Cultivated methanotrophs associated with rhizospheres of traditional rice landraces from Western India belong to *Methylocaldum* and *Methylocystis*

Monali C. Rahalkar¹ · Sanjay Patil² · Prashant K. Dhakephalkar¹ · Rahul A. Bahulikar²

Received: 23 August 2017 / Accepted: 25 May 2018 / Published online: 1 June 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Aerobic methanotrophs associated with Indian rice plants have rarely been cultivated. In the present study, we cultured aerobic methanotrophic bacteria from the rhizosphere regions of rice plants. Rhizospheric soils from seven rice landraces traditionally grown and maintained by tribal people in Jawhar region belonging to part of the Western Ghats in India, were used. Seven methanotrophic cultures were isolated from the last positive dilution (10^{-4}). Methanotrophs were identified by analyzing the partial methane monooxygenase gene, *pmoA* gene and three of these belonged to the genus *Methylocaldum* (gammaproteobacterial, Type I methanotrophs) and four belonged to the genus *Methylocystis* (alphaproteobacterial, Type II methanotrophs). We present here the first report on the cultivation of methanotrophs from Indian traditional rice landraces originating from a biodiversity hotspot.

Keywords Methanotrophs · Rice rhizosphere · Aerobic · Methane-oxidizing bacteria · Cultivation · *PmoA* · Rice landraces · Western Ghats

Methane is generated in flooded rice fields and is the second most important greenhouse gas. Thereby, rice fields are one of the major anthropogenic sources of global methane (Conrad 2009). India leads in terms of rice production area, and therefore, methane emissions from rice fields remain a concern (Dubey 2001). It has been estimated that methanotrophs or methane-oxidizing bacteria dwelling in rice fields can oxidize 10–30% of the methane and prevent it from being

released into the environment (Shrestha et al. 2010). Very few studies have focused on the cultivation of methanotrophs from rice fields, and most of the work done in this area is using culture-independent approaches (Qiu et al. 2008; Reim et al. 2012; Shrestha et al. 2008, 2010). Study of the properties of the indigenous methanotrophs would be important as they might have biotechnological and environmental applications. Additionally, they may also serve as models to study methanotrophy associated with rice agriculture. In our previous work, we investigated methanotrophic communities present in rice rhizospheres from two rice fields in Western India (Pandit et al. 2016). Both of these studies indicated the presence of Type I and Type II methanotrophs, and in one of the studies few putative novel species and a genus, were isolated (Pandit et al. 2016).

According to a recent report, rhizosphere bacterial communities associated with wild rice varieties differ in complexity (species richness) and composition compared with cultivated rice rhizospheres (Shenton et al. 2016). Differences were observed explicitly in methanotrophic communities associated with the wild rice (Shenton et al. 2016). Since the landraces in the present study have been grown and developed for a particular geographical location and have been maintained for many generations, the microbial

Monali C. Rahalkar and Rahul A. Bahulikar contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-018-1306-z>) contains supplementary material, which is available to authorized users.

✉ Monali C. Rahalkar
monalirahalkar@aripune.org

✉ Rahul A. Bahulikar
bahulikar@gmail.com

¹ Bioenergy Group, MACS Agharkar Research Institute, G.G. Agarkar Road, Pune, Maharashtra 411004, India

² Central Research Station, BAIF Development Research Foundation, Uruli Kanchan, Maharashtra, India

community associated with the roots might be stable. Each landrace was unique with respect to the grain character, plant morphology, etc. (Supplementary Fig. 1). The rice plants were maintained under natural conditions with no fertilizer or pesticide inputs by tribal people in Jawhar region, Palghar district, Maharashtra. Jawhar region belongs to the Western Ghats, which is one of the world biodiversity hotspots in India. In our study, we focused on the culturable aerobic methanotrophs which were associated with the rhizospheres of seven rice landraces.

The seven rice landraces used in this study were: Dhaval Bhat, Najar Bhat, Kamal Bhat, Surati Kolam, Juna Kolam, Bangalya and Sukalya (Table 1). These landraces can be differentiated by their grain size, grain color, and other morphological characters and have traditional names (Supplementary Fig. 1, Supplementary Table 1). Rice roots with the soil attached to it were uprooted, stored in sterile plastic bags and immediately transported on the same day to the laboratory. In the laboratory, soil was shaken off from the roots by beating on a hard surface for 8–10 times followed by brief washing to remove the extra attached soil. The soil tightly attached to the roots was scraped and collected in sterile Petri plates. This soil was used for the isolation and cultivation of methanotrophic bacteria. A modified nitrate mineral salt (NMS) medium, dilute NMS medium, containing one-fourth concentration of KNO_3 compared to the original NMS medium (Whittenbury et al. 1970) was used for the enrichment of methanotrophs as described before (Pandit et al. 2016). The samples were serially diluted in microtitre plates up to 10^{-8} dilutions in duplicates. These plates were incubated in glass desiccators at 28 °C and the gas atmosphere was maintained as: ~20% methane, ~25% air and ~55% nitrogen. Bacterial growth was monitored every 2 weeks for turbidity or pellicle formation and the cells were observed under a phase contrast microscope. Once turbidity was observed, a subsample was streaked from the last positive dilution on 2% agarose containing (MP Biomedicals, India) dilute NMS medium Petri plates (Pandit et al. 2016). The purification of methanotrophs was carried out as described

before (Pandit et al. 2016). DNA extraction from colonies was done using GenElute™ Bacterial Genomic DNA kit (Sigma). The partial methane monooxygenase gene, *pmoA* gene was amplified from the DNA samples using the universal *pmoA* primer pair A189f-mb661r (Costello et al. 2002). All amplifications were carried out in 25 µl total volume in ABI thermal cycler (Veriti) using Promega PCR premix. Purified PCR products were sent to First Base Laboratory for sequencing, using both the primers. All forward and reverse sequences were assembled using the Lasergene software. A NCBI BLAST search was performed using GenBank (<http://www.ncbi.nlm.nih.gov/>) (Altschul et al. 1990). Partial *pmoA* sequences of methanotrophic type strains that showed the closest match to query sequences were included in the phylogenetic analysis. Online alignments were done using the MAFFT aligner (<http://mafft.cbrc.jp/>), and the phylogenetic tree was constructed using the maximum likelihood algorithms as implemented in MEGA 6.0 with 1000 bootstraps (Tamura et al. 2013). The partial *pmoA* sequences from this study have been submitted to Genbank under the accession numbers: MF614616–MF614622.

After approximately 8 weeks, all the samples showed growth in the form of pellicle or turbidity from 10^{-1} to 10^{-4} . The last positive enrichments, i.e., growth from 10^{-4} dilution were streaked on dilute NMS agarose medium (Pandit et al. 2016). Approximately, 2–6 weeks were required for obtaining visible colonies. After two to three rounds of repeated streaking, seven methanotrophic cultures were obtained. Colonies of the methanotrophs 64 and 79 isolated from the landraces ‘Najar Bhat’ and ‘Bangalya’ were pink in color and the cells were slightly spiral (Table 1). Morphologically, the methanotrophs represented diverse forms of cells ranging from spiral rods to large globular to coccoidal forms (Supplementary Fig. 2). Strains 10, 117 and 321 showed the presence of large globular cells in packets. Four strains showed the presence of single cells which were either spiral rods (79 and 64) or coccoidal (41 and 47) (Supplementary Fig. 2). All the cultures grew in liquid and on solid medium with methane supplied in the gas phase. Sequences of *pmoA*

Table 1 Cultivated methanotrophs from various landraces

Number	Rice variety	Methanotroph culture	NCBI accession number of the <i>pmoA</i> gene	Colony color	Nearest cultured relative based on <i>pmoA</i> gene
1	Dhaval Bhat	10	MF614617	Brown-yellow	<i>Methylocaldum tepidium</i> U89304.1 99%
2	Surati Kolam	41	MF614618	Cream	<i>Methylocystis hirsuta</i> strain CSC1, DQ364434.1 98%
3	Juna Kolam	47	MF614619	Cream	<i>Methylocystis parvus</i> strain OBBP AF533665.1 94%
4	Najar Bhat	64	MF614620	Pink	<i>Methylocystis hirsuta</i> strain CSC1 DQ364434.1 97%
5	Bangalya	79	MF614621	Pink	<i>Methylocystis hirsuta</i> strain CSC1 DQ364434.1 97%
6	Sukalya	117	MF614622	Brown-yellow	<i>Methylocaldum gracile</i> U89301.1 97%
7	Kamal Bhat	321	MF614616	Brown-yellow	<i>Methylocaldum gracile</i> U89301.1 98%

The *pmoA* gene accession number is mentioned and the closest phylogenetic affiliate is mentioned

gene were of good quality reflecting that each culture had a single methanotroph. When NCBI BLAST was performed, all of these seven methanotrophs were found to belong to *Methylocystis* (4 strains) or *Methylocaldum* genus (3 strains) (Table 1). The percent homology of the *pmoA* sequence with the sequences from valid methanotrophic species was 94–98%. In the *pmoA* phylogenetic tree (Fig. 1), methanotrophs isolated from the Kolam rice variety ('Surati' and 'Juna') grouped with *Methylocystis* spp. The aerobic methanotrophs cultivated from 'Bangalya' and 'Najar Bhat' were pink in color and these grouped with *Methylocystis rosea*, a pink-pigmented species of *Methylocystis*. The three methanotrophs obtained from 'Kamal Bhat', 'Sukalya' and 'Dhaval Bhat' belonged to *Methylocaldum* species and grouped with *Methylocaldum tepidum* or *Methylocaldum gracile*. Thus, to summarize, seven methanotrophic cultures belonging to the genera *Methylocystis* and *Methylocaldum* were obtained as a result of this investigation.

Methylocaldum and *Methylocystis* are two of the most important methanotrophic genera reported from Indian rice rhizospheres (Pandit et al. 2016; Vishwakarma and Dubey 2010) as well as from other rice fields in the world (Shrestha et al. 2008; Dianou et al. 2012; Bodelier et al. 2000). In Jawhar, summer temperatures can rise to 43 °C and the soil temperatures are predicted to be even higher. Therefore, methanotrophs thriving under such conditions should be

resistant to heat and dry climate prevalent during summer. *Methylocaldum* is a thermotolerant genus, and *Methylocystis* species produce desiccation resistant cysts (Garrity et al. 2005; Whittenbury et al. 1970a, b). Apart from the monsoonal rains, Jawhar is mostly dry throughout the year, and therefore, species such as *Methylocaldum* and *Methylocystis* which can survive under dry and hot conditions may prevail. *Methylocystis* species have been reported to resist protozoan grazing due to the cell walls that are harsher to break (Murase et al. 2011). In our recent studies, when we used metagenomics approach for investigating methanotrophic communities from rice roots of a urea-fertilized field in India, *Methylocystis* and *Methylococcus*–*Methylocaldum* were two important members of the community (Pandit et al. 2016). In a rice field which was organically fertilized using compost, (Pandit et al. 2016a, b) we found the dominance of Type I methanotrophs of a novel taxon in addition to the presence of sequences from *Methylocaldum* and *Methylocystis*. Type II methanotrophs and *Methylococcus*–*Methylocaldum* group have also been particularly observed in rice fields with alternate dry and wet cycles (Leng et al. 2015; Ma et al. 2013).

Since India occupies the largest rice cultivation area in the world and rice fields are supposed to be a significant source of anthropogenic methane emissions (Dubey 2005), studies on indigenous methanotrophs are much needed for

Fig. 1 Maximum-likelihood tree of the *pmoA* gene sequence of strains obtained from rice landraces in comparison with other methanotrophic cultured members. GenBank accession numbers are given in parentheses. Bar represents 5% sequence divergence

exploring answers to reduce global warming. The cultures obtained in the present study would be further explored as novel bio-inoculants for inoculation in rice fields which can mitigate more methane. Further, these also could be explored for other biotechnological applications such as single cell protein, carotenoid or biodiesel production (Pandey et al. 2013; Strong et al. 2015). This work will also provide a basis for the future work aimed towards methane mitigation from flooded rice fields.

Acknowledgements We thank Pranitha S. Pandit MACS, Agharkar Research Institute for the help during DNA isolation of the cultures.

Author contributions MCR and RAB planned the work, wrote the manuscript and prepared figures and tables. SP maintained the rice landraces at Jawhar and also did the sampling. RAB performed initial processing of samples and helped in phylogenetic analysis. MCR did all the microbiological and molecular biological work. PKD reviewed the work and helped in writing the manuscript.

Funding This work was partially supported by DBT BioCARE program (BT/BioCARE/06/840/2012) sanctioned to MCR. We thank both the institutes for providing institutional funds.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Bodelier PL, Roslev P, Henckel T, Frenzel P (2000) Stimulation by ammonium-based fertilizers of methane oxidation in soil around rice roots. *Nature* 403(6768):421–424. <https://doi.org/10.1038/35000193>
- Conrad R (2009) The global methane cycle: recent advances in understanding the microbial processes involved. *Environ Microbiol Rep* 1(5):285–292
- Costello AM, Auman AJ, Macalady JL, Scow KM, Lidstrom ME (2002) Estimation of methanotroph abundance in a freshwater lake sediment. *Environ Microbiol* 4(8):443–450
- Dianou D, Ueno C, Ogiso T, Kimura M, Asakawa S (2012) Diversity of cultivable methane-oxidizing bacteria in microsites of a rice paddy field: investigation by cultivation method and fluorescence in situ hybridization (FISH). *Microbes Environ* 27(3):278–287. <https://doi.org/10.1264/jsme2.ME11327>
- Dubey SK (2001) Methane emission and rice agriculture. *Curr Sci* 81(4):345–346
- Dubey SK (2005) Microbial ecology of methane emission in rice agroecosystem: a review. *Appl Ecol Environ Res* 3(2):1–27
- Garrry G, Brenner DJ, Krieg NR, Staley JR (2005) *Bergey's Manual® of Systematic Bacteriology*, vol 2. Volume two the proteobacteria part C the alpha-, beta-, delta-, and epsilonproteobacteria, 2nd edn. Springer, New York
- Leng L, Chang J, Geng K, Lu Y, Ma K (2015) Uncultivated *Methylocystis* species in paddy soil include facultative methanotrophs that utilize acetate. *Microb Ecol* 70(1):88–96. <https://doi.org/10.1007/s00248-014-0540-0>
- Ma K, Conrad R, Lu Y (2013) Dry/wet cycles change the activity and population dynamics of methanotrophs in rice field soil. *Appl Environ Microbiol* 79(16):4932–4939. <https://doi.org/10.1128/AEM.00850-13>
- Murase J, Hordijk K, Tayasu I, Bodelier PL (2011) Strain-specific incorporation of methanotrophic biomass into eukaryotic grazers in a rice field soil revealed by PLFA-SIP. *FEMS Microbiol Ecol* 75(2):284–290. <https://doi.org/10.1111/j.1574-6941.2010.01007.x>
- Pandey VC, Singh JS, Singh DP, Singh RP (2013) Methanotrophs: promising bacteria for environmental remediation. *Int J Environ Sci Technol* 11(1):241–250. <https://doi.org/10.1007/s13762-013-0387-9>
- Pandit PS, Ranade DR, Dhakephalkar PK, Rahalkar MC (2016a) A pmoA-based study reveals dominance of yet uncultured Type I methanotrophs in rhizospheres of an organically fertilized rice field in India. *3 Biotech* 6(2):135. <https://doi.org/10.1007/s13205-016-0453-3>
- Pandit PS, Rahalkar M, Dhakephalkar P, Ranade DR, Pore S, Arora P, Kapse N (2016b) Deciphering community structure of methanotrophs dwelling in rice rhizospheres of an Indian rice field using cultivation and cultivation independent approaches. *Microb Ecol* 71:634–644. doi:<https://doi.org/10.1007/s00248-015-0697-1>
- Qiu Q, Noll M, Abraham WR, Lu Y, 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(6):602–614. <https://doi.org/10.1038/ismej.2008.34>
- Reim A, Luke 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(11):2128–2139. <https://doi.org/10.1038/ismej.2012.57>
- Shenton M, Iwamoto C, Kurata N, Ikeo K (2016) Effect of wild and cultivated rice genotypes on rhizosphere bacterial community composition. *Rice* 9(42):1–12. <https://doi.org/10.1186/s12284-016-0111-8>
- 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(2):400–412. <https://doi.org/10.1111/j.1462-2920.2007.01462.x>
- 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(12):1545–1556. <https://doi.org/10.1038/ismej.2010.89>
- Strong PJ, Xie S, Clarke WP (2015) Methane as a resource: can the methanotrophs add value? *Environ Sci Technol* 49:4001–4018
- Tamura K, Stecher G, Peterson D, Filipksi A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30(12):2725–2729. <https://doi.org/10.1093/molbev/mst197>
- Vishwakarma P, Dubey SK (2010) Diversity of methanotrophs in urea-fertilized tropical rice agroecosystem. *Indian J Microbiol* 50(2):205–211. <https://doi.org/10.1007/s12088-010-0040-y>
- Whittenbury R, Davies S, Davey JF (1970a) Exospores and cysts formed by methane-utilizing bacteria. *J Gen Microbiol* 61:219–226
- Whittenbury R, Phillips KC, Wilkinson JF (1970b) Enrichment, isolation and some properties of methane utilising bacteria. *J Gen Microbiol* 61:205–218