Tracking of methanotrophs and their diversity in paddy soil: A molecular approach

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Methanotrophs, the inhabitants of irrigated rice soils, were monitored using molecular tools. Methanol dehydrogenase coded by *mxaF* locus has been used as the genus-specific locus. The presence of the locus has been demonstrated in DNA extracted from soil samples as well as in methanol utilizing isolates derived from those samples. Further, ARDRA pattern and cluster analysis revealed the following closely related genera – type-I methanotrophs *Methylomonas* and/or *Methylocaldum* in rhizosphere soil and co-existence of both type-I (*Methylomonas* and/or *Methylocaldum*) and type-II methanotrophs *Methylobacterium* and/or *Methylocella* in non-rhizosphere soil.

METHANE, a greenhouse gas, is present at a level of about 1.8 ppmV in the atmosphere and its amount is increasing at the rate of 1% per year[1,2]. Out of the total observed methane load in the atmosphere, at least 30% is attributed to rice cultivation[3]. Methanotrophs, a unique group of organisms associated with the rhizosphere of paddy plants[4–7], can use methane as the sole source of carbon and energy[8]. Methanotrophs fix methane in a reaction catalysed by methane monooxygenase to methanol, which enters into the cell metabolism as formate via methanol dehydrogenase (MDH). The *mxaF* gene encoding the large sub-unit of MDH[9] is present in all Gram negative methanotrophs[10] and has been used for tracking methanotrophs in natural habitat[11,12]. In rice ecosystems, spatio-temporal variations in microbial environments occur which might result in shifts in the methanotrophic community, and would influence overall methane oxidation activity. Therefore, a better understanding of the methanotrophic community structure in paddy field is important to know the mechanistic basis of methane oxidation in soil. Recently, a number of researchers have conducted molecular investigations of methanotrophs in different environments[10,13–15]. Dubey *et al.* have reported the occurrence and activities of methanotrophs in dry land[5,7] and flooded rice fields[6]. In this study, a PCR-based approach was used to investigate the existence of methanotrophs in terms of their population size and community structure with type and genera in both rhizosphere and non-rhizosphere (between the two hills of rice plants) soils of rice fields vegetated by the rice (*Oryza sativa*) cultivar, Swarna (MTU.7029).

Soil samples were taken from the rice fields (vegetated by rice cultivar, Swarna MTU.7029) of the agriculture farm of the Banaras Hindu University, Varanasi, India (25° 18′ N, 83° 3′ E, 129 m asl). This site has been used repeatedly to study the microbial ecology of rice fields[16]. The soil (10 cm depth, WHC 44%) is well-drained Inceptisol, pale brown, silty loam (sand 32%, silt 65% and clay 3%) with 0.61–0.74% organic C, 0.07–0.11% total N and pH 6.8–7.5.

Experimental design and soil sampling was done as reported earlier[5], except that the sampling was carried out at the grain-filling stage and irrigation was done whenever required. The moist soil was sieved with a 2 mm sieve in order to ensure homogeneity and the number of anoxic microsites. The samples collected were aseptically placed in serum bottles and transported to the laboratory and stored at 4°C until further use.

Methane oxidizing bacteria (MOB) were enumerated from fresh soil samples (5 g) in 50 ml of nitrate mineral salt (NMS) medium incubated overnight at 4°C, and shaken at 260 rpm. NMS contained [g l^{-1} distilled water at pH 6.8][17]: KNO_3, 1.0; KH_2PO_4, 0.54; $Mg_2SO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.015. $CaCl_2$, Mg_2SO_4 and trace elements were added after autoclaving[18]. This suspension served as inoculum. The inoculated tubes were incubated under 20% (by volume) CH_4 in air at 25°C in the dark for 3 weeks and then tested for bacterial growth against a control. For control, culture tubes were prepared with soil inoculum under CH_4-free air. The MPN was calculated from the dry weight of the soil, dilution factor and tables for three parallel dilution series based on a statistical treatment of counting methods[19]. The cultures were then plated on NMS agar medium with known dilutions (10^{-2} to 10^{-9}). After 24 h, the individual colonies were transferred to the NMS medium (pH 6.8) with 1000 ppm methanol as the sole carbon source[20,21]. The cultures were harvested after 2 to 3 days by centrifugation[22], and the cell pellets were used for making NT-DNA (NaOH-Tris treated DNA) using mild alkali treatment[23].

One gram of soil from each sample (rhizosphere and non-rhizosphere) was used for extracting DNA. The extraction protocol used was based on cell lysis with 10% sodium dodecyl sulphate, followed by DNA purification with ammonium acetate precipitation and isopropanol precipitation. The procedure is described in detail by Padmanabhan *et al.*[24]. The DNA was further purified by ultra-centrifugation and ethidium bromide was removed by washing with *n*-butanol, according to the standard protocol.

The same approach for tracking of genotype has been used, as reported earlier for different environmental niches[25,26]. PCR reactions were performed in a PTC-200

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thermocycler (MJ Research Inc., Watertown, MA) with Taq DNA polymerase (Gibco BRL, USA). The temperature cycles used for amplification of *mxaF* locus were as follows: 94°C for 5 min; 30 cycles consisting of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min followed by 72°C for 5 min. The 16S rDNA amplicon was amplified using the following temperature cycles: 94°C for 5 min; 35 cycles consisting of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min followed by 72°C for 5 min. Following PCR, 10 µl of each reaction mixture was analysed on a regular agarose gel (1.5%) by electrophoresis.

The DNA was amplified using the primer set *mxaF* 1003f (5′-GCG GCA CCA ACT GGG GCT GGT-3′) and *mxaF* 1561r (5′-GGG CAG CAT GAA GGG CTC CC-3′) for the α-subunit of the methanol dehydrogenase gene, as reported earlier[10,12]. In a 200 µl thin-walled tube, 5 µl of NT-DNA or 1 : 100 diluted total DNA from soil extract or 5 µl H_2O (negative control) was denatured for 5 min at 95°C. Reactions were performed as reported earlier with 1 µM primers to yield a 550 bp product[12,27].

PCR for eubacterial 16S rDNA genes from DNA isolates was performed using primers 27f(5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r(5′-TACGGYTACCTTGTTACGACTT-3′)[28]. The NT-DNA from the isolates (5 µl) was used as template to yield 1450 bp products. The resulting amplicons were digested with *Hae*III (Amersham) as per manufacturer's protocol. The discrete band patterns obtained for the isolates by Amplified Ribosomal DNA Restriction Analysis (ARDRA) were analysed using hierarchial cluster analysis in order to depict the diversity amongst the isolates. The reported sequences of *Methylobacter*, *Methylomicrobium*, etc. available in the NCBI database were downloaded and ARDRA patterns were generated using Laser gene software. Ten possible band patterns were treated as attributes and their presence or absence in different isolates was observed. A binary type data set was generated for each isolate, with 1.0 indicating the presence of band and 0 indicating its absence. Likewise, data were generated for the observed 12 different ARDRA patterns. The average linkage method resulted in a dendogram giving the topology of relationship amongst the isolates based on the ARDRA pattern, using SPSS-6 package.

The MPN results revealed that the culturable population size of MOB was higher ($5.5 \pm 0.7 \times 10^8$ cells g^{-1}) in rhizosphere than in non-rhizosphere ($2.8 \pm 0.4 \times 10^6$ cells g^{-1}) soils; the same trend that was observed in earlier studies, thus confirming that rhizosphere provides the most favourable site for the occurrence and multiplication of MOB[5–7]. The O_2-supplying potential under such conditions in rice roots is the major factor for multiplication, growth and sustenance of MOB. The supply of both CH_4 and O_2 would thus favour the MOB population[17] to develop in the rhizosphere than in the non-rhizosphere soils. Most quantitative data on MOB population size rely on MPN methods. The limitations of this method are well known[29]. Therefore, in this study molecular tools have been used for tracking and analysing the diversity associated with the methanotrophic community in paddy soils.

Methanotrophs were monitored using *mxaF*, which has been reported by Murrell *et al.*[12] as a highly conserved locus and indicator of the presence of methane/methanol utilizing organisms in natural habitat. Initially, DNA of high molecular mass was extracted from 1 g of soil (rhizosphere and non-rhizosphere). The *mxaF*-specific product was amplified with predicted size (550 bp) from both the soil samples tested at a dilution of 1 : 10 and 1 : 100 with parallel control. Concurrently, we have used the DNA derived from 12 representative isolates selected

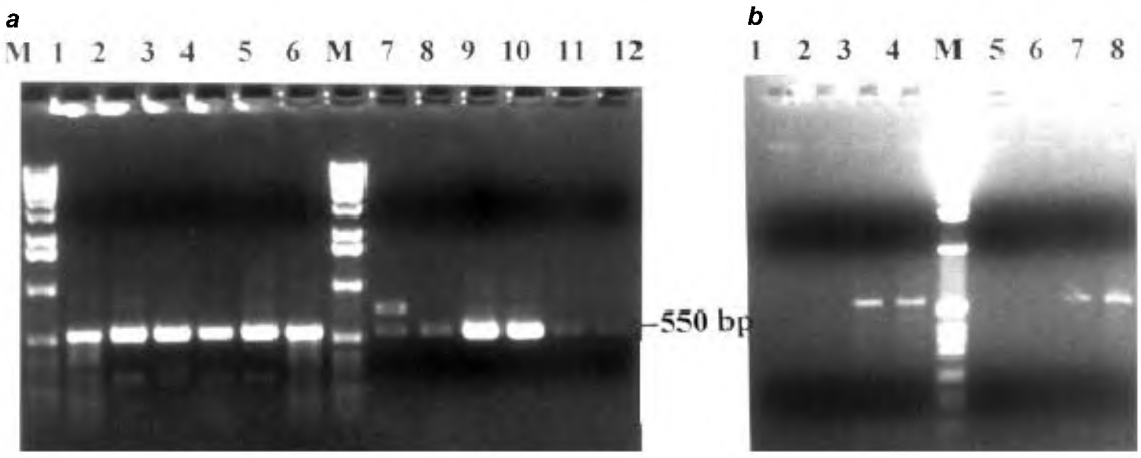

Figure 1. Agarose gel electrophoresis of PCR products from 12 representative bacterial isolates (methanotrophs) and two soil samples (rhizosphere and non-rhizosphere) amplified with *mxa*F primer pairs with expected amplicon 550 bp: ***a***, Lanes M 1 kb molecular weight markers; lanes 1–12, Isolates (1–6 isolates from rhizosphere and 7–12 those from non-rhizosphere); ***b***, Lane M 1 kb molecular weight marker; lanes 1–4, Rhizosphere soil DNA samples with different dilutions (1, Neat; 2, 1 : 10 dilution; 3, 1 : 100; 4, 1 : 1000); lanes 5–8, Non-rhizosphere soil DNA samples with different dilutions (5, Neat; 6, 1 : 10 dilution; 7, 1 : 100; 8, 1 : 1000).

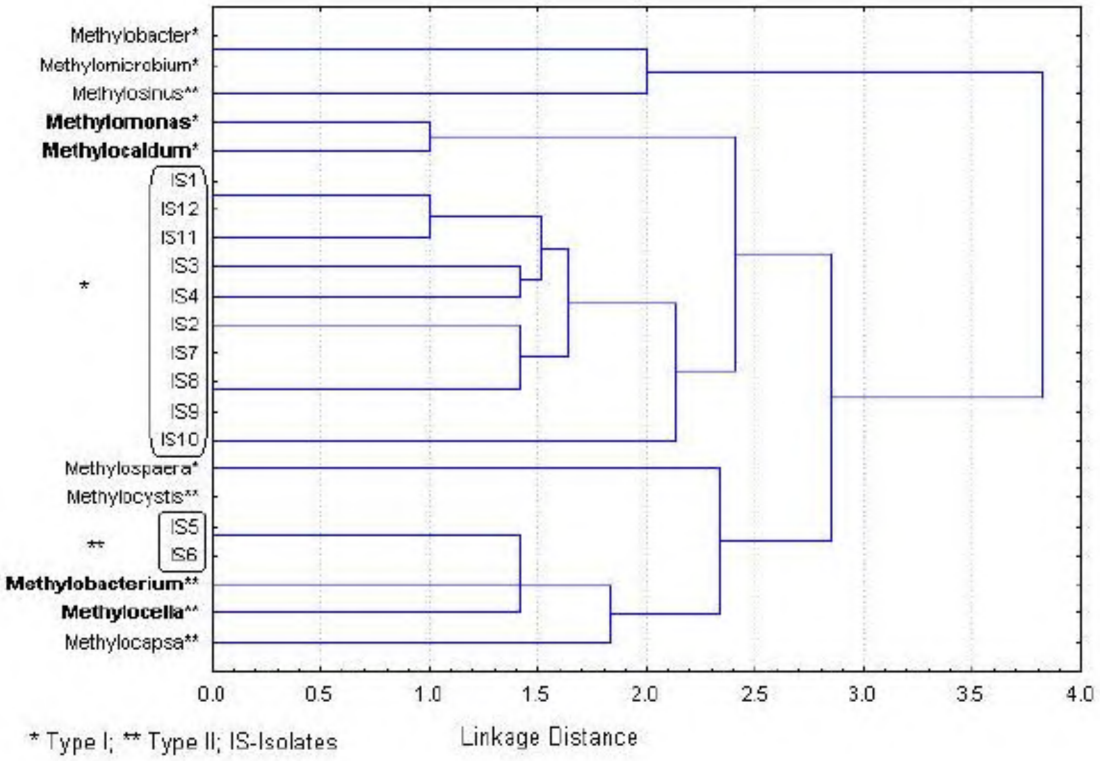

Figure 2. Cluster analysis of ARDRA pattern.

after enrichment on methanol using inoculum from the above soil samples. All the representative isolates and soil samples gave the *mxaF*-specific product, as shown in Figure 1 *a* and *b*.

The community analysis was carried out using ARDRA patterns, which were recorded for all the representative isolates (cultures S1, S2, S3, S7, S8 and S9, rhizosphere and S4, S5, S6, S10, S11 and S12, non-rhizosphere). Simulated ARDRA patterns were obtained for the known methanotrophs using the same *Hae*III restriction digestion as shown in Figure 2. On cluster analysis, the ARDRA patterns revealed that both type-I and type-II MOB were present in rice field soils. Further, type-I MOB was dominant in rhizosphere soil, whereas both-type-I and type-II MOB were present in non-rhizosphere soil. Bodelier *et al.*[13] have reported that type-II methane oxidizers dominated methane metabolism in unplanted soil and that type-I species were distinctly greater in the rhizosphere soil than in non-rhizosphere soil, indicating that the presence of rice plant is an essential factor for type-I methanotrophs to proliferate. According to Conrad[30], type-II methanotrophs frequently found in the soil, are able to adapt to change in methane concentration by changing their K_m. On comparing the ARDRA results with the existing gene patterns by cluster analysis, it was found that the type-I MOB more or less matched with genus *Methylomonas* and *Methylocaldum*, and type-II MOB with genus *Methylobacterium* and *Methylocella*.

We conclude that molecular biological methodology can be used to characterize and detect the community structure and function of methanotrophs (type-I and type-II bacteria). This can be beneficial to understand the mechanistic ability of methanotrophs for methane oxidation at the molecular level in irrigated rice fields.

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MEETINGS/SYMPOSIA/SEMINARS

Hands-on Training Course on Molecular Markers: Tools for Genetic Variability Analysis

Date: 3–15 November 2003
Place: Lucknow

The training will cover theoretical and practical aspects of a range of molecular markers, bioinformatic tools and data analysis for population genetic studies. The training is open to researchers working in the area.

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International Workshop on Genomics and Proteomics (IWGP)

Date: 10–13 September 2003
Place: Sivakasi

The aim of this workshop is to give the participants hands-on training and exposure to modern instruments like PCR, Sequence, etc., and the computer-assisted online practicals with the help of biological databases. The programme will focus on computer operation, handling, maintenance and troubleshooting of biological databases along with plenary lectures cum discussions pertaining to the current status of Genomics and Proteomics which will help to realize the need of the present day.

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