Ecological and molecular analyses of the rhizospheric methanotroph community in tropical rice soil: effect of crop phenology and land-use history

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To study the effect of crop phenology and cultivation practices on methanotrophic communities, two tropical rice fields located in the upper Gangetic plain of India with similar soil type and different cropping history were selected. A laboratory incubation experiment for the enumeration of methanotrophs and for the measurement of CH_4 oxidation potential was conducted on a parallel basis. The methanotroph population size was found to be significantly higher in the Banaras Hindu University (BHU), Varanasi soil than the Indian Institute of Vegetable Research (IIVR), Varanasi soil. The population size increased with the age of the plant for both the sites. The CH_4 oxidation potential was higher with the BHU soil compared to the IIVR soil. The CH_4 oxidation rate increased significantly from tillering to flowering to grain-filling stages, and finally there was no significant difference between the grain-filling and the grain-maturation stages. A diagnostic microarray targeting the *pmoA* gene and a 16S rRNA denaturing gradient gel electrophoresis (DGGE)-based approach were applied to assess the diversity of the methanotrophic community for the two sites. A broad diversity of methanotrophs was detected at both sites, including type I and type II methanotrophs of the genera *Methylobacter*, *Methylomonas*, *Methylosarcina*, *Methylosphaera*, *Methylomicrobium* and *Methylocystis*. Type II methanotrophs were found in higher abundance as compared to type I methanotrophs at both the sites. DGGE analysis indicated that the methanotroph community in BHU soil was more or less stable, while little variation was found in IIVR soil during crop growth.

Keywords: Crop phenology, land-use history, methanotrophs, microarray, rice rhizosphere.

RICE ecosystems are regarded as one of the largest sources of atmospheric methane, contributing up to 20% (20–100 Tg CH_4 yr^{-1}) of global methane emissions[1]. Out of 18.6 Tg of total Indian methane emissions, rice fields[2] contribute 4 Tg. Methane-oxidizing bacteria have the ability to utilize CH_4 as a sole source of carbon and energy[3]. Based on evolutionary relatedness and differences in morphology, biochemistry and physiology, methanotrophs are categorized as type I and type II, belonging to the *Gammaproteobacteria* and *Alphaproteobacteria* respectively. The type I methanotrophs represent the Methylococcaceae family and currently include the following ten genera: *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosarcina*, *Methylosphaera*, *Methylosoma*, *Methylohalobium*, *Methylococcus*, *Methylocaldum* and *Methylothermus*[4,5]. Type II methanotrophs are represented by four genera: *Methylosinus*, *Methylocystis*, *Methylocella* and *Methylocapsa*[4,6], with the first two representing the *Methylocystaceae* family and the other two belonging to the *Beijerinckiaceae* family. Methanotrophs have adapted to most habitats where methane and oxygen are present, including wetland environments, where high concentrations of methane are produced in anaerobic zones by methanogenesis, and in upland soils where atmospheric methane is oxidized[3,7]. All the known methanotrophs, with the exception of the acidophilic *Methylocella* species[8], possess particulate methane monooxygenase (pMMO), and the *pmoA* gene, which encodes a subunit of the enzyme, has been frequently used as a molecular marker to detect methanotrophs in environmental samples[9,10].

The rhizospheric microbial community undergoes a perceptible influence due to soil type, plant age and cultivation pattern[11]. It is well known that only a small proportion of the microbial community is recovered by cultivation[12]. This can be overcome by the direct analyses of genes from environmental samples, including the genes encoding enzymes involved in the oxidation of methane by methanotrophs[10]. For this purpose, Bodrossy *et al.*[13] have developed a *pmoA* gene microarray that enables the rapid, reproducible and semi-quantitative identification of methanotrophs in environmental samples.

It is unclear how different agricultural practices, such as crop rotation and irrigated upland rice cultivation

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Table 1. Soil characteristics

Soil property	BHU agricultural farm	IIVR agricultural farm
Texture	Sand 32%, silt 65%, clay 3% (Inceptisol)	Sand 30%, silt 70%, clay 2% (Inceptisol)
pH	7.04 ± 0.14	6.71 ± 0.12
WHC (%)	44.00 ± 2.30	39.87 ± 3.60
Total N (%)	0.12 ± 0.03	0.09 ± 0.06
Organic C (%)	0.75 ± 0.12	0.73 ± 0.18

affect the fluxes of CH_4 from these systems. Here we have studied the methanotrophic population size, diversity and capacity of the soil for CH_4 oxidation in two irrigated rice field soils which differ only in the crop rotation pattern. The diversity of methanotrophs in tropical irrigated paddy soils is poorly characterized and is limited to some studies of soils from South East Asia[14,15]. This study provides the first *pmoA* diversity data for Indian rice agroecosystems.

Materials and methods

Experimental sites

Soil samples were collected from two tropical rice agroecosystems in India that are part of the Gangetic plain. One experimental site was located at the agricultural farm of Banaras Hindu University (BHU), Varanasi (25°18′N lat., 83°03′E long. and 129 m amsl). The second site was at the agricultural farm of the Indian Institute of Vegetable Research (IIVR), Varanasi (25°08′N lat., 83°03′E long. and 90 m amsl). The annual rainfall during 2005 was 916 mm, of which 753 mm occurred during the experimental period (July to December), and the temperature ranged between a minimum of 18°C and a maximum of 38°C. Both sites have been used for intensive agricultural production, especially the BHU site for rice–wheat production and IIVR site for vegetable production. Characteristics of the soils for both the sites are summarized in Table 1.

Crop cultivation, fertilizer application and soil sampling

Twenty-one-day-old seedlings of rice (*Oryza sativa*) varieties Swarna (MTU-7029) and Pusa-121 were transplanted to the BHU and the IIVR sites respectively. At the time of ploughing, a basal treatment of KCl + P_2O_5 + farmyard manure was applied at a rate of 60 : 60 : 1000 kg ha^{-1}. Chemical fertilizer in the form of urea was applied at the rate of 30 kg N ha^{-1} in two split doses at the time of tillering, and before grain filling. The fields were irrigated as required. Soil sampling[16] was performed at the tillering, flowering, grain-filling and grain-maturation stages of the rice plants at both sites. Field moist rhizospheric soil samples were mixed, sieved and stored in black polyethylene bags at 4°C for subsequent analyses.

Enumeration of methanotrophs by most probable number

The population size of methanotrophs was measured by a most probable number (MPN) technique with tenfold dilutions in three replicates, as described earlier[17]. The cultivation was performed using nitrate mineral salts (NMS) medium containing (g l^{-1} distilled water) 1.0 g $MgSO_4$, 1.0 g KNO_3, 0.717 g $Na_2HPO_4 \cdot 12H_2O$, 0.272 g KH_2PO_4, 0.2 g $CaCl_2 \cdot 2H_2O$ and 0.005 g EDTA (ferric)[18]. The pH of the NMS was adjusted to 6.8 and trace elements were added after autoclaving. As controls, culture tubes were prepared without added CH_4.

Methane oxidation capacity of soils

Methane oxidation capacity of the soil was studied in laboratory incubation assays using a protocol reported elsewhere[17]. In brief, 10 g of field-moist samples, after an equilibration period of 24 h at 25°C, were transferred in triplicate into gas-tight 135 ml Erlenmeyer flasks sealed with a rubber stopper, and incubated at 30°C for 10 days in the dark. Headspace samples (0.1 ml) were taken with a gas-tight glass syringe after 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h of incubation. The samples were analysed for CH_4 on a gas chromatograph (Nucon, 5765); a flame ionization detector was used with a Porapak N column and helium as carrier gas (flow rate 40 ml min^{-1}). For calculation of methane oxidation rate (in μmol l^{-1} h^{-1} g^{-1} dw of soil) across different stages of the plant at both sites, a first-order kinetic equation was used.

Ammonium-N, plant biomass and statistical analysis

Ammonium-N (NH_4^+-N) was extracted by 2 M KCl and analysed using the phenate method[19]. On each sampling date, rice hills with soil were harvested in triplicate as blocks (15 × 20 × 15 cm) using rectangular open-top plastic cylinders. Plant biomass was determined according to the methods described by Dubey and Singh[16]. Statistical tests were performed using the SPSS-12.0 package. To analyse the effect of sampling sites and crop phenology on the methanotroph population, CH_4 oxidation rates of soils and NH_4^+-N content, ANOVA was used. Tukey's HSD

test was performed to determine the significance of differences between means. Simple linear regression analysis was also used whenever required.

Extraction of DNA from soil

DNA from soil samples (0.5 g) was extracted using the Fast DNA® spin soil kit (Q-Biogene, Inc.), according to the manufacturer's instructions. Additional purification of DNA was performed by electrophoresis through 1% agarose gel in Tris-borate EDTA buffer. Large molecular weight DNA (> 10 kb) was recovered from agarose using the QIAquick® Gel Extraction Kit (Qiagen). The DNA was eluted in a final volume of 50 µl EB (Qiagen) and stored at –20°C. DNA quantification was performed using a NanoDrop® ND-1000 Spectrophotometer.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed using the DCode™ system (Bio-Rad). Bacterial 16S rRNA genes were amplified from the soil using the PCR primers 341F-GC (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG) and 907R (CCG TCA ATT CMT TTG AGT TT)[20]. PCRs were performed in 50 µl reaction volumes containing approximately 10 ng of purified soil DNA, 1 × PCR buffer (Fermentas), 200 µM of each dNTP (Fermentas), 50 pmol of each primer and 2.5 units of Taq DNA polymerase (Fermentas). Cycling was performed using a Biometra T3000 instrument with a 5 min initial denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and then by a final extension at 72°C for 10 min. Samples of PCR product (5 µl) were loaded onto a 6% polyacrylamide (37.5 : 1 acrylamide : bisacrylamide) (Bio-Rad) gel containing a linear gradient of the denaturants urea and formamide (30 to 70%), as described elsewhere[20,21]. Electrophoresis was performed in TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA (pH 7.6)) for 16 h at 60°C and 100 V. The gels were stained with SYBR green I and visualized using a phosphorimager (Fuji-Film).

Microarray analysis

Approximately 10 ng of soil DNA was used as template for amplification of *pmoA* genes. A semi-nested PCR protocol was used exactly as described by Horz *et al.*[22,23], except that a T7 transcription initiation site was included in the mb661 primer[13]. Primers were custom synthesized by Invitrogen (UK) and *Taq* DNA polymerase was purchased from Fermentas Inc. The PCR buffer, composition and cycling programme were used as described by Horz *et al.*[23]. The triplicate *pmoA* PCR products were pooled and used for hybridization according to Bodrossy *et al.*[13]. The hybridized slides were scanned at 10 mm resolution with a GenePix 4000 A laser scanner (Axon, Foster City, CA, USA) at 435 wavelengths of 532 and 635 nm for Cy3 and Cy5 respectively. Results were normalized to a positive control with probe mtrof173, and triplicate subarrays were analysed and averaged per assay to minimize operation errors.

Results

Ammonium-N

Ammonium-N concentration was lower at the BHU site (2.8–7.1 µg g^{-1} soil) as compared to the IIVR site (4.0–8.3 µg g^{-1} soil; Table 2). Differences due to sites were significant ($F_{1,16} = 51.81$; $P = 0.000$). NH_4^+-N content declined at both sites from tillering to flowering to grain filling to grain maturation ($F_{3,16} = 63.49$; $P = 0.000$).

Methanotroph population size

Methanotroph population sizes, as determined by MPN counts, were in the range 1.1×10^8 to 5.5×10^8 cells g^{-1} soil at the BHU site and 6.2×10^7 to 3.9×10^8 cells g^{-1} soil at the IIVR site (Table 2). ANOVA indicated significant differences ($F_{1,16} = 39.99$; $P = 0.000$) in the methanotroph population size due to the sites. Higher values were counted at the grain-maturation stage followed by grain filling, flowering and tillering in both the sites. The effect of crop phenology on methanotroph population size was significant ($F_{3,16} = 67.29$; $P = 0.000$), although the HSD test indicated that differences between the grain filling and grain maturation were not significant at both the sites.

CH_4 oxidation capacity of soils

Consumption rates of CH_4 by the soils at different stages of rice plant growth are shown in Figure 1 and Table 2. ANOVA revealed no significant differences for variations in CH_4 uptake rates with respect to sampling sites ($F_{1,23} = 1.38$; $P = 0.253$). The rate of methane consumption was higher at the grain-filling stage followed by grain maturation, flowering and tillering, with significant variations ($F_{3,23} = 1456.30$; $P = 0.000$). The trend was similar for both the sites. The HSD test did not detect significant variations between the grain-filling and grain-maturation stages.

Plant biomass

The number of tillers, root biomass and shoot biomass were higher at the BHU site as compared to the IIVR site (Table 2). The effect of the site on the number of tillers

Table 2. Parameters, including the most probable number (MPN) count of methanotrophs, methane oxidation rate, NH_4^+-N concentration, tiller density, root and shoot biomass of plants in BHU and IIVR sites across the phenological stages of the rice plants. Values are means of three replicates ±1 SE

Parameter	Site	Crop phenology			
		Tillering	Flowering	Grain filling	Grain maturation
MPN count ($\times 10^6$ cells g^{-1} soil)	BHU	105.3 ± 4.0	302.4 ± 25.0	482.0 ± 20.8	551.8 ± 58.1
	IIVR	61.5 ± 5.0	155.4 ± 28.2	303.9 ± 18.1	388.6 ± 32.0
CH_4 oxidation (μmol l^{-1} h^{-1} g^{-1} dw of soil)	BHU	0.06 ± 0.02	0.22 ± 0.03	0.40 ± 0.07	0.32 ± 0.08
	IIVR	0.04 ± 0.01	0.17 ± 0.03	0.31 ± 0.07	0.25 ± 0.03
NH_4^+-N (μg g^{-1} soil)	BHU	7.1 ± 0.4	4.3 ± 0.4	3.8 ± 0.3	2.8 ± 0.6
	IIVR	8.3 ± 0.3	6.6 ± 0.2	5.2 ± 0.2	4.0 ± 0.1
Tillers (m^{-2})	BHU	110 ± 10.0	190.0 ± 26.5	250.0 ± 10.0	240.0 ± 17.3
	IIVR	100.0 ± 10.0	170.0 ± 10.0	210.0 ± 17.3	180.0 ± 34.6
Root biomass (g m^{-2})	BHU	74.9 ± 6.0	123.9 ± 13.0	181.5 ± 20.6	174.3 ± 8.4
	IIVR	61.1 ± 2.6	90.3 ± 22.9	124.2 ± 10.8	119.1 ± 98
Shoot biomass (g m^{-2})	BHU	464.9 ± 26.7	1427.1 ± 188	1999.6 ± 29	1983.9 ± 49.9
	IIVR	363.4 ± 65.6	943.4 ± 51.5	1532.0 ± 268.6	1500.5 ± 272.0

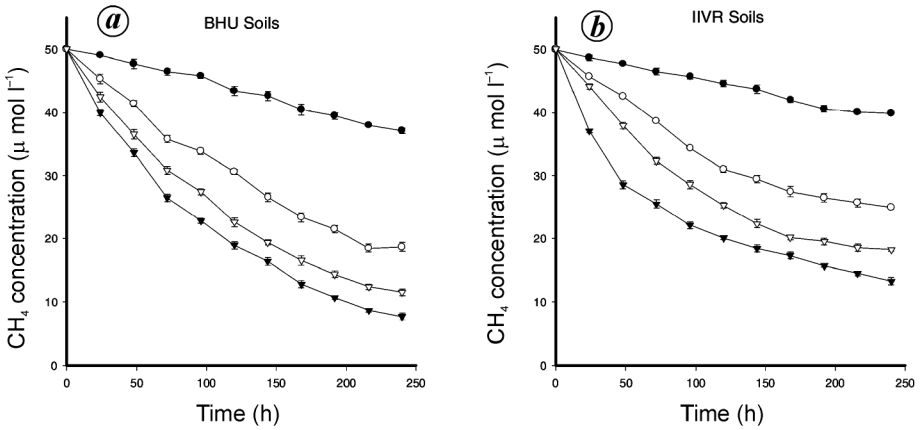

Figure 1. Time series concentration of CH_4 in the headspace of flasks containing rhizospheric soils. (***a***) BHU soil and (***b***) IIVR soil. CH_4 uptake is shown for each of the stages of rice plant development; tillering (filled squares), flowering (open squares), grain filling (filled triangles) and grain maturation (open triangles).

($F_{1,16} = 4.64$; $P = 0.040$), root biomass ($F_{1,16} = 16.13$; $P = 0.001$) and shoot biomass ($F_{1,16} = 12.08$; $P = 0.003$) was significant. The effect of crop phenology on plant variables was also found to be significant, such as the number of tillers ($F_{1,16} = 14.71$; $P = 0.000$), root biomass ($F_{1,16} = 15.84$; $P = 0.000$) and shoot biomass ($F_{1,16} = 33.01$; $P = 0.000$). The HSD test revealed no significant differences among plant variables between the grain-filling and grain-maturation stages.

Denaturing gradient gel electrophoresis

DGGE fingerprinting of bacterial 16S rRNA genes revealed a complex banding pattern for two soils at different phenological stages of the rice plant (Figure 2). DGGE primers targetting methanotroph 16S rRNA genes showed that type I and type II methanotrophs were present in both the soils. Diversity amongst type I, type II and Eubacteria was similar at all stages of the rice plant from the BHU site; however, differences in the communities were detected at different stages of the rice plant for the IIVR soil.

pmoA microarray analyses

pmoA gene PCR products from each of the samples were hybridized against a microarray containing 137 probes (Table 3), which has been continuously updated since the original description[13] and covers the known diversity of *pmoA* sequences. The analysis revealed a wide diversity of

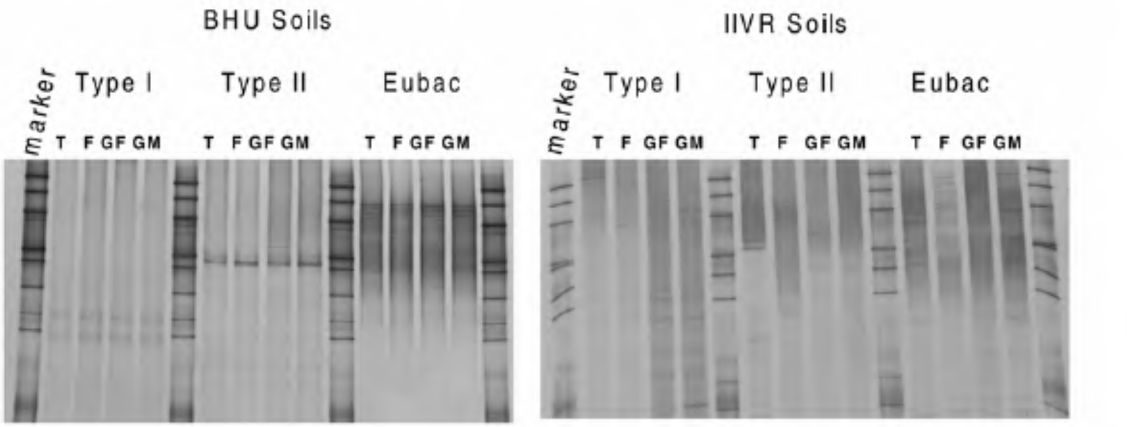

Figure 2. Denaturing gradient gel electrophoresis band patterns of type I, type II and Eubacterial amplification products from soil samples of two different sites across the age of rice plants. T, Tillering; F, flowering; GF, Grain filling, and GM, Grain maturation.

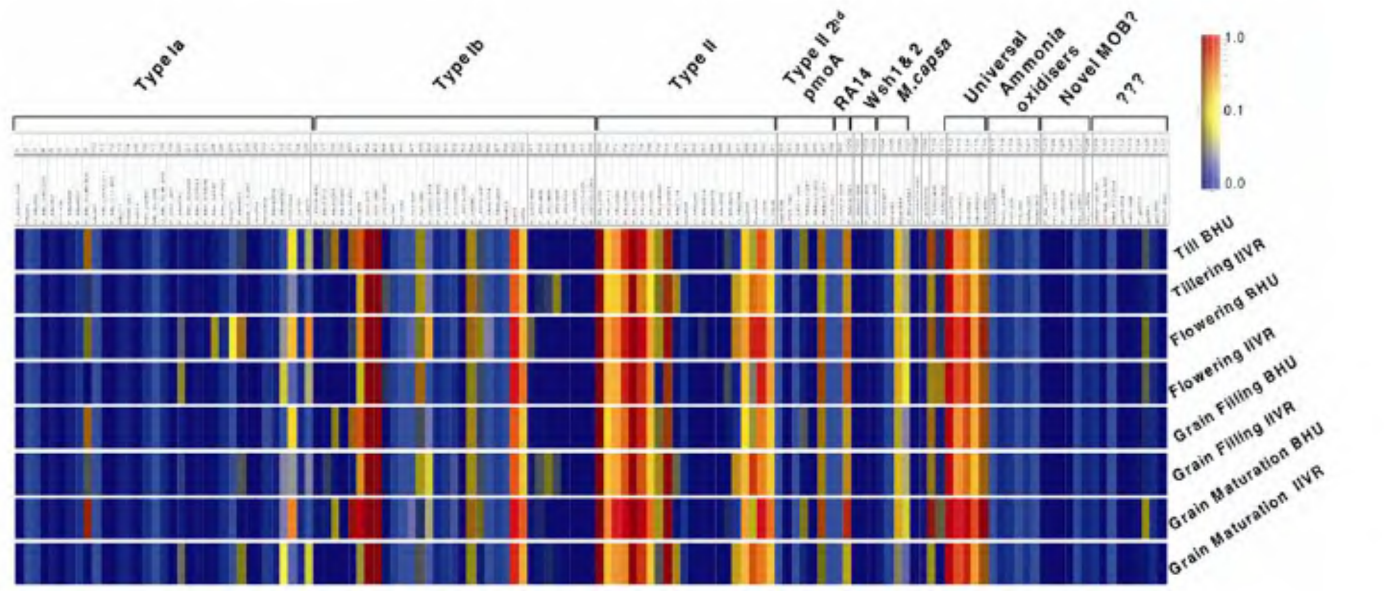

Figure 3. Summary of results for the *pmoA*-based methanotrophic community analysis using the microarray. PCR products of *pmoA* gene amplified from soil DNA of BHU and IIVR soils at different phenological stages of the rice plants. Data for each of the probes are organized into target groups, which are identified above the columns (numbered from 1 to 137)[+]. Probes labelled 'Novel MOB?' target environmental *pmoA* sequences that are likely to belong to methanotrophs. Probes '???' correspond to those targeting environmental sequences related to *pmoA*, but for which it is difficult to assign the functional or phylogenetic affiliation. Colour coding bar on the right side represents achievable signal for an individual probe (1 indicates maximum signal obtained, 0.1 indicates that 10% signal, i.e. only 10% hybridization to that probe and 0 indicates no signal). [+]List of probes in the respective order is given in Table 3.

pmoA genes, with numerous methanotroph genera represented in the soils (Figure 3). *Methylocystis* sequences were highly abundant and found in all samples. In contrast, *Methylosinus* sequences were almost completely absent. Furthermore, the weak signals with some *Methylosinus* probes may have resulted from cross-hybridization with *Methylocystis* sequences, which are closely related. The probe for peat-associated *Methylocystis* sequences (Peat264) hybridized with all samples, although it seemed to hybridize more strongly with those from the IIVR site. The type Ib probes, which target the thermotolerant and thermophilic genera *Methylococcus*, *Methylothermus*, *Methylocaldum* and related, uncultivated clades, were also highly abundant. Notably, there was some hybridization with probes targetting tropical upland soil cluster-associated *pmoA* sequences (probes TUSC409 and TUSC502).

Type Ia probes revealed a generally low abundance of the targetted genera *Methylobacter*, *Methylomonas*, *Methylosarcina*, *Methylosphaera* and *Methylomicrobium*. One *Methylobacter* sequence related to sequences from a soda lake and represented by probe Mb_SL#3-300, was found in all the BHU samples; only a weak signal with this probe was observed for the grain-filling stage from

Table 3. Probes used for microarray analysis. Column numbers correspond to the order in which the probes are arranged for the microarray analysis as shown in Figure 3

Column Number	Probe name	Column number	Probe name	Column number	Probe name	Column number	Probe name
1	O_BB51-299	36	P_JRC4-432	71	O_Mcy522	106	B2all343
2	Mb292	37	P_MclT272	72	P_Mcy264	107	O_B2all341
3	O_Mb282	38	P_MclG281	73	P_Mcy270	108	pmoAMO3-400
4	P_Mb_URC278	39	P_MclE302	74	P_Mcy459	109	P_ESR-579
5	P_Mb267	40	P_MclS402	75	O_Mcy255	110	P_TUSC409
6	511-436	41	Mcl408	76	P_McyM309	111	P_TUSC502
7	P_MbA486	42	P_501-375	77	P_McyB304	112	mtrof173
8	P_MbA557	43	O_501-286	78	P_MsT214	113	mtrof362-I
9	P_Mb_SL#3-300	44	P_USC3-305	79	P_MsT343	114	mtrof661
10	Mb460	45	Mc396	80	P_MM_MsT343	115	mtrof662-I
11	P_Mb_LW12-211	46	Fw1-639	81	Msi520	116	mtrof656
12	P_Mb_C11-403	47	O_fw1-641	82	O_Msi269	117	NmNc533
13	Mb271	48	P_fw1-286	83	P_MsS314	118	Nsm_eut381
14	PS80-291	49	P_LW21-374	84	P_MsS475	119	PS5-226
15	Est514	50	P_LW21-391	85	P_Msi263	120	PI6-306
16	Mm_pel467	51	P_OSC220	86	P_Msi423	121	NsNv207
17	Mb_SL-299	52	P_OSC300	87	O_Msi294	122	NsNv363
18	O_Mb_SL#1-418	53	P_JRC3-535	88	Msi232	123	P_Nit_rel471
19	P_DS1_401	54	P_LK580	89	Peat264	124	Nit_rel223
20	P_Mm531	55	P_JRC2-447	90	O_II509	125	P_ARC529
21	P_Mm_ES294	56	O_M90-574	91	O_II630	126	Nit_rel470
22	P_Mm_ES543	57	O_M90-253	92	xb6-539	127	Nit_rel351
23	P_Mm_ES546	58	Mth413	93	LP21-190	128	Nit_rel304
24	P_Mm_M430	59	Ib453	94	O_LP21-260	129	M84P105-451
25	P_Mm_MV421	60	Ib559	95	P_NMcy1-247	130	WC306_54-385
26	Mm275	61	P_DS3-446	96	P_NMcy2-262	131	M84_P22-514
27	P_Mm451	62	P_JR2-409	97	P_NMsiT-271	132	gp23-454
28	peat_1_3-287	63	P_JR2-468	98	LP21-232	133	MR1-348
29	Jpn284	64	P_JR3-505	99	O_RA14-594	134	P_gp619
30	Mmb303	65	P_JR3-593	100	P_RA14-591	135	gp391
31	P_Mmb259	66	Nc_oce426	101	P_Wsh1-566	136	gp2-581
32	O_Mmb562	67	P_USCG-225	102	P_Wsh2-491	137	RA21-466
33	LP20-644	68	P_USCG-225b	103	P_Wsh2-450		
34	O_Ia193	69	P_Mcy233	104	O_B2rel251		
35	O_Ia575	70	O_Mcy413	105	B2-400		

the IIVR site. Probe LP20-644 indicated in all samples the presence of a deep branching and so far uncultivated type Ia clade, detected by DGGE in rice field soils[22].

Discussion

Methanotroph population sizes

Methanotroph population sizes during the entire period of the rice crop ranged from 61.5 to 581.8×10^6 cells g^{-1} soil. Takeda *et al.*[24] found by MPN that the methanotroph population ranged between 10^5 and 10^6 g^{-1} in flooded and drained subtropical rice soils. Our study showed that the methanotroph population size distinctly increased with the age of the rice plant at both the sites (Table 2). The increasing trend in the methanotroph population size during the development of the plant is in agreement with other studies[17,25,26]. The population size of methanotrophs (Y, 10^5 cells g^{-1}) had an inverse linear relationship with NH_4^+-N concentration (X, μg g^{-1}) according to $Y = 743.7 - 86.0X$ ($R^2 = 0.69$; $P = 0.001$) at BHU. For the IIVR site the relationship was $Y = 697.4 - 76.9X$ ($R^2 = 0.87$; $P = 0.000$). Dubey and Singh[16] have reported an inverse relationship between methanotroph population size and NH_4^+-N concentration. The increase in methanotroph numbers could be due to a continued decline in NH_4^+-N concentration (Table 2). The inhibition mechanism of NH_4^+ may arise from the competitive inhibition of methane monooxygenase by NH_4^+ and potentially augmented also by inhibition of methanotrophs by the nitrite that is produced by NH_4^+ oxidation, especially at elevated methane concentrations[27].

Furthermore, increase in the number of tillers, root biomass and shoot biomass during the plant growth period (Table 2) would be another possible reason for variations in the methanotroph population size. Some studies have shown that increase in plant biomass influenced the methanotroph population by enlarging the vol-

ume of aerenchyma[25] and the number of tillers[16], allowing for more effective O_2 transport from the atmosphere to the rhizosphere, which is considered as the most active site for growth and multiplication of methanotrophs[25,28].

Methane oxidizing capacity of soils

In the present study, the rate of CH_4 oxidation (μmol l^{-1} h^{-1} g^{-1} dw of soil) was not found to be statistically different between the sites (Table 2). Although NH_4^+-N content, methanotroph population size and plant biomass were significantly different in both the sites, one possible reason for insignificant differences in methane oxidation rates between the sites could be due to the similarity in soil texture, as both the soils were inceptisols. The difference in methanotroph population size between the sites suggests that the relative activities of methanotrophs differed between the sites. Methane oxidation rates were low during the early phase of rice plant growth (tillering stage); the oxidation rate increased gradually with the age of the plant and reached a maximum at the grain-filling stage (Table 2). The results exhibited a strong impact of crop phenology on the methane oxidation capacity of the soils. These results were similar to those as described for two different tropical rice agroecosystems[17]. This finding may be explained by several factors, including greater methanogenesis, a larger methanotroph population and lower NH_4^+-N concentration. Rice roots release an increasing amount of organic compounds with age, which provide a substrate for methanogenesis, and hence increased methanotrophic activity towards maturity due to substrate availability[29]. It is apparent that at the grain-filling stage, maximum biomass availability provides a suitable habitat and niche in the rice rhizosphere for methanotrophs to oxidize methane, which is in agreement with the present study.

Diversity of methanotrophs

Microarray analyses targetting *pmoA* genes showed the presence of both type I and type II methanotrophs in rice soils (Figure 3). Type II methanotrophs (in particular the genus *Methylocystis* and the uncultivated peat-associated type II clade) were the most abundant in all samples analysed. Type Ib methanotrophs were also detected with a high abundance in all samples, reflecting a bit broader variation between sites and sampling times. Detected type Ib methanotrophs included the genus *Methylocaldum*; the uncultivated clades 501, fw1 and LW21, all associated with various sediment type environments[30,31]; and JR2 and JR3, described from Japanese rice soil samples (unpublished). Type Ia methanotrophs (*Methylobacter*, *Methylomonas*, *Methylosarcina*, *Methylosphaera* and *Methylomicrobium*) were found to be scarce in comparison to type II and type Ib methanotrophs at both the sites. These findings are consistent with earlier studies which have stated that type II methanotrophs exist in higher abundance than type I methanotrophs in the rice rhizosphere[32,33]. The presence of tropical upland soil-cluster sequences associated with atmospheric methane oxidation suggests that it may be due to cultivation practices. In the eastern Indo-Gangetic plain of India during May–June, before the arrival of the monsoon, the rice fields remain uncultivated and relatively dry. Such environments probably provide a possible niche for high-affinity methanotrophs to utilize atmospheric methane. The tropical upland soil cluster methanotrophs may remain as a reservoir in these fields and are engaged in the utilization of atmospheric CH_4 during intermittent dry spells.

The methanotrophic population and its activity has shown similar pattern at both the sites during the growth of the rice plant. Community analysis by DGGE revealed differences in the methanotrophic community between both the sites (Figure 2). DGGE analysis of type I and type II methanotroph 16S rRNA genes from the BHU soil samples revealed that the banding pattern was stable over the entire season of the rice plant except at the grain-filling stage. On the other hand, the IIVR samples with the same primers showed differences in both the type I and type II methanotroph communities at the different stages of the rice plant. The BHU site has been regularly vegetated by rice–wheat–rice crops in the recent past and therefore, the present study suggests that the methanotrophic community is well adapted to the rice cultivation environment. The IIVR soil had been exposed to rice cultivation for the first time and therefore the community in the soil underwent a significant adaptation to the rice crop environment. It has been reported in earlier studies that the microbial community structure can change in response to changes in the long-term cultivation and environmental variations[34,35].

Conclusion

A microarray approach for the analysis of methanotrophs revealed the presence of type I and type II groups of methanotrophs in the rice soils. Type II methanotrophs were found in higher abundance than type I methanotrophs. The present study suggests that land-use history and plant age are important factors that lead to changes in the methanotrophic communities in rice agroecosystems. More research will be required to examine the activity of different methanotroph groups in response to crop phenology and soil conditions.

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