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ENVIRONMENTAL MICROBIOLOGY

Uncultivated *Methylocystis* Species in Paddy Soil Include Facultative Methanotrophs that Utilize Acetate

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Abstract Methanotrophs are crucial in regulating methane emission from rice field systems. Type II methanotrophs in particular are often observed in high abundance in paddy soil. Some cultivated species of *Methylocystis* are able to grow on acetate in the absence of methane. We hypothesize that the dominant type II methanotrophs in paddy soil might facultatively utilize acetate for growth, which we evaluate in the present study. The measurement of methane oxidation rates showed that the methanotrophic activity in paddy soil was inhibited by the addition of acetate compared to the continuous supplementation of methane, but the paddy soil maintained the methane oxidation capacity and recovered following methane supplementation. Terminal restriction fragment length polymorphism analysis (T-RFLP) combined with cloning and sequencing of *pmoA* genes showed that *Methylocystis* was enriched after incubation with added acetate, while the type I methanotrophs *Methylocaldum*/*Methylococcus* and *Methylobacter* were enriched by methane supplementation. A comparison of *pmoA* sequences obtained in this study with those in the public database indicated that they were globally widespread in paddy soils or in associated with rice roots. Furthermore, we performed stable isotope probing (SIP) of *pmoA* messenger RNA (mRNA) to investigate the assimilation of ^{13}C-acetate by paddy soil methanotrophs. RNA-SIP revealed that *Methylocystis*-related methanotrophs which shared the same genotype of the above enriched species were significantly labelled. It indicates that these methanotrophs actively assimilated the labelled acetate in paddy soil. Altogether, these results suggested that uncultivated *Methylocystis* species are facultative methanotrophs utilizing acetate as a secondary carbon source in paddy soil.

Keywords Acetate utilization · Facultative methanotrophs · Paddy soil · SIP

Introduction

Rice fields are considered as one of the most important sources of atmospheric methane. The aerobic methanotrophs, as a biofilter of methane into atmosphere, are crucial for the attenuation of methane emission from rice fields. At present, all known aerobic methanotrophs belong to two bacterial phyla, the *Proteobacteria* and the *Verrucomicrobia*. The verrucomicrobial methanotrophs are restricted to geothermal environments and therefore are not relevant to rice fields [1, 2]. In contrast, the proteobacterial methanotrophs have been proven to play major roles in methane oxidation in paddy soils, and their community structures, abundances and activities have been studied extensively [3–6].

The proteobacterial methanotrophs are generally classified into two groups, type I (belonging to *Gammaproteobacteria*) and type II (belonging to *Alphaproteobacteria*) [7, 8]. Type II methanotrophs, especially *Methylocystis*-like species, are often found to be more abundant than type I methanotrophs in paddy soils [4, 9–11]. In contrast, accumulating evidences showed that the active methanotrophs in paddy soils are type I groups rather than type II [6, 12–14]. So, the ecophysiology of type II methanotrophs in paddy soil remains largely unknown.

For a long time, it was believed that all methanotrophs were obligately methanotrophic, meaning they were able to metabolize methane but not multi-carbon substrates as their sole

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carbon and energy source. However, the last decade has seen the description of several facultative methanotrophs [15]. Species of the genus *Methylocella* isolated from acidic *Sphagnum* peat were the first to be recognized as able to use a variety of multi-carbon compounds, including acetate [16]. Recently, *Methylocapsa aurea* KYG^T from forest soil and some species of the genus *Methylocystis* from peat or bog samples were reported to have the capacity for facultative growth on acetate [17–19]. In addition, a labelling study showed that the atmospheric methane oxidizers (USC alpha clade) in forest soil assimilate ^{13}C-acetate [20]. Altogether, these findings showed that facultative methanotrophs are more diverse than previously thought, although all currently known facultative species belong to the *Alphaproteobacteria* methanotrophs. Additionally, facultative methanotrophs might be widespread in many natural environments [21].

Acetate is the most important intermediate in the anaerobic decomposition of organic matter in paddy soil. Its concentration can reach several millimolar or even higher, especially when rice straw or root is anaerobically degraded [3, 22]. Therefore, we hypothesize that the dominant type II methanotrophs in paddy soil might have the facultative capacity to use acetate for growth. In the present study, the methanotrophs in a Chinese paddy soil were enriched by supplying methane and/or acetate. The structure and abundance of methanotrophic community were analysed by *pmoA*-based terminal restriction fragment length polymorphism (T-RFLP), cloning, sequencing and quantitative polymerase chain reaction (qPCR), respectively. In addition, stable isotope probing of *pmoA* messenger RNA (mRNA) was performed to investigate the assimilation of ^{13}C-acetate by the active methanotrophs in paddy soil.

Materials and Methods

Site Description

Soil was collected from a rice field at Sanjiang Experimental Station of Wetland Ecology, Chinese Academy of Sciences (47° 35′ N, 133° 31′ E), which is located in Sanjiang Plain in the eastern part of Heilongjiang province, China. The Sanjiang Plain, formerly the largest area of the freshwater wetlands in China, experienced incredible land-use changes during the past half century. Considerable areas of natural marsh (more than 3 million ha) have been reclaimed for agricultural purposes [23], and the area of rice fields increased especially since the 1990s and now accounts for one third of total cultivated area [24].

Soil was sampled after rice harvest in late autumn 2011. In total, six replicate cores (height, 20 cm; diameter, 5 cm) of the plow layer soil were collected, and the distance between two cores was about 10 m. The soils were mixed, air-dried, sieved (2-mm mesh size) and stored at room temperature. The soil was classified as Hydric Medihemists with the following basic characteristics: pH 5.8, organic matter of 4.0 %, total N of 1.0 g kg^{-1}, total P of 0.54 g kg^{-1} and a texture of silty clay. The climatic conditions and agricultural practices, such as rice variety, water regime and fertilization, were described previously [25].

Experiment 1: Soil Incubation

Soil (10 g dry weight) was amended with 20 ml M1 medium in 50-ml serum bottles capped with butyl stoppers. The M1 medium was prepared according to published protocols [26]. The air headspace was supplemented with 10 % (about 100,000 ppm by volume) methane. All the bottles were incubated in the dark at 25 °C, with shaking at 120 rpm. After pre-incubating for 6 days, all the bottles were divided into three groups by supplying different substrates. The first group of bottles was continuously supplemented with 10 % methane until the end of incubation (referred as ME treatment). The second group was alternately supplemented with 10 mM acetate from days 6 to 12, days 16 to 21 and days 28 to 33, and 10 % methane in the remaining days (referred as AC treatment). The third group was the control relative to AC treatment by replacing acetate with the same volume of water (referred as CK treatment; Fig. 1). Each treatment was carried out in 12 replicate bottles. The original unincubated soil was sampled and referred to as ‘day 0’. On days 21 and 39, four bottles from each treatment were destructively sampled. Soil slurries were mixed thoroughly and then centrifuged at 5,000 rpm for 10 min. The precipitated soils were stored frozen at -20 °C until DNA extraction.

During the incubation, the depletion of methane in the headspace was measured every 12 h by gas chromatography using a flame ionization detector (GC-FID) [27]. When the methane concentration dropped below 1 %, the bottle headspace was flushed with air and supplemented again to 10 % methane. Methane oxidation rates were calculated from the slopes of regression lines of methane depletion with time and expressed as micromoles $CH_4 h^{-1}$ per gram (dry weight) (gdw^{-1}) of soil. The consumption of acetate in the slurry, with an average rate of 0.28 μmol h^{-1} gdw^{-1}, was measured by high-performance liquid chromatography (HPLC) every 2 days. When acetate was consumed, the bottle was flushed with air and the new acetate was supplied to reach the concentration of 10 mM.

Experiment 2: Labelling Incubation

Incubation for stable isotope probing with acetate was performed by using the soil slurry from the above AC treatment on day 39. About 3 ml slurry was amended with a final concentration of 10 mM acetate (^{12}C or ^{13}C), dissolved with

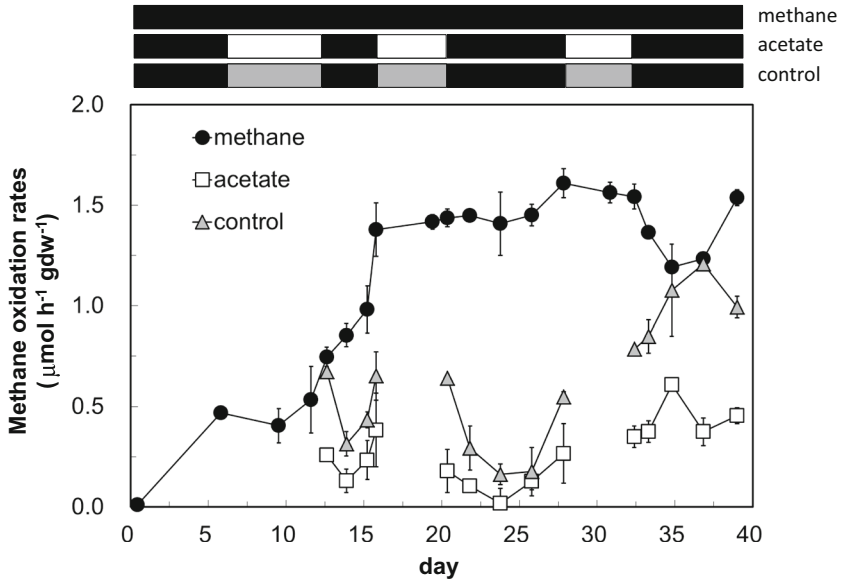

Fig. 1 Methane oxidation rates of paddy soil under different incubation conditions. *Bars* represent standard errors (n=4). *ME* continuously supplemented with methane, *AC* alternately supplemented with acetate and methane, *CK* alternately supplemented with water and methane

1 ml M1 medium (4×) in 12-ml serum bottles. The fully labelled acetate ($^{13}CH_3{}^{13}COOH$, 99 % ^{13}C) was purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Each labelling treatment was performed in triplicate. The bottles were incubated in the dark at 25 °C, shaking at 120 rpm. The consumed acetate was renewed to a final concentration of 10 mM every 2 days according to the results of a preliminary experiment, and the bottles were flushed with air at the same time. After labelling for 16 days, the soil slurry was sampled and frozen within 15 min using liquid nitrogen and stored at −80 °C until RNA extraction.

Nucleic Acid Extraction and SIP Fractionation

DNA was extracted by the Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, USA) following the manufacturer's instructions [27]. RNA was extracted using a bead-beating method following the detailed procedures described previously [28]. In brief, 0.5 g soil was extracted first with TPMS buffer once, and then twice with phenol-based lysis buffer. The pooled supernatants were further extracted with water-saturated phenol, phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. The total nucleic acids were precipitated with cold ethanol, washed with cold 70 % ethanol and dissolved in TE buffer. The DNA in crude extracts was digested by RNase-free DNase (Takara, Dalian, China), and RNA was further purified by RNeasy® Mini Kit (Qiagen, Hilden, Germany). The quality and purity of DNA and RNA were checked by 1 % agarose gel electrophoresis and NanoDrop1000 spectrophotometry (NanoDrop Technologies, Wilmington, USA).

The purified RNA extracts from three replicates were pooled before gradient centrifugation. The gradient centrifugation of RNA was carried out in cesium trifluoroacetate (CsTFA) as described previously [29]. After centrifugation at 39,000 rpm for 65 h at 20 °C, the gradients were fractionated and the density of each fraction was determined by refractometry. RNA was precipitated with cold ethanol and 3 M sodium acetate (pH 5.2), washed with cold 70 % ethanol and finally resuspended in 30 μl of nuclease-free water. Then the PrimeScript™ 1st Strand complementary DNA (cDNA) Synthesis Kit (Takara) was used to synthesize the cDNA from RNA samples of each fraction by random priming according to the manufacturer's instructions.

T-RFLP Analysis, Cloning, Sequencing and qPCR

DNA and cDNA samples were used for PCR amplification of fragments of *pmoA* using the primer set A189 and mb661. For the analyses of terminal restriction fragment length polymorphism (T-RFLP), the forward primer was labelled with 6-carboxyfluorescein (FAM). For cloning and sequencing, PCR amplification was performed using the same primers without a FAM label. T-RFLP, cloning and sequencing were performed according to previous protocols [11, 27]. Four clone libraries were constructed: three for *pmoA* genes (one soil on day 0 and two soils of AC and CK treatments on day 39, respectively) and one for *pmoA* transcripts (heavy fraction of labelled sample). In total, 177 randomly selected clones were sequenced. The operational taxonomic unit (OTU) cut-off value of 13 % based on nucleotide sequence identity was used to reflect methanotroph species [30]. One representative sequence from each OTU was chosen to construct the phylogenetic tree. The detailed procedures of phylogenetic analysis were described previously [27]. Sequences were deposited in the GenBank database under accession numbers KM924554 to KM924730.

The real-time (quantitative) PCR of *pmoA* fragments was performed in a 7500 real-time PCR system (Applied Biosystems) using the primer set A189 and mb661. Prior to qPCR, DNA preparations were diluted 10:1 to minimize possible inhibition. The DNA standard was prepared from purified plasmid DNA of a *pmoA* clone with a concentration ranging from 1.0×10^{1} to 1.0×10^{8} copies μl^{-1} [27]. The thermal cycles and fluorescence signal acquisition followed the protocol described previously [31]. Each measurement was performed in triplicate. For all assays, the amplification efficiency ranged between 89 and 94 % and r^2 values were 0.96 to 0.98.

Statistical Analysis

One-way ANOVA was performed to test for significant differences between treatments using SAS software (SAS Institute, Cary, USA). Other procedures related to data analyses have been described previously [11].

Results

Methane Oxidation Rate

In ME treatments, the methane oxidation rate increased to a peak on day 16, and then stabilized between 1.2 to 1.6 μmol h^{-1} gdw^{-1} until the end of the incubation (Fig. 1). In the CK treatment, the first and second stops of methane supply resulted in a significant decrease in methane oxidation rate, which recovered from day 35 to 37 to a level comparable to the ME treatment. The methane oxidation rate in the AC treatment was lower than in the ME and CK treatments. The rate decreased to almost zero on day 24. Surprisingly, the third addition of acetate did not cause a decrease. The rate maintained from 0.35 to 0.61 μmol h^{-1} gdw^{-1} after day 32, which was slightly higher than the rates from day 13 to 28. The oxygen concentration decreased rapidly after flushing with air, but was never lower than 54 μmol l^{-1}, which is equivalent to 1 % (Fig. S1). Therefore, the incubation conditions remained oxic to prevent the possibility of anaerobic methane production by acetoclastic methanogenic archaea.

Quantification of *pmoA* Genes

The quantitative PCR of *pmoA* genes showed an obvious growth of methanotrophs during the incubation (Fig. 2). The highest growth was observed in the ME treatment, increasing from 6.8×10^{8} copies gdw^{-1} on day 0 to 1.6×10^{11} copies gdw^{-1} on day 39. The copy numbers in AC and CK treatments also increased rapidly from days 0 to 21, but maintained stable from days 21 to 39. The differences among

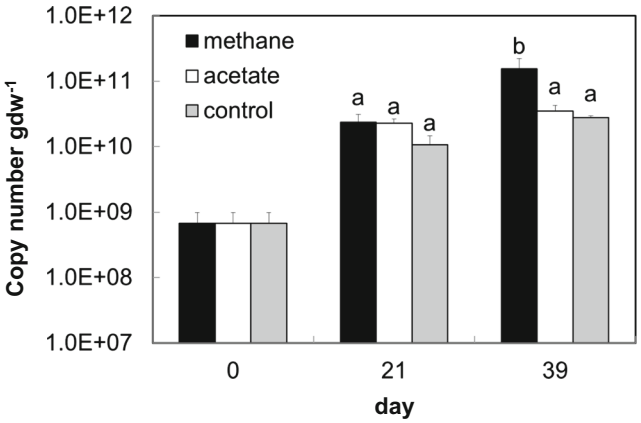

Fig. 2 Number of *pmoA* gene copies in paddy soil under different incubation conditions. *Different letters* on the same sampling day indicate a significant difference ($p<0.05$)

three treatments on day 21 were not significant ($p>0.05$), although the copy number in the CK treatment was slightly lower. At the end of incubation (day 39), the copy numbers were significantly lower in AC and CK treatments than in the ME treatment ($p<0.01$). In addition, the quantitative PCR of *mcrA* genes showed that the copy number decreased during the incubation, indicating that methanogens did not grow (Fig. S2).

Methanotrophic Community Structure

The structure of methanotrophic community was investigated by a combination of T-RFLP analysis with cloning and sequencing of *pmoA* genes. In the pattern of original soil (day 0), the T-RF of 244 bp was found to be dominant with 80 % contribution, while other T-RFs of 79, 437 and 505 bp accounted for less than 20 % (Fig. 3). The 47 sequences in clone library for soil sample on day 0 were divided into six OTUs (Table 1; Table S1). Seventy-two per cent of sequences were related to the type II methanotroph *Methylocystis* (T-RF

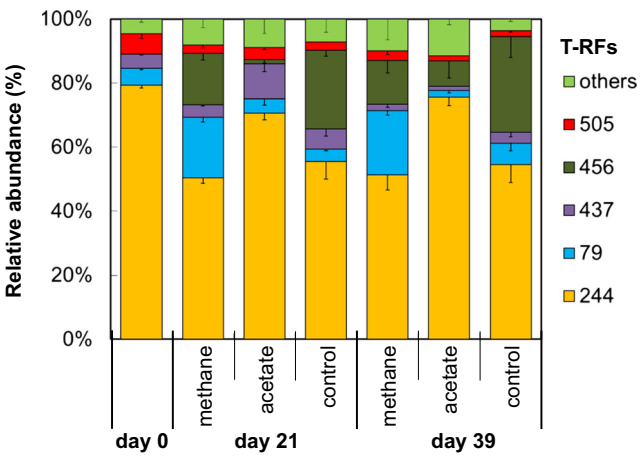

Fig. 3 Structures of methanotrophic communities in paddy soil under different incubation conditions by T-RFLP-based analysis of *pmoA* genes

Table 1 Analyses of four clone libraries of *pmoA* genes or transcripts

OUTs	Phylogenetic affiliation	Day 0			Day 39 (AC)			Day 39 (CK)			SIP		
		N	P	RF (bp)	N	P	RF (bp)	N	P	RF (bp)	N	P	RF (bp)
OTU1	*Methylocystis*	34	72.3	244	39	95.1	244	27	58.7	244	31	72.1	244
OTU2	*Methylobacter*	4	8.5	456 (1), 505 (3)				14	30.4	505			
OTU3	*Methylosarcina*	3	6.4	505 (2), 437 (1)									
OTU4	*Methylomonas*	2	4.3	505 (1), 437 (1)	1	2.4	505				12	27.9	505
OTU5	*Methylocaldum/Methylococcus*	3	6.4	79				4	8.7	79			
OTU6	*Methylocaldum/Methylococcus*	1	2.1	79	1	2.4	79	1	2.2	79			
Total		47	100		41	100		46	100		43	100	

The number in the brackets indicates the number of clones whose restriction fragment is assigned to different T-RFs

N number of clones in each OTU, *P* percentage accounting for the whole clone library, *RF* restriction fragments of the sequences

of 244 bp), and 28 % were related to type I methanotrophs. The type I groups consisted of *Methylobacter* (T-RF of 505 bp), *Methylosarcina* (T-RFs of 505 or 437 bp), *Methylomonas* (T-RFs of 505 or 437 bp) and *Methylocaldum*/*Methylococcus* (T-RF of 79 bp). Neither *Methylocella*- nor *Methyloferula*-related sequences were detected in a clone library of *mmoX* genes (Fig. S3), indicating that this paddy soil did not contain *Methylocella* or *Methyloferula*.

The phylogenetic analysis of *pmoA* genes showed that sequences of OTU 1 were most closely related (99.7 % nucleotide sequence similarity, Fig. 4) to *Methylocystis* sp. LW2 which was isolated from the sediment of Lake Washington [32]. Among taxonomically described methanotrophs, *Methylocystis echinoides* IMET10491T displayed the highest identity with OTU 1 (96.3 % similarity of nucleotide sequence). *M. echinoides* IMET10491T, which was isolated from the sludge of sewage treatment plant [33], was able to slowly grow on acetate in the absence of methane [18]. An analysis by BLAST against *pmoA* sequences in the NCBI database (Table 2) indicated that OTU 1 were closely related (>99 % nucleotide sequence similarity) to numerous environmental sequences retrieved from paddy soils [37–40], rice roots [38, 41, 42] and other soils [43-45].

The methanotroph community compositions changed in the ME treatment after the incubation with methane (Fig. 3). The relative abundances of the 244 bp T-RF (*Methylocystis*) decreased whereas the T-RFs of 79 bp (*Methylocaldum*/*Methylococcus*) and 456 bp (*Methylobacter*) clearly increased. Similarly, the methane supplementation resulted in the decrease of the 244 bp T-RF (*Methylocystis*) and an increase of the 456 bp T-RF (*Methylobacter*) in the CK soil samples (Fig. 3, Table 1).

In contrast, the alternating supplementation with methane and acetate showed a different effect on methanotrophic compositions (Fig. 3). Compared with the T-RFLP pattern of the original soil, the relative abundance of T-RF of 244 bp in the AC treatment did not change much, but the T-RFs of 437 and 456 bp increased on days 21 and 39, respectively. The clone library for the soil sample from the AC treatment on day 39 showed that OTU 1 (*Methylocystis*, T-RF of 244 bp) became significantly dominant, accounting for 95 % of all sequences (Table 1).

Activity of Methanotrophs

Stable isotope probing of *pmoA* mRNA was performed for the ^{13}C-acetate labelled soil and the unlabelled soil. The T-RFLP patterns among all fractions from the unlabelled soil sample were similar to each other (Fig. 5a). In contrast, the T-RFLP patterns from labelled soil differed significantly between heavy and light fractions. The T-RFs of 244 and 437 bp dominated in heavy and light fractions, respectively (Fig. 5b). In the clone library retrieved from the heavy fraction of labelled sample, the sequences belonging to OTU 1 (*Methylocystis*, T-RF of 244 bp) and OTU 4 (*Methylomonas*, T-RF of 505 bp) accounted for 72 and 28 %, respectively (Table 1).

Significant labelling of the bacterial 16S rRNA transcripts was observed, indicating the strong utilization of acetate directly or CO_2 indirectly due to cross-feeding by soil bacteria (Fig. S4). Sequence analysis of 37 clones retrieved from the heavy fraction of the labelled sample showed that the active bacteria belonged to *Pseudomonadales* and *Burkholderiales* (Figs. S5 and S6). No sequences related to methanotrophs were observed, probably due to the insufficient numbers of clones or the low abundance of methanotrophs.

Discussion

To the best of our knowledge, this was the first study to investigate the activity and community structure of methanotrophs of Sanjiang Plain paddy soil. This paddy soil

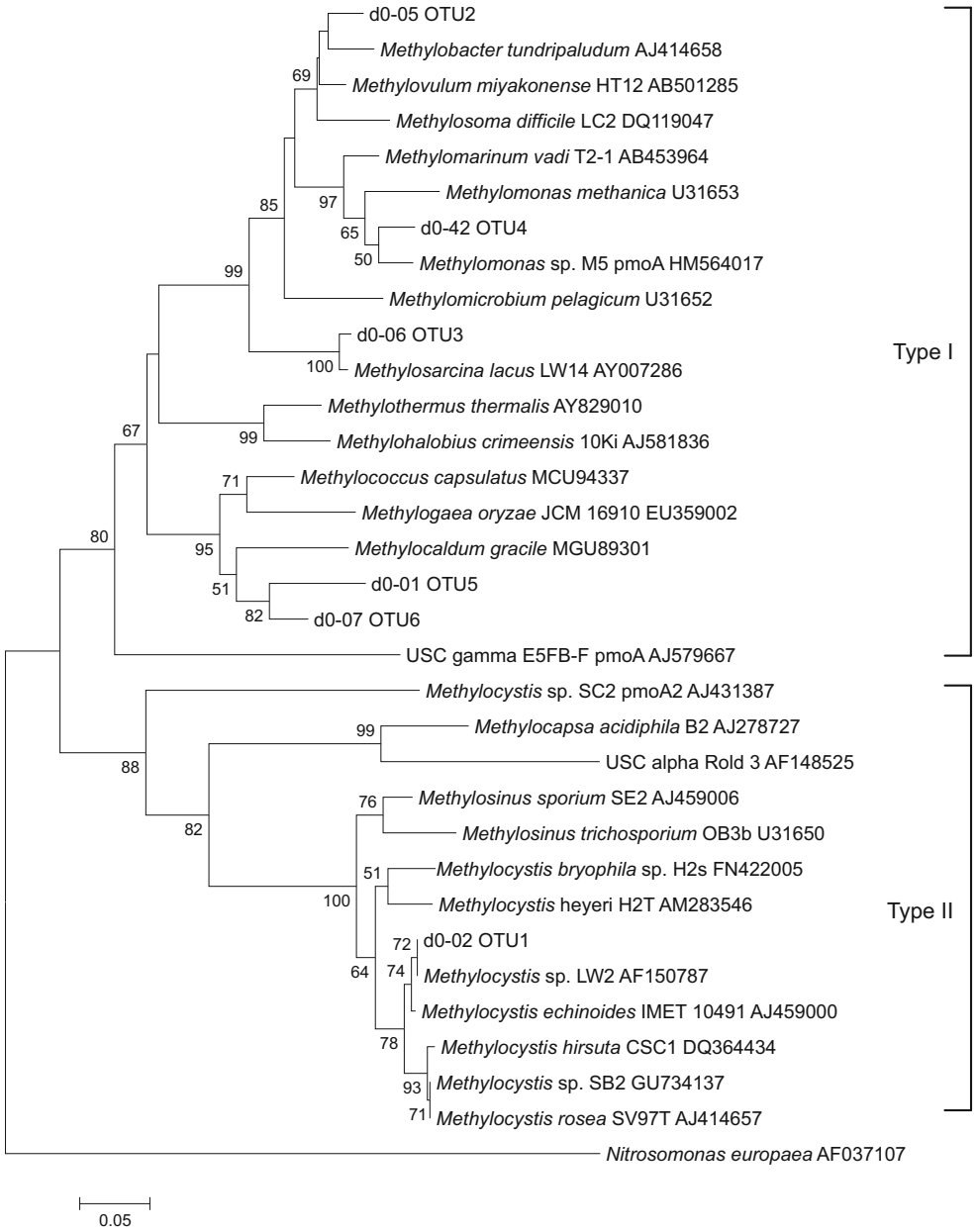

Fig. 4 Phylogenetic tree of representative clone sequences of *pmoA* genes and transcripts retrieved from paddy soil. One representative sequence from each OTU was chosen to construct the phylogenetic tree

has two distinctive features, which are the highest latitude location and the lowest mean annual temperature among paddies around the world. Until now, most studies on paddy methanotroph diversity used the subtropical or tropical paddies as the model systems [43]. Therefore, the present study provides new information about the microbial methane oxidation of a paddy soil from a cold temperate zone. The methane oxidation rate of this paddy soil is relatively high and comparable to the rates of other subtropical paddy soils [6]. This indicated that the low mean annual temperature in Sanjiang Plain did not influence the potential activity of soil methanotrophs.

The analysis of community structure showed the presence of both type I and type II proteobacterial methanotrophs in Sanjiang Plain paddy soil, including members of the genera *Methylobacter*, *Methylosarcina*, *Methylomonas*, *Methylocaldum*/*Methylococcus* and *Methylocystis*. Such methanotrophic diversity has been detected in many other paddy soils [4, 27]. Furthermore, type II methanotrophs, especially the *Methylocystis* populations, were found to be more abundant than type I methanotrophs. Recently, a systematic study investigating methanotroph diversity in paddy soils from different temperate regions also revealed that *Methylocystis* are typically most abundant [43]. It seems that the predominance of type II methanotrophs is a common feature for paddy soils [9, 11], probably due to their special features [44]. For example, type II methanotrophs are able to form exospores or cysts during starvation and other forms of

Table 2 The environmental *pmoA* sequences highly identical to the sequences of OTU 1

Isolation source	Location	Representative clones			References
		Clone name	Identity (%)	GenBank number	
Paddy soil					
	Cixi, China	20-2000B-661r	>99	JN591240	[40]
	Taoyuan, China	JH-TY32	>99	EU193297	[37]
	Tsukuba, Japan	FL25pmo	98.3	AB500818	[39]
	Vercelli, Italy	RS-PK57-271	>99	FN649469	[38]
	Fuyang, China	pmoA-101	>99	JQ671305	unpublished
Rice root					
	Fuyang, China	R20dO7-37	>99	FM986118	[41]
	Vercelli, Italy	18f_12H	>99	FN600008	[42]
	Vercelli, Italy	RT-PK57-485	>99	FN649550	[38]
Other soils					
Oil field soil	Tianjin, China	DG1.5-10	>99	GU056149	[34]
Lake sediments	Brandenburg, Germany	D1_14	>99	HM216860	[35]
Littoral wetland soil	Siilinjärvi, Finland	E07_29A	>99	FN597133	[36]

stress [45]. Therefore, they can survive adverse conditions and seem to be the major reservoir (or seed bank) of the methanotrophic community for the next season [46, 47]. This might also explain why type II methanotrophs are often detected in high abundance in the anoxic bulk soil [10].

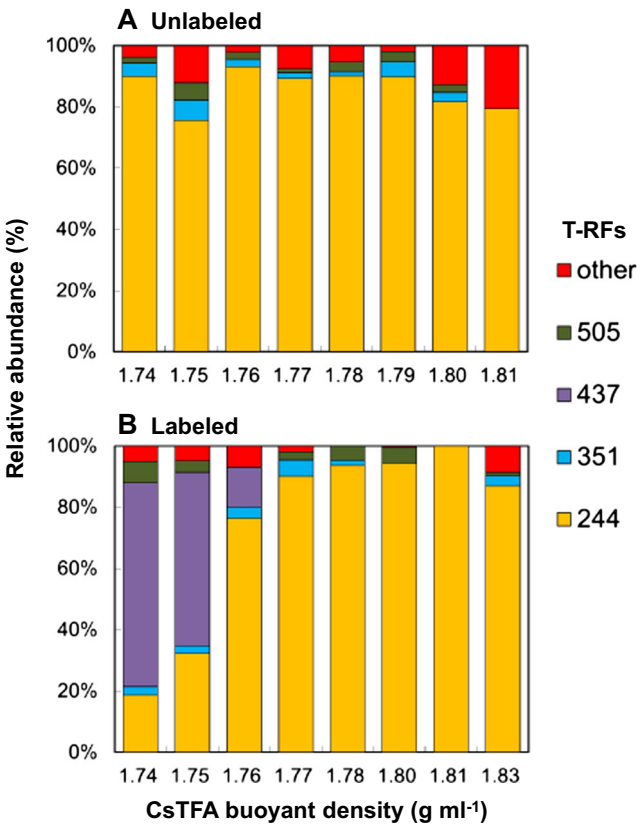

Fig. 5 T-RFLP fingerprints of *pmoA* transcripts retrieved from unlabelled (**a**) and labelled (**b**) samples in RNA-SIP

Another reason for the predominance of type II methanotrophs is that they are able to use alternative carbon sources to support their growth when methane is limited [15]. The capacity to utilize multi-carbon compounds by facultative methanotrophs was first proven by the study of pure cultures. To date, there are four isolates of *Methylocystis* that are able to grow on acetate, namely *Methylocystis bryophila* H2s, *Methylocystis heyeri* H2T, *M. echinoides* IMET10491T [18, 48] and *Methylocystis* strain SB2 [19]. For example, *Methylocystis* strain H2s was shown to grow using acetate with a specific growth rate of 0.006 h^{-1} (equivalent to a doubling time of 115 h) [18]. Acetate utilization, as an important survival strategy for *Methylocystis* in the soils, has also been proven in environmental studies including peatlands [18] and fen soil [49]. The present study provides evidence of acetate utilization by *Methylocystis* in paddy soil. Firstly, we found that one genotype (OTU 1) closely related to *M. echinoides* IMET10491T was significantly enriched by acetate addition. In contrast, the type I methanotrophs *Methylocaldum*/*Methylococcus* and *Methylobacter* were enriched by the methane supplementation. Secondly, RNA-SIP of the *pmoA* gene transcripts revealed that OTU 1 was dominant in the heavy fraction of labelled samples, indicating that these *Methylocystis*-like methanotrophs actively assimilated the labelled acetate in paddy soil. Thirdly, the comparative analysis of environmental sequences from NCBI database showed that these uncultivated *Methylocystis* species (OTU 1-related) are widespread in paddy environments, including both bulk soil and rice root habitats.

Although the facultative methanotrophs were detected in paddy soil, the methanotrophic activity was significantly inhibited by acetate addition. Our results showed that the initial two additions of acetate did not influence the growth of methanotrophs, but significantly inhibited the methane oxidation activity. This is consistent with the findings from studies with pure culture [18], as well as with a forest soil [20] and fen soil [49]. This strongly suggests that methane is the preferred substrate for the facultative methanotrophs *Methylocystis*. Surprisingly, the paddy soil undergoing three periods of acetate addition still had methane oxidation activity, suggesting that the methanotrophs maintain the methane oxidation machinery by utilizing acetate in the absence of methane [18].

In summary, the present study showed that facultative methanotrophs belonging to the genus *Methylocystis* could use acetate as a secondary carbon source in paddy soil. This observation helps to explain the predominance of *Methylocystis* in paddy soils. Future efforts should aim to isolate these *Methylocystis* strains from paddy soil in order to expand our understanding of the diversity of facultative methanotrophs and their ecophysiological functions.

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References

1. Op den Camp HJM, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S, Jetten MSM, Birkeland NK, Pol A, Dunfield PF (2009) Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. Environ Microbiol Rep 1:293–306
2. Sharp CE, Smirnova AV, Graham JM, Stott MB, Khadka R, Moore TR, Grasby SE, Strack M, Dunfield PF (2014) Distribution and diversity of *Verrucomicrobia* methanotrophs in geothermal and acidic environments. Environ Microbiol 16:1867–1878
3. Conrad R (2007) Microbial ecology of methanogens and methanotrophs. Adv Agron 96:1–63
4. Horz HP, Yimga MT, Liesack W (2001) Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA*, *mmoX*, *mxaF*, and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. Appl Environ Microbiol 67:4177–4185
5. Lüke C, Frenzel P (2011) Potential of *pmoA* amplicon pyrosequencing for methanotroph diversity studies. Appl Environ Microbiol 77: 6305–6309
6. Ma K, Conrad R, Lu YH (2013) Dry/wet cycles change the activity and population dynamics of methanotrophs in rice field soil. Appl Environ Microbiol 79:4932–4939
7. Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60:439–471
8. Semrau JD, DiSpirito AA, Yoon S (2010) Methanotrophs and copper. FEMS Microbiol Rev 34:496–531
9. Macalady JL, McMillan AMS, Dickens AF, Tyler SC, Scow KM (2002) Population dynamics of type I and II methanotrophic bacteria in rice soils. Environ Microbiol 4:148–157
10. Wu LQ, Ma K, Lu YH (2009) Rice roots select for type I methanotrophs in rice field soil. Syst Appl Microbiol 32:421–428
11. Ma K, Lu YH (2011) Regulation of microbial methane production and oxidation by intermittent drainage in rice field soil. FEMS Microbiol Ecol 75:446–456
12. Qiu QF, Noll M, Abraham WR, Lu YH, Conrad R (2008) 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*. ISME J 2:602–614
13. Shrestha M, Abraham WR, Shrestha PM, Noll M, Conrad R (2008) Activity and composition of methanotrophic bacterial communities in planted rice soil studied by flux measurements, analyses of *pmoA* gene and stable isotope probing of phospholipid fatty acids. Environ Microbiol 10:400–412
14. Reim A, Lueke C, Krause S, Pratscher J, Frenzel P (2012) One millimetre makes the difference: high-resolution analysis of methane-oxidizing bacteria and their specific activity at the oxic-anoxic interface in a flooded paddy soil. ISME J 6:2128–2139
15. Semrau JD, DiSpirito AA, Vuilleumier S (2011) Facultative methanotrophy: false leads, true results, and suggestions for future research. FEMS Microbiol Lett 323:1–12
16. Dedysh SN, Knief C, Dunfield PF (2005) *Methylocella* species are facultatively methanotrophic. J Bacteriol 187:4665–4670
17. Dunfield PF, Belova SE, Vorob'ev AV, Cornish SL, Dedysh SN (2010) *Methylocapsa aurea* sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase, and emended description of the genus *Methylocapsa*. Int J Syst Evol Microbiol 60:2659–2664
18. Belova SE, Baani M, Suzina NE, Bodelier PLE, Liesack W, Dedysh SN (2011) Acetate utilization as a survival strategy of peat-inhabiting *Methylocystis* spp. Environ Microbiol Rep 3:36–46
19. Im J, Lee SW, Yoon S, DiSpirito AA, Semrau JD (2011) Characterization of a novel facultative *Methylocystis* species capable of growth on methane, acetate and ethanol. Environ Microbiol Rep 3:174–181
20. Pratscher J, Dumont MG, Conrad R (2011) Assimilation of acetate by the putative atmospheric methane oxidizers belonging to the USC alpha clade. Environ Microbiol 13:2692–2701
21. Rahman MT, Crombie A, Chen Y, Stralis-Pavese N, Bodrossy L, Meir P, McNamara NP, Murrell JC (2011) Environmental distribution and abundance of the facultative methanotroph *Methylocella*. ISME J 5:1061–1066
22. Rui JP, Peng JJ, Lu YH (2009) Succession of bacterial populations during plant residue decomposition in rice field soil. Appl Environ Microbiol 75:4879–4886
23. Huang Y, Sun WJ, Zhang W, Yu YQ, Su YH, Song CC (2010) Marshland conversion to cropland in northeast China from 1950 to 2000 reduced the greenhouse effect. Glob Chang Biol 16:680–695
24. Li T, Huang Y, Zhang W, Yu YQ (2012) Methane emissions associated with the conversion of marshland to cropland and climate change on the Sanjiang Plain of Northeast China from 1950 to 2100. Biogeosciences 9:5199–5215
25. Jiang CS, Wang YS, Hao QJ, Song CC (2009) Effect of land-use change on CH4 and N2O emissions from freshwater marsh in Northeast China. Atmos Environ 43:3305–3309
26. Dedysh SN, Panikov NS, Tiedje JM (1998) Acidophilic methanotrophic communities from *Sphagnum* peat bogs. Appl Environ Microbiol 64:922–929
27. Ma K, Qiu QF, Lu YH (2010) Microbial mechanism for rice variety control on methane emission from rice field soil. Glob Chang Biol 16:3085–3095
28. Ma K, Conrad R, Lu YH (2012) Responses of methanogen *mcrA* genes and their transcripts to an alternate dry/wet cycle of paddy field soil. Appl Environ Microbiol 78:445–454
29. Lueders T, Manefield M, Friedrich MW (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. Environ Microbiol 6:73–78

30. Degelmann DM, Borken W, Drake HL, Kolb S (2010) Different atmospheric methane-oxidizing communities in European beech and Norway spruce soils. Appl Environ Microbiol 76:3228–3235
31. Kolb S, Knief C, Stubner S, Conrad R (2003) Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl Environ Microbiol 69:2423–2429
32. Auman AJ, Stolyar S, Costello AM, Lidstrom ME (2000) Molecular characterization of methanotrophic isolates from freshwater lake sediment. Appl Environ Microbiol 66:5259–5266
33. Bowman JP, Sly LI, Nichols PD, Hayward AC (1993) Revised taxonomy of the methanotrophs: description of *Methylobacter* gen.nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. Int J Syst Bacteriol 43:735–753
34. Zhang F, She YH, Zheng Y, Zhou ZF, Kong SQ, Hou DJ (2010) Molecular biologic techniques applied to the microbial prospecting of oil and gas in the Ban 876 gas and oil field in China. Appl Microbiol Biotechnol 86:1183–1194
35. Dumont MG, Pommerenke B, Casper P, Conrad R (2011) DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment. Environ Microbiol 13:1153–1167
36. Siljanen HMP, Saari A, Krause S, Lensu A, Abell GCJ, Bodrossy L, Bodelier PLE, Martikainen PJ (2011) Hydrology is reflected in the functioning and community composition of methanotrophs in the littoral wetland of a boreal lake. FEMS Microbiol Ecol 75:430–445
37. Zheng Y, Zhang LM, Zheng YM, Di HJ, He JZ (2008) Abundance and community composition of methanotrophs in a Chinese paddy soil under long-term fertilization practices. J Soils Sediments 8:406–414
38. Shrestha M, Shrestha PM, Frenzel P, Conrad R (2010) Effect of nitrogen fertilization on methane oxidation, abundance, community structure, and gene expression of methanotrophs in the rice rhizosphere. ISME J 4:1545–1556
39. Mayumi D, Yoshimoto T, Uchiyama H, Nomura N, Nakajima-Kambe T (2010) Seasonal change in methanotrophic diversity and populations in a rice field soil assessed by DNA-stable isotope probing and quantitative real-time PCR. Microbes Environ 25:156–163
40. Ho A, Lueke C, Cao ZH, Frenzel P (2011) Ageing well: methane oxidation and methane oxidizing bacteria along a chronosequence of 2000 years. Environ Microbiol Rep 3:738–743
41. Qiu QF, Conrad R, Lu YH (2009) Cross-feeding of methane carbon among bacteria on rice roots revealed by DNA-stable isotope probing. Environ Microbiol Rep 1:355–361
42. Lüke C, Krause S, Cavigiolo S, Greppi D, Lupotto E, Frenzel P (2010) Biogeography of wetland rice methanotrophs. Environ Microbiol 12:862–872
43. Lüke C, Frenzel P, Ho A, Fiantis D, Schad P, Schneider B, Schwark L, Utami SR (2014) Macroecology of methane oxidizing bacteria: The β-diversity of *pmoA* genotypes in tropical and subtropical rice paddies. Environ Microbiol 16:72–83
44. Ho A, Kerckhof F-M, Lüke C, Reim A, Krause S, Boon N, Bodelier PLE (2013) Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies. Environ Microbiol Rep 5:335–345
45. Whittenbury R, Davies SL, Davey JF (1970) Exospores and cysts formed by methane-utilizing bacteria. J Gen Microbiol 61:219–226
46. Eller G, Krüger M, Frenzel P (2005) Comparing field and microcosm experiments: a case study on methano- and methylo-trophic bacteria in paddy soil. FEMS Microbiol Ecol 51:279–291
47. Ho A, Frenzel P (2012) Heat stress and methane-oxidizing bacteria: effects on activity and population dynamics. Soil Biol Biochem 50: 22–25
48. Belova SE, Kulichevskaya IS, Bodelier PLE, Dedysh SN (2013) *Methylocystis bryophila* sp nov., a facultatively methanotrophic bacterium from acidic *Sphagnum* peat, and emended description of the genus *Methylocystis* (ex Whittenbury et al. 1970) Bowman et al. 1993. Int J Syst Evol Microbiol 63:1096–1104
49. Wieczorek AS, Drake HL, Kolb S (2011) Organic acids and ethanol inhibit the oxidation of methane by mire methanotrophs. FEMS Microbiol Ecol 77:28–39