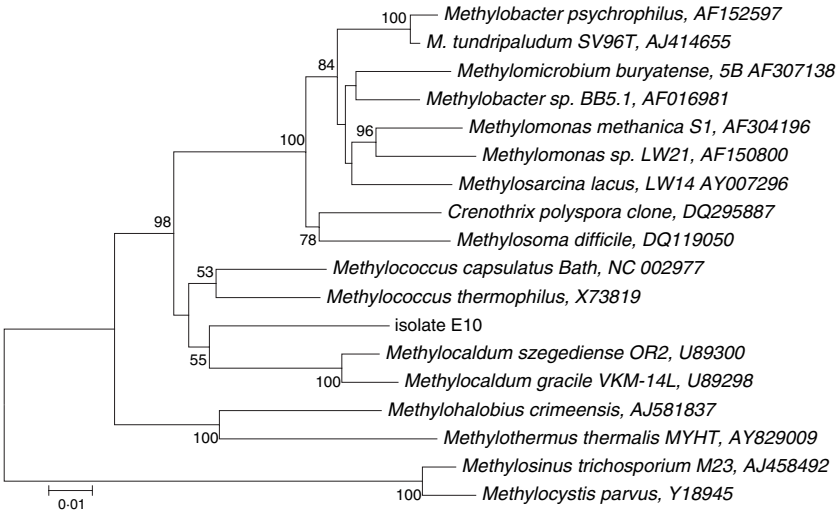


Figure 3 Neighbour-joining tree of the 16S rRNA sequence of strain E10 in comparison with other cultured methanotrophs. GenBank accession numbers are written by each name. Bootstrap values ≥50% (500 data re-samplings) are given.

Table 1 Relative abundance of methanotrophic groups from fertilized rhizospheric soil and soil–water interface based on frequencies of *pmoA* cloned genes arranged according to RFLP patterns and sequence similarity

		Rhizospheric soil		Soil–water interface		
Phylogenetic Group	Clone group*	No. of clones	Relative abundance (%)	No. of clones	Relative abundance (%)	Closest relative (% identity)
Type I	1	73	83·0	13	15·1	Clone MLM4, AY571999, (97–98%), Clone 1HA9, AY550697, (96–98%)
	2	3	3·4	2	2·3	Clone D2, AF368367, (94%) *Methylobacter sp.* SV96, AJ414658, (93%)
	3	4	4·5	38	44·2	*Methylomonas* sp. LW21 and LW15, AF150801 and AF150795, (92–93%, 91%)
	4	6	6·8	28	32·5	Clone MCR-A, AB222680, (86–87%)
	5	0	0	4	4·7	Clone FW-M, AB222692, (88%) Clone JH-TY19, EU193284, (90%) *Methylococcus capsulatus*, L40804, (81%)
Type II	6	2	2·3	1	1·2	*Methylosinus* sp. strain LW2, AF150787, (96–98%)

*Clone groups 1–6 contain all clones obtained in this study arranged into 15 OTUs according to RFLP patterns and are based on at least 90% sequence identity within one group of representative clones from each OTU.

using a threshold of 90% sequence identity (Pester *et al.* 2004). Clones related to *Methylobacter pmoA* sequences were the most dominant sequences retrieved from the rhizospheric soil library (86·4%), while a minor relative abundance (17·4%) of these sequences was found in the SWI library (Table 1). These *Methylobacter*-related OTUs sub-grouped into two clades (groups 1 and 2). One clade (group 1) clustered with *Methylobacter psychrophilus* and was closely related to *pmoA* clones from soda lake sediments (Lin *et al.* 2004, 2005) and freshwater lake sediments (Nercessian *et al.* 2005). The other clade, group 2, had *pmoA* sequences that closely grouped with a clone from a Danish soil (Bourne *et al.* 2001) with *Methylobacter tundripaladum* SV96 as its closest cultured relative. Clone group 3 (44·2% of all SWI clones and 4·5% of all rhizospheric soil clones) clustered within the *Methylomonas* species. Clones from group 4 (32·5% and 6·8% of all SWI and rhizospheric soil clones, respectively) were not closely related to any cultured methanotroph but related to clones from microcrustaceans in rice floodwater (Niswati *et al.* 2004). Clone group 5 (4·7% of all SWI clones) was only distantly related to known methanotrophs but grouped close to the branch where isolate E10 and clones from microcrustaceans in rice floodwater and Chinese paddy fields were present (Niswati *et al.* 2004). Finally, clones from group 6 (1–2% of all clones in both soils) were related to *Methylosinus* sp. strain LW2 and isolate H8 (Table 1).

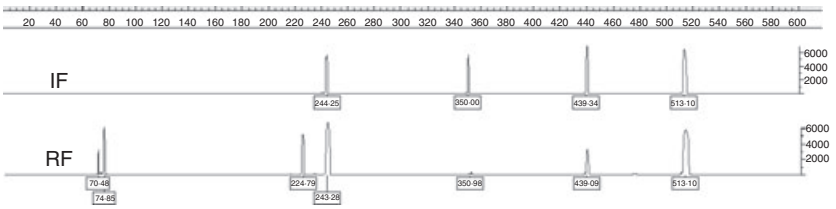


Figure 4 *pmoA*-based *Msp*I T-RFLP representative profiles of soil from fertilized rice soil–water interface (IF) and fertilized rice rhizospheric soil (RF). The fragment sizes in base pairs are labelled, while peak heights are shown as fluorescent units detected.

T-RFLP fingerprinting

The tetrameric restriction enzyme *Msp*I was selected for *pmoA*-based T-RFLP analysis of fertilized rhizospheric soil and SWI methanotrophic communities. Previous studies in rice soils and plants have used this enzyme to study the diversity of methanotrophic bacteria (Horz *et al.* 2001; Hoffmann *et al.* 2002). T-RFLP analysis with *Msp*I of type culture strains of selected genera of methanotrophs produced T-RFs matching those predicted by in silico analysis of the GenBank *pmoA* sequence database (data not shown). An exception to the former was the experimentally derived 75 bp T-RF for *Methylococcus. capsulatus* ATCC 33009 which differed from the predicted value of 79 bp. In line with this, Osborn *et al.* (2000) have acknowledged a variation greater than 2 bp between experimentally derived and predicted T-RF sizes for the smaller T-RFs. The *pmoA* sequences retrieved from the clone libraries were used to predict in silico the identity of peaks in the T-RFLP analyses (Fig. 2). Independent replicate plots produced virtually identical T-RFLP profiles. The community T-RF profiles of the rhizospheric soil and the SWI showed both similarities and differences in the presence of major T-RFs (Fig. 4). Three major peaks were 244, 439 and 513 bp in both sites, the 244 bp peak reflected the presence of clone group 6 and isolates C9, H8 and B8. As expected, this specific peak was indicative for the presence of type II methanotrophs (Horz *et al.* 2001). Another prominent peak was 439 bp, which corresponded to the majority of sequences from clone group 3 affiliated with members of the genus *Methylomonas*. The 513 bp peak, which represents the undigested *pmoA* sequence types without the *Msp*I recognition site (Horz *et al.* 2005), possibly reflected the presence of certain clones in groups 1 and 3 which did not show in silico any *Msp*I restriction site. In contrast, the 351-bp T-RF representing clone group 4 which formed a separate phylogenetic cluster displayed only a distinct peak in the SWI sample. Further differentiation between the T-RFLP patterns of both sites was observed in the detection of T-RFs of 71-, 75- and 225-bp length in the rhizospheric soil. The T-RF of 75-bp from the rhizospheric soil could be correlated to the *pmoA* sequence restriction site from isolate E10 although the latter was obtained from the SWI. This strain produced a 75-bp T-RF which slightly differed from that predicted based on the sequence information (79-bp) but matched that experimentally derived by *Msp*I digestion of the *pmoA* sequence from *Methylococcus capsulatus* ATCC 33009 (data not shown). The 71- and 225-bp T-RFs could not be affiliated with the obtained *pmoA* sequences although fragments of this size were detected in tubes of the MPN counts (data not shown). The former T-RF could not be assigned to any of the *pmoA* sequence types deposited in public-domain databases and could thus correspond to novel *pmoA* sequence types. The latter 225-bp fragment could be correlated to *pmoA* amplicons from rice fields soils in the Philippines which grouped within the *Methylococcus–Methylocaldum* group (Hoffmann *et al.* 2002).

Discussion

Central to the study of microbial diversity is the issue of how to obtain the best objective picture of the microbial community present. Clearly, all methods in microbial ecology, classical and molecular have their limitations. Hence, the importance of pairing both approaches to provide a comprehensive understanding of microbial processes as well as revealing the 'true' nature of the diversity present. In this case study, we describe and compare the methanotrophic microbial community of rice soil–water interface layer and rhizospheric soil, something which has not been done until now. Cultivation yielded predominantly MOB of the *Methylocystis–Methylosinus* group (type II MOB) from both microsites. Several attempts have been assayed to optimize the recovery of the numerically dominant MOB strains in different environments by modification of the composition of the widely used Whittenbury's medium (Wise *et al.* 1999; van Bodegom *et al.* 2001; Bussmann *et al.* 2001). However, there is still an enormous gap between the diversity of the cultivated MOB and the diversity of methanotrophs in ecosystems such as rice fields. In fact, the preferential cultivation of the *Methylocystis–Methylosinus* group (type II MOB) in rice soils does not mirror the diverse community structure that through molecular analyses this and other studies have revealed where both type I and type II MOB are numerically important (Horz *et al.* 2001; Hoffmann *et al.* 2002; Macalady *et al.* 2002). Nevertheless, we succeeded in this study to isolate strain E10 which represents

a novel lineage in the phylogenetic tree of type I MOB, on a branch close to that of thermotolerant and moderately thermophilic methanotrophs belonging to the *Methylococcus–Methylocaldum* group (Fig. 3). The *pmoA* sequence is also novel, clustering with clone group 5 and more related (97%) to a *pmoA* clone isolated from a Chinese rice paddy field.

On the other hand, both cultivation-independent approaches with *pmoA* as a molecular marker (cloning and T-RFLP fingerprinting) detected a broader diversity of type I methanotroph-like sequences in both microsites. Among the type I MOB, our T-RFLP community profiles indicated the presence of several of its members. For instance, the T-RF peak at 351 represented clone group IV. This clade branched separately from cultured MOB and as the closest relative was an environmental *pmoA* sequence (86% identity) originated from rice floodwater, these *pmoA* sequences might represent a novel methanotrophic species adapted to the floodwater rice environment.

Certain disparities concerning the detection of *pmoA* species by the two approaches were also observed. Noticeably, a lower proportion of type II methanotroph *pmoA* sequence types was detected in the clone libraries than expected based on the high relative signal intensity of the 244-bp T-RF. Similar inconsistencies between cultivation-based approach, *pmoA* clone libraries and *pmoA*-based T-RFLP analysis have been reported earlier (Bussmann *et al.* 2003; Horz *et al.* 2005). At present, no simple explanation for this difference is at hand although PCR or cloning bias of certain *pmoA* sequence types can be alleged.

According to the results obtained from the MPN counts, no preference of MOB for rhizospheric soil or SWI was observed in this ecosystem. Previous studies have disagreed about the microsites where CH_4 oxidation predominates. While a series of studies dealing with model systems based on rice field soil from northern Italy revealed lower numbers of methanotroph populations in bulk soil than in association with rice roots, a study in California rice paddies showed that methanotroph growth occurred primarily close to the SWI (Macalady *et al.* 2002). Comparisons of our results with MOB counts from other studies are difficult due to differences in the environmental and culturing conditions. Unlike the MPN counts of the fertilized plots, the corresponding methane oxidation potential in the rhizospheric soil slurries was higher than in the SWI. Although few studies have addressed the study of the CH_4 oxidation potential in the oxic surface layer, it has been suggested that it is probably lower than in the soil surrounding the rice roots (Bosse and Frenzel 1997), in agreement with our data. Noticeably, in the unfertilized plots a nearly four-fold higher value of the MOR was observed in the rhizospheric soil than in the SWI. However, ammonium addition to soil slurries from unfertilized plots, substantially enhanced the methane consumption rate of the planted SWI. This may point to the size of the active methanotroph population at the enzymatic level, to the variability between the types of methanotrophs present or to a differential availability of N for methanotrophs in each of the environments investigated. The role of mineral N with respect to the MOB community remains controversial. In the past, several authors have reported that N fertilization limits methane oxidation (Bosse *et al.* 1993; Cai and Mosier 2000) while more recent studies acknowledge a stimulating effect in densely rooted rice soil (Bodelier *et al.* 2000a,b). In this work, the application of ammonium-based fertilization stimulated rather than inhibited methane-oxidizing activity in both the rhizospheric soil and the oxic surface of the soil.

In our study, the rice SWI supported a relatively complex community including type I methanotrophs affiliated with the genera *Methylobacter*, *Methylomonas* and *Methylococcus* and a novel type I methanotroph sublineage. In contrast, previous studies of rice bulk soils have revealed a much less diverse MOB community where type II MOB predominate (Eller and Frenzel 2001). Interestingly, to our knowledge the isolation of a Type I MOB has not been previously reported in the rice ecosystem. Thus, the cultivation strategy employed appears to enlarge the diversity of culturable methanotrophs. With our present study we broadened our knowledge about the MOB community activity and structure in the SWI, a poorly studied ecological microniche in rice paddies characterized by spatiotemporal variations in substrate concentrations. Further study is required to elucidate methane oxidation dynamics in the different microsites of flooded rice fields to develop better ways of mitigating CH_4 emissions.

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