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Evaluation of methane oxidizer bacteria in the rice soil in Malaysia

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Abstract: Methane is known as a powerful greenhouse gas due to its global warming potential (GWP = 21). Rice fields are methane producers because of the flooding irrigation system. Two microbial communities are involved in methane cycle in the soil including methanogens and methanotrophic bacteria which are responsible for methane production and methane oxidation respectively. Methanotrophic bacteria as aerobic unicellular microorganisms dominantly exist in soil oxic area (e.g. surface of the soil and the rhizosphere). These microorganisms can regulate the methane emission from rice soil. This experiment applied PCR-DGGE to detect methane oxidizer bacteria (MOBs) within the rice soil from two depths 0-5 cm and 5-10 cm in different rice growth stages and cultivation systems. Consequently, several MOBs from type I and type II could be identified. However, type I was detected in depth of 0-5 cm and drained condition rather than 5-10 cm and flooding condition.

Keywords: Methane oxidizer bacteria (MOB), Rice, Denaturing gradient gel electrophoresis (DGGE), Tropical soil.

Introduction

Methane is a strong greenhouse gas with global warming potential 21 times more than carbon dioxide. Rice fields because of the flooding irrigation system are main source for this gas (Li et al. 2011) [1]. Two microbial communities are involved in methane cycle in flooded soils, methanogens as producers and methanotrophs as oxidizers of methane. Methanotrophs are unicellular organisms including aerobic methanotrophic bacteria and anaerobic methanotrophic archaea. Methanotrophs have been studied in various environments and by different methods (Fazli et al. 2013) [2]. Known methanotrophic bacteria categorize into three types (I, II and X), under 14 genera (Wu et al. 2009 [3], Semrau et al. 2010 [4], Vishwakarma et al. 2010 [5] and Rosenzweig and Ragsdale, 2011) [6]. Types I and II have been identified in rice soil with different niches depending on oxygen and methane concentration (Hoffmann et al. 2002 [7], Vishwakarma et al. 2009 [8] and 2010 [9]). Type I methanotrophs are more active in a higher oxygen and lower methane environment compared to type II (Mayumi et al. 2010) [10]. The aim of this study was identifying the microbial diversity of methane-oxidizer bacteria in Malaysia rice soil by culture independent microbial detection technique (PCR-DGGE).

Material and Method Used

A. Soil Description

Soil samples were taken from Tanjung Karang paddy field located at 30° 25′ to 30° 45′ N latitude and 100° 58′ to 101° 15′ E longitude in the state of Selangor Malaysia. The soil was of Jawa series with 51% clay and 43% silt in top 10 cm and, 53% clay and 42% silt in sub soil. The soil carbon and nitrogen contents were 6.38% and 0.62%, respectively.

B. Rice Cultivation System

Three rice cultivation systems were studied including, conventional method, original system of rice intensification (SRI-O) (Uphoff, 2008) [11] and oblong-triangular system of rice intensification (SRI-T) (Zheng et al. 2004) [12] (Table 1).

Table 1: Cultivation practices generally recommended for SRI compared to conventional methods (Adapted from Fazli et al. 2012) [13].

Practices	SRI methods		Conventional methods
	Original SRI	Oblong-Triangular SRI	
Nursery bed	One of the paddy tanks was allocated to nursery purpose		
Seed Variety	MR219	MR219	MR219
Seedling age at Transplanting	8-12 days at transplanting	8-12 days at transplanting	21-30 days at transplanting
Seedling no.	1 seedling in each hill transplanted at 1–2 cm depth;	transplanting 3 seedlings per hill separated by 7 cm	3-5 seedlings in each hill, plunged into soil
Spacing	25×25 cm with regular distances	40×45cm with regular distances	15–20 cm at random intervals
Irrigation			
Vegetative growth stage	Intermittent irrigation with wet-dry cycle; only shallow standing water during wet periods (±2 cm)		Continuous irrigation, keeping ±10 cm of standing water on fields
Reproductive stage	Continuous irrigation, keeping 2-5 cm of standing water		Continuous irrigation, keeping ±10 cm deep standing water
Weeding Method	Rotary weeder, weeding tools, or manual weeding every 10-12 days starting 10-12 days after transplanting		Use of weeding tools, or manual weeding whenever its needed
Fertilizer use Type	Chemical fertilizer used by farmers (Urea, Compound Fertilizer, Mixed Fertilizer)		

C. Soil Sampling Scheme

A total of 90 soil samples were taken in different rice growth stages including rice transplanting day (0 day after transplanting (DAT)), vegetative stage (Vs) (42 DAT), panicle initiation stage (PIs) (62 DAT), heading stage (Hs) (80 DAT) and harvest stage. Eh of less than -200 mV was selected as an indicator for methane formation zone to determine the boundary depth between aerobic and anaerobic condition in the soil (Chen and Avnimelech, 1986) [14]. Therefore, the depth of 10-12 cm was identified as the boundary. Subsequently, soil samples were prepared from two ranges of depth 0-5 cm and 5-10 cm in triplicate. In addition, soil sampling points were selected from both interplant and rhizosphere areas. Three replicates of soil samples were mixed and then, subjected to the process of homogenisation by air drying, separating the plant litters and residues, sieving (2 mm) and mixing thoroughly. Afterwards, soil samples were stored at -20°C for further microbial analysis or immediately transferred to the laboratory for subjecting to DNA extraction.

D. Extraction of Total Deoxyribonucleic Acid (DNA) from Soil Samples

The soil type was hard to lysis; therefore, some modification in lysis section and the last step of the manufacturer's instructions of PowerSoil® DNA Isolation Kit-MO BIO was applied for DNA extraction. Consequently, the lysis section procedure performed as follows: 200 µl of bead solution was removed from the tube and then 200 µl of phenol: chloroform: isoamyl alcohol pH 7-8 (PCI) was added in. This step was followed by adding 60 µl of solution C1. Then, the samples were vortexed for 15 to 20 minutes. Centrifuge was run to pellet for 1 minute at full speed. Afterwards, all steps were done according to the manufacturer's instructions until step 20. However, solutions C2 and C3 each were applied at 100 µl. At the end, two rounds of adding 50 µl of solution C6 was to the center of the filter membrane and incubation for 5 minutes were carried out. After each round, centrifuge was run for 30s at full speed. Extracted DNA samples were stored at -20°C before Polymerase Chain Reaction (PCR) analysis.

E. PCR amplification of pmoA genes

Regarding amplification of methanotrophic DNA, two oligonucleotide primer sets were selected to amplify a 500 bp conserved region of the particulate methane monooxygenase (pMMO) gene including, A189f/A682r and A189f/mb661r (Table 2). To amplify pmoA gene sequences, 50µl of PCR reactions were carried out on thermocycler (eppendorf) using the following reaction mix: 10ul of DNA template, 1×of PCR buffer (MgCl2, 2 mM), 200µM of dNTP, 200 nM of each primer, 2.5 U of EX Taq DNA polymerase (Takara, Japan). Then re-amplification performed by following program: Initial denaturation was performed at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and the final extension at 72 °C for 6 min (Holmes et al., 1995) [15].

Table 2: The oligonucleotide primer sets were used to detect the methanotrophic bacteria.

Primer	Sequence (5′─3′)	Amplicon length (bp)	Target	Reference
A189f	GGnGACTGGGACTTCTGG	565	pmoA ; pMMO/ AMO	Holmes et al. 1995 [15]
A682r	GAAsGCnGAGAAGAAsGC	525		
mb661R	CCGGmGCAACGTCyTTACC	-		Lin et al. 2005 [16] Wu et al. 2009 [3] Yun et al. 2010 [17]
GC-clamp	CCCCCCCCCCCCCCGCCCCCCGCCCCCCGC CCCCGCCGCCC			Tuomivirta et al. 2009 [18]

F. Denaturing gradient gel Electrophoresis (DGGE)

PCR amplicons of soil DNA samples (565 bp) from four rice growth stages were subjected to gel electrophoresis by 1% (w/v) agarose gel. Then the PCR products with clear bands were applied for DGGE analysis after being concentrated. DGGE was performed. For this purpose, about 15 µl of the PCR product was loaded and separated by the Bio Rad DCode Universal Mutation Detection System / Electrophoresis (Bio-Rad Lab Los Angeles) applying an 8% (wt/vol) polyacrylamide gel (40% acrylamide-bisacrylamide [37:5:1] which had a denaturant gradient of 30–70% (100% denaturant equal to 7 M urea, 40% formamide [vol/vol], and 8% acrylamide). Then DGGE gel electrophoresis was run with 1×TAE buffer (40mM Tris, 20mM acetic acid, and 1mM EDTA, pH 8.0) at 60°C and 100 V for 16 h. Gel staining was carried out by SYBR® Safe DNA Gel Stain solution. Selected DGGE bands were eluted in 60 µl of MilliQ water after excising from the gel. Then, the eluted DNA samples were incubated at 4 °C overnight. Fifty µl of the eluted DNA samples were subjected to PCR and re-amplified by GC Clamp-A189f/mb661r primer set using following program: Initial denaturation was performed at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and the final extension at 72 °C for 6 min (Holmes et al., 1995) [15]. Sequencing of DNA samples was carried out by a capillary ABI Prism 3100 sequencer. Obtained pmoA gene sequences were compared to nucleic acid sequences of pmoA genes in the GenBank database applying the BLAST program (www.ncbi.nlm.nih.gov/BLAST) by the Blastn search option.

Results and Discussion

A. Diversity of MOBs based on the pmoA genes

Some PCR products did not give bands or gave a smear band by gel electrophoresis (1% agarose gel) (Table 3). These samples mostly were taken during flooded condition from soil depth of 5-10 cm (Fig. 1). Consequently, only PCR products which produced clear band through gel electrophoresis supplied for DGGE (Fig. 2).

Table 3: Presentation of band qualities from taken samples

Rice Growing Stage	Treatments					
	Conventional method	SRI-O	SRI-T	Conventional method	SRI-O	SRI-T
Soil depth	0-5 cm			5-10 cm		
0 DAT	2	2	2	2	2	2
Vs	0	1	1	0	1	1
PIs	1	1	1	0	0	1
Hs	1	2	2	0	1	1
Harvest	2	2	2	2	2	2

Note:- 0 = no band; 1= smear band; 2 = clear band
SRI-O= Original system of rice cultivation; SRI-T= Oblong-triangular system of rice cultivation.
DAT=Day After Transplanting; Vs= Vegetative Stage; PIs= Panicle Initiation Stage; Hs= Heading Stage.

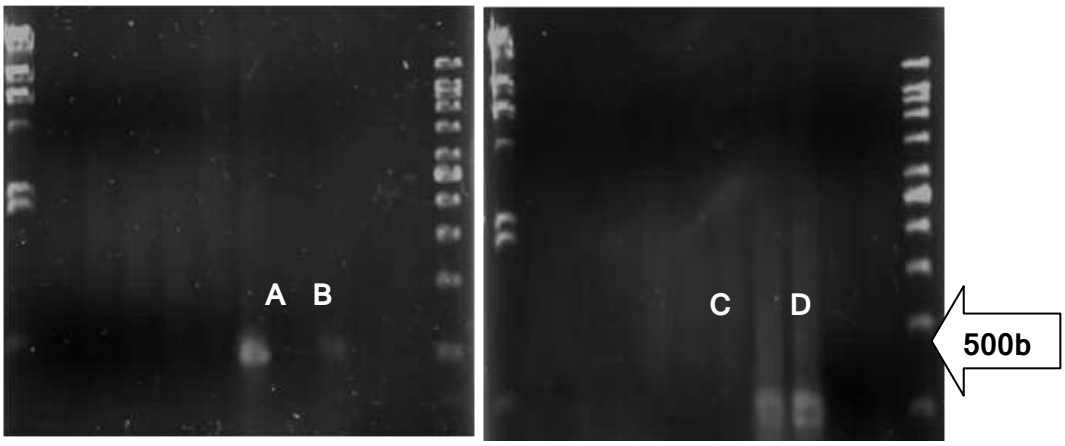

Figure 1: Samples without band or with smear band belong to flooding condition; A: SRI-O, 5-10 cm depth, 42 DAT; B: SRI-T, 5-10 cm depth, 42 DAT; C: SRI-T, 5-10 cm depth, 62 DAT; D: SRI-O, 5-10 cm depth, 80 DAT.

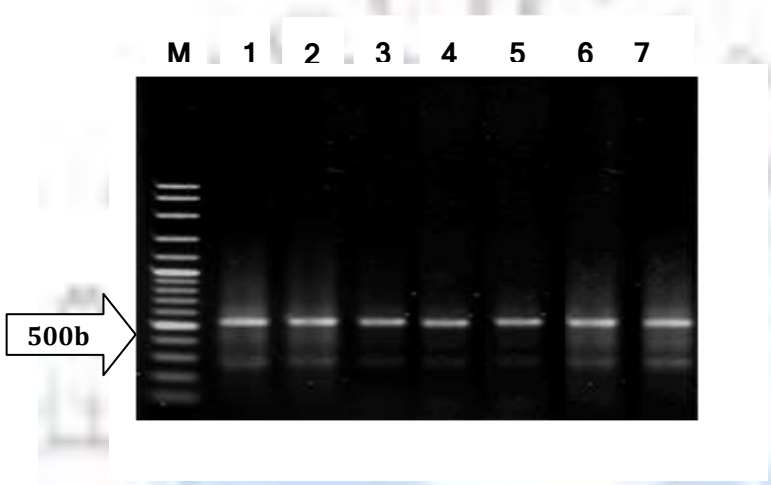

Figure 2: Some of the samples which produced clear bands mostly from 0-5 cm depth of soil and drained periods. M: 100 bp DNA ladder; 1: Conventional method, 0 DAT, 0-5 cm depth; 2: SRI-O, 0 DAT, 0-5 cm depth; 3: SRI-T, 0 DAT, 0-5 cm depth; 4: SRI-T, 80 DAT, 0-5 cm depth 5: SRI-O, 80 DAT, 0-5 cm; 6: SRI-T, 110 DAT, 5-10 cm; 7: SRI-O, 110 DAT, 5-10 cm.

The DGGE profile revealed only a few dominant bands so that a same size band (C) was repeated in all samples (Fig. 3). However, it was smear for 5-10 cm depth compared to 0-5 cm depth at flooded condition. Amplification of pmoA gene sequences of 15 bands resulted in identification of 101 clones of MOBs in 0-5 cm depth for SRI treatments, 37 clones of MOBs in 0-5 cm for Conventional method, and 33 clones of MOBs in 5-10 cm depth samples. The MOBs community structure was same at both depths (Liebner et al. 2009) [19]. Nevertheless, the diversity of MOBs was higher at 0-5 cm depth of SRIs compared to other treatments. This difference might be due to depth and irrigation management so that it has been indicated that methanogens may display different community structure in soil depth profile (Bodelier et al. 2005) [20]. In addition, the irrigation pattern of SRI (alternate wetting and drying the soil) could change the composition, population and transcriptional activities of soil microbial communities (e.g. methanogenic archaea) (Watanabe et al. 2010) [21].

The most dominant band (C) in all samples belonged to α-proteobacteria and uncultured bacterium clones particulate methane monooxygenase alpha subunit (pmoA) gene, partial cds (531 bp). Other bands represented uncultured bacterium clones ammonia monooxygenase/particulate methane monooxygenase-like (amoA/pmoA) gene (B-590 bp), Methylocystaceae (D-481 bp), and uncultured ammonia-oxidizing bacterium (A-670 bp). Furthermore, type I methanotrophs including, γ-proteobacteria, Methylococcales, Crenotrichaceae and type II methanotrophs including Methylocystaceae (481 bp), uncultured methanotrophic bacterium (496 bp), and uncultured methanotrophic proteobacterium were detected at 0-5 cm depth (Table 4 and 5).

Methylocystaceae (Vishwakarma et al. 2009 [8], Ma and Lu, 2011 [22]) and Methylococcales (Hoffmann et al. 2002 [7], Yun et al. 2010 [17] and Ma and Lu, 2011 [22]) were detected in rice soil by several groups.

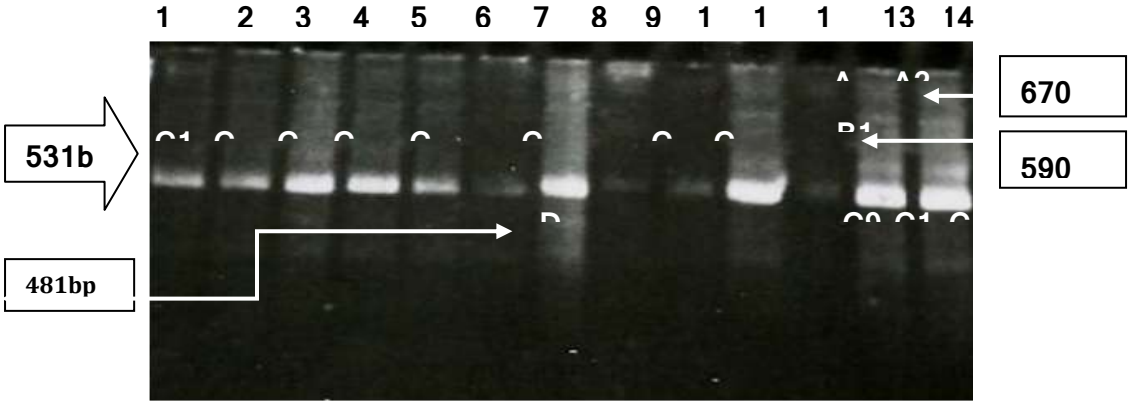

Figure 3: DGGE profile of pmoA gene sequences of MOBs from rice soil samples in different rice growth stages and depths (There; 1,2,3.....14 are the different rice growth stages and depths in GE profile of pmoA gene sequences of MOBs from rice soil samples).

Table 4: Report on operational taxonomic units.

Bacteria	
0-5 cm of the depth of soil	
1. Proteobacteria	1.1 γ -proteobacteria (T1[a]); (Methylococcales)
	1.1.1 Crenothrix polyspora,
	1.1.2 Uncultured Crenothrix sp.
	1.2 α -proteobacteria (T2[b])
	1.2.1 Environmental samples
	1.2.1.1 Uncultured αproteobacterium, Uncultured methanotrophic α -proteobacterium
	1.2.2 Unclassified Methylocystaceae
	1.2.2.1 methanotroph K3-21, methanotroph K2-14, methanotroph K3-17
	1.3 Uncultured methanotrophic proteobacterium
2. Environmental Samples	2.1 Uncultured bacterium
	2.2 Uncultured bacteria gp ensemble
	2.3Uncultured methanotrophic bacterium
	2.4 Uncultured ammonia-oxidizing bacterium
5-10 cm of the depth of soil	
1.Environmental samples (T2[b])	1.1 Uncultured bacterium, Uncultured bacterium gp22 (ammonia-oxidizing bacteria)
2.Uncultured α -proteobacterium (T2[b])	2.1 Proteobacteria, α proteobacteria

Note:- T1[a] means Type I methanotrophs and T2[b] means Type II methanotrophs

Table 5. Affiliation of excised bands of DGGE.

Band	Nearest relative	Accession	Soil depth (cm)	Band size	Similarity (%)	Phylogeny
A1	Uncultured ammonia-oxidizing bacterium	JQ735299	0-5	670	99	Bacteria
A2	Uncultured ammonia-oxidizing bacterium	JQ735350	5-10	670	99	Bacteria
B1	Uncultured bacterium	DQ008438	5-10	590	99	Bacteria
C1	α-proteobacteria	DQ367741	0-5	531	99	Bacteria
C2	Uncultured bacterium	JN591214	0-5	531	100	Bacteria
C3	Uncultured alpha proteobacterium	DQ367742	0-5	531	89	Bacteria
C4	Crenothrix polyspora	DQ295904	5-10	531	99	Methylococcales
C5	Crenothrix polyspora	DQ295903	5-10	531	99	Methylococcales

C6	Uncultured bacterium	JN591215	0-5	531	100	Bacteria
C7	Uncultured bacterium	JN591201	5-10	531	100	Bacteria
C8	Crenothrix polyspora	DQ295899	0-5	531	99	Methylococcales
C9	Crenothrix polyspora	DQ295900	0-5	531	99	Methylococcales
C10	NC10 bacterium enrichment culture	JF706214	5-10	531	100	Bacteria
C11	Uncultured alpha proteobacterium	DQ367742	5-10	531	84	α-proteobacteria
D1	Methanotroph K2-14	AF547177	0-5	481	80	Methylocystaceae

Generally, all identified genera of MOBs by researchers have hitherto been classified into two groups, type I and type II (Rosenzweig and Ragsdale, 2011) [6]. Type I MOBs are gamma-proteobacteria phylogenetically and assimilate one-carbon compounds via the ribulose monophosphate cycle and type II are alpha-proteobacteria phylogenetically and assimilate C1 intermediates via the serine pathway (Rosenzweig and Ragsdale, 2011) [6]. In addition, Hanson and Hanson, (1996) reported type X as other group of methanotrophs. From identified MOBs, Methylococcus is of Type-X, because, these microorganisms occupy an intermediate position (Hanson and Hanson, 1996 [23], Bowman, 2006 [24] and Dubey, 2005 [25]). In fact, type X can be considered as a split from type I. Because, in spite of some differences between the members of group X and other methanotrophs (e.g. differences in phylogeny, chemotaxonomy, internal ultrastructure, carbon assimilation pathways, and certain other biochemical aspects), there are some similarities such as possessing low levels of enzymes of the serine pathway (Bowman, 2006 [24]). However, group X members grow at higher temperatures than type I and type II. They possessed DNA with higher moles percent G + C content (56–65) compared to most type I (43–60) but less than type II (60–67) (Hanson and Hanson, 1996 [23] and Bowman, 2006 [24]). After all, in recent reports MOBs consist of two subgroups, type I and type II so that the genera of type X is categorized under type I MOBs (Wu et al. 2009 [3]; Semrau et al. 2010 [4] ; Vishwakarma et al., 2010 [5, 8 - 9] and Dubey, 2005 [25]; Rosenzweig and Ragsdale, 2011 [6]).

In current study, type I methanotrophs were obtained only in SRI treatments at 0-5 cm depth. On the other hand, type II was mostly dominant in samples of 5-10 cm depth. This finding was in agreement with previous reports (Mayumi et al. 2010 [10]). Moreover, Type I methanotrophs are more sensitive to environmental condition compared to type II. Thus, this group tend to be in more favorable condition especially regarding oxygen availability (Wu et al. 2009 [3]; , Semrau et al., 2010 [4], Vishwakarma 2010 [5] and Dubey 2005 [25]). Also, it has been reported that flooding condition has decreasing effect on methanotrophs' population (Yue et al. 2007[26]). Accordingly, in this study, MOBs showed better presence at 0-5 cm depth in SRI treatments during flooding periods rather than 5-10 cm depth. In conventional method, MOBs exhibited weaker presence even at 0-5 cm depth compared to SRI. This difference could be due to the level of standing water which was higher in conventional method (10 cm) compared to SRI treatments (1-2 cm) by influencing the oxygen level in the soil. In SRI treatments, MOBs could be identified strongly for both depths during drained periods. The irrigation pattern in SRI treatments can provide oxic-condition periodically. Thus, MOBs could enhance their population intermittently.

Conclusions and Final Remarks

In conclusion, diversity of MOBs was higher under drained condition. Oxygen availability was a determining factor for the type of MOBs in the soil. Type II MOBs showed higher dominancy compared to type I especially at 5-10 cm depth because these MOBs can be active in less oxygen concentrate rather than type I. In contrast with SRI treatments, conventional rice cultivation system because of applying flooding continuously as the water management could have suppressing effect on these microorganisms. Accordingly, higher MOBs diversity (esp. Type I) has been identified in SRI treatments. Therefore, SRI treatments are stimulating cultivation system for MOBs.

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