



Isolation and Characterization of Novel Methanogens and sMMO Producing Methanotrophs from Rice Paddy Soil

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ABSTRACT

In the present study, the diversity of two functional microbial communities; methanogenic archaea and soluble methane monooxygenase (sMMO) producing methanotrophic bacteria from rice paddy soil of different regions of Punjab, Pakistan were studied using phylogenetic analysis. Morphological and biochemical analysis showing the presence of different species of novel methanogenic archaea and sMMO-producing methanotrophic bacteria in rice paddy soil samples. The evolutionary analysis indicated a phylogram with five monophyletic groups (MPG-I to MPG-V). Results revealed that three isolated species (*Sphingomonas* sp. MG-1, *Sphingomonas* sp. MG-2 and *Sphingomonas* sp. MT-2) were in a close evolutionary relationship while the fourth isolated species (*Sphingomonas* sp. MT-1) appeared in MPG-II and showed strong evolutionary relationships with *Sphingopyxis terrae* and *Novosphingobium capsulatum*. The appearance of *Sphingomonas* sp. MT-1 in a different clade was also evidence of its distant evolutionary relationships with the other three isolated strains of *Sphingomonas* sp. (MG-2, MG-1 and MT-2). The strain designated as MT-1 was found to be rod-shaped with μ_{max} 0.065 ± 0.25 h⁻¹ and 13.59 ± 0.25 U mL⁻¹ sMMO. The optimum pH and temperature for the growth of MT-1 and production of sMMO were 6.3 and 37 °C, respectively. The concentration of MG-1 and MT-1 was significantly higher in the soil of Faisalabad region (FSD) as compared to MG-2 and MT-2 in soil of Sargodha region (SGD). Overall FSD soil was observed to be more suitable for methanogenic and methanotrophic growth than SGD soil according to the conditions needed for microbial growth in soil.

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Authors' Contribution

SK designed and executed the study. TR did sampling, processed the samples and wrote manuscript. GM compiled phylogenetic part of manuscript. SR analyzed the data statistically. SAB and SK reviewed the manuscript.

Key words

Evolutionary analysis, Gram-negative, Methanogenic archaea, Rice paddy soil, *Sphingomonas*

INTRODUCTION

Methane as being reduced form of carbon plays a vital role in the global economy as a fuel and greenhouse gas. Methane exhibits almost 34 times greater heat retention capacity as compared to CO₂ and has become a major threat to climate change as its atmospheric abundance (approximately 1857 ppb in 2018) is about 2.6 times greater than in preindustrial times (Saunois *et al.*, 2016). The main sources of methane include thermogenic, biogenic and abiogenic microbes, agricultural production systems and wastewater treatment plants (Nazaries *et al.*, 2013). Agricultural activities contribute greater than 50%

of overall methane emission by anthropogenic means amongst which, 20% of methane emissions are contributed by rice fields. Unfortunately, processes required to sequester CH₄ and CO₂ need costly catalytic systems, high pressure (~30 MPa), and elevated temperature (~450°C) with the release of toxic by-products such as carbon monoxide; thus presenting an expensive and non-sustainable technology. A major portion of methane is reduced before its release into the atmosphere by natural means that involve methane-oxidizing bacteria (MB) employing featured sets of enzymes for utilization of methane as carbon and energy source; hence converting it into useful metabolites (Krause and Townsend, 2016; Tilche and Galatola, 2008). MB and enzymes present an attractive alternative as biocatalysts for the sequestration of CH₄ and CO₂ owing to the milder operating conditions without the release of toxic by-products (Tsapekos *et al.*, 2020). Methanotrophs having different types of methane monooxygenases (MMO) can remediate various types of organic pollutants such as diverse groups of halogenated organic compounds like trichloroethylene and aliphatic hydrocarbons with their halogenated derivatives (Kumar *et al.*, 2020). Except few, all methanotrophs produce particulate methane

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monooxygenases (pMMO) whereas methanogens utilize their ability of organic acid degradation and conversion into methane. Methanogens can be used as a cost-effective and easy approach to degrade industrial effluents (Kour *et al.*, 2021). Though methanogens have proved useful for preserving aquatic biodiversity for the ecosystem they pose a very serious threat as methanogenic prevalence can lead to a significant increase in methane emissions which results in the greenhouse effect. This problem can be resolved by employing methanotrophs that consume methane as an energy source (Mao *et al.*, 2020). Both soluble and particulate methane monooxygenases (sMMO and pMMO, respectively) enzymes are responsible for the conversion of methane into methanol in the an-aerobic type of methane oxidation.

The present study aimed to ascertain the diversity of sMMO-producing methanotrophs using NMS media and to isolate methanogenic archaea from different rice paddy soils. Characterization of some representative methanogenic and methanotrophic bacteria from different rice paddy soils was also undertaken at the morphological, biochemical and molecular levels.

MATERIALS AND METHODS

The methanogenic and methanotrophic bacteria were isolated from rice paddy fields of Faisalabad and Sargodha (Pakistan). Soil samples were collected from Chak No. 248/Rb Bismillah Pur Faisalabad, Pakistan, where rice crop was being cultivated for 8 years and Chak No. 128/NB in Sillanwali Tehsil of Sargodha region where rice was being cultivated for 5 years.

Samples were collected randomly from 0-30 cm of depth for methanogenic and methanotrophic bacteria (Seo *et al.*, 2014) in two layers. The upper layer (0-15cm) was preferred for the isolation of methanogens (MG-1 and MG-2) while the lower layer (15-30cm) was selected for the study of methanotrophs (MT-1 and MT-2). Soil electrical conductivity (EC) was determined by EC meter. The amount of available phosphorus and potassium in the soil was determined with LaMotte soil test kit. Soil pH was determined by pH meter after its mixing with distilled water and allowing particles to settle down (Seo *et al.*, 2014). The dry oven method was used for measuring soil saturation and organic matter was measured by loss on ignition (Bisutti *et al.*, 2004; Waqas *et al.*, 2018).

Isolation of pure bacterial culture

The basal media used for the isolation of MB consisted of the following composition per 100 mL of distilled water: 400 μ L of 20% yeast extract solution, 400 μ L of 25% sodium acetate solution, NaHCO₃, 0.24g,

nutrient agar, 5g; Wolfe's vitamin solution, 400 μ L and Wolfe's mineral solution, 400 μ L (Onderko *et al.*, 2019). The NMS (Nitrate mineral salt) media used for the isolation of methanotrophs consisted of 0.85 g/L NaNO₃, 0.17 g/L K₂SO₄, 0.04 g/L MgSO₄·7H₂O, 0.01 g/L CaCl₂·2H₂O supplemented with a trace element solution (0.29 mg/L ZnSO₄·7H₂O, 0.17 mg/L MnCl₂·6H₂O, 0.06 mg/L H₃BO₃, 0.05 mg/L Na₂MoO₄·2H₂O, 0.05 mg/L CoCl₂·6H₂O, 0.08 mg/L KI, 0.13 mg/L CuSO₄·5H₂O), 50 μ M CuSO₄·5H₂O, and 40 μ M FeSO₄·7H₂O at pH 6.3 (Smith *et al.*, 2011).

Serial dilution of soil samples was done by using 0.9% saline. The streak plate method was used to grow MB on the surface of the growth media with composition: 400 μ L of 20% yeast extract solution, 0.24g of 25% sodium acetate solution, 5g of nutrient agar, 400 μ L of Wolfe's vitamin solution, 400 μ L of Wolfe's mineral solution (volume was made up to 100 mL) (Onderko *et al.*, 2019) and methanol vapors as a carbon source. Individual colonies of bacteria were isolated and tested (Meruvu *et al.*, 2020). For MB, Petri plates were placed in anaerobic jars consisting of a gas pack to provide anaerobic conditions (Pappachan *et al.*, 2019). The NMS (Nitrate mineral salt) media used for the isolation of methanotrophs consisted of 0.85 g/L NaNO₃, 0.17 g/L K₂SO₄, 0.04 g/L MgSO₄·7H₂O, 0.01 g/L CaCl₂·2H₂O supplemented with a trace element solution (0.29 mg/L ZnSO₄·7H₂O, 0.17 mg/L MnCl₂·6H₂O, 0.06 mg/L H₃BO₃, 0.05 mg/L Na₂MoO₄·2H₂O, 0.05 mg/L CoCl₂·6H₂O, 0.08 mg/L KI, 0.13 mg/L CuSO₄·5H₂O), 50 μ M CuSO₄·5H₂O, and 40 μ M FeSO₄·7H₂O at pH 6.3 (Smith *et al.*, 2011).

Characterization of bacterial isolates

Gram staining technique was used to differentiate bacteria as Gram-negative or Gram-positive (Moyes *et al.*, 2009). For biochemical analysis, a carbohydrate fermentation test was done by using phenol carbohydrate media (10 g trypticase, 5 g NaCl, 0.018 g phenol red, 10 g carbohydrate source in 1000 mL distilled water) (Kali *et al.*, 2015). An oxidase test was performed to detect the presence of a cytochrome oxidase system, present in aerobic bacteria. The microorganisms that contain cytochrome C as part of their respiratory chain are oxidase positive and turn the reagent into blue or purple color (Deutzmann *et al.*, 2014).

Determination of growth rate

To determine the growth rate of both methanogenic archaea and methanotrophs, cells having initial OD= 0.1 at 600nm were allowed to grow in 1L sealed gastight Erlenmeyer flasks consisting of 150 mL nitrate mineral salt media (For MT-1 and MT-2) and basal media (for MG-1 and MG-2) in anaerobic conditions. The methane-

air mixture (2:6) was flushed into methanotrophic isolates and kept in incubator shaker at 30°C. After intervals of 2h optical density was measured and sMMO activity was noted through naphthalene oxidation assay (Miller *et al.*, 2002).

I6S rRNA gene amplification

Amplification of genomic DNA was done by using the I6S rRNA gene. DNA from methanogens and methanotrophs was extracted using the WizPrep™ gDNA Mini Kit (Wizbiosolutions) following the protocol of the manufacturer. DNA extracted products were confirmed by using 1.2% agarose gels leading to PCR for amplification (Tabani *et al.*, 2017). Eubacterial-specific primers (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTACCTTGTTACGACTT-3') (Lane, 1991) were used to amplify 16S rRNA genes. The PCR mixture contained 13 µl MM, 1 µl of each reverse and forward primer, 3 µl of DNA sample, 7 µl of distilled water. The optimized protocol was followed. Run of PCR products for 30 min at 100V was done by using 1.2% (w/v) agarose electrophoresis in 1x TAE. DNA bands were visualized through a UV transilluminator. 1Kb plus DNA ladder was used as a marker (Thermo Fisher scientist) (Zheng *et al.*, 2017).

Phylogenetic analysis

The nucleotide sequences of four isolated species (MT-1, MT-2, MG-1 and MG-2) were analyzed using BLAST (Basic Local Alignment Search Tool) available on the NCBI website (<http://www.ncbi.nlm.nih.gov/>) against rRNA/ITS database of 16S ribosomal RNA sequences (Bacteria and Archaea) (Altschul *et al.*, 1990). Along with nucleotide sequences of four isolated species, thirty-seven sequences of 16S rRNA from various species were retrieved from GenBank for phylogenetic systematics. All sequences were aligned using Clustal X and imported into the MEGA6 program for manual alignment. Neighbor-Joining (NJ) phylogenetic tree was reconstructed using MEGA6 with 100 bootstrap replicates (Kumar *et al.*, 2016).

RESULTS

Soil characteristics

Characteristics of soil samples of Faisalabad and Sargodha at the level of 0-15 cm and 15-30 cm are presented in Table I. The soil of both regions was found loamy with pH ranges between 8.3-8.4 which is fit for methanogens and methanotrophic growth. Most of the soils in natural wetlands like paddy rice fields have alkaline nature (Minasny *et al.*, 2016). Loam soils have SP values between 20-35%. Soil texture is usually characterized by saturation percentage. The texture of soil samples used in the present study is loamy (a mixture of clay, sand and soil), with appropriate water holding capacity. The saturation of soil samples in this study ranges from 32-34, which is best for the bacterial community in soil (Kalyuzhnaya *et al.*, 2019). The EC results showed highly significant variation among both regions. MG-1, MG-2, MT-1 and MT-2 samples have EC of 4.98, 2.57, 3.09 and 2.48, respectively. Organic matter content also varied highly significantly among the two regions and was found to be 0.98, 0.42, 0.56 and 0.28% among MG-1, MG-2, MT-1 and MT-2 respectively, thus best for microbial growth. The analysis of variance regarding P and K indicated highly significant differences between the two regions and samples. The available P and K for MG-1, MG-2, MT-1 and MT-2 soil samples is 10.8, 3.2, 6.5, 2.3 ppm, and 200, 80, 180, 60 ppm, respectively. The saturation of both soil samples varied highly significantly and ranged between 32-34%. The moisture level is needed to be maintained to support microbial growth (Szafranek-Nakonieczna *et al.*, 2019).

Characteristics of bacterial isolates

Isolates obtained by enrichment and by streak plating were screened for sMMO assay using naphthalene oxidation assay and CH₄ production potential (Li *et al.*, 2021; Zheng *et al.*, 2017). The maximum growth rates (μ_{max}) for all isolates were nearly similar (values of μ_{max} between 0.041 to 0.065 h⁻¹; Table II). These values are typical for methanogens and methanotrophs are grown in batch culture having sMMO expression and CH₄ emission.

Table I. Soil properties from the regions of Faisalabad and Sargodha district.

Soil sample	Depth (cm)	Sample name	EC dS m ⁻¹	Soil pH	org.matter (%)	P (ppm)	K (ppm)	Saturation (% age)	Texture
Faisalabad	0-15	MG-1	4.98 ± 0.25 ^a	8.41 ± 0.05 ^a	0.98 ± 0.001 ^a	10.8 ± 0.50 ^a	200 ± 0.05 ^a	34 ± 0.02 ^a	Loam
	15-30	MT-1	3.09 ± 0.00 ^b	8.31 ± 0.20 ^b	0.56 ± 0.05 ^b	6.5 ± 0.70 ^b	180 ± 0.25 ^b	32 ± 0.00 ^b	Loam
Sargodha	0-15	MG-2	2.57 ± 0.05 ^c	8.30 ± 0.00 ^b	0.42 ± 0.25 ^c	3.2 ± 0.05 ^c	80 ± 0.00 ^c	32 ± 0.00 ^b	Loam
	15-30	MT-2	2.48 ± 0.01 ^c	8.29 ± 0.03 ^b	0.28 ± 0.05 ^d	2.3 ± 0.40 ^d	60 ± 0.02 ^d	32 ± 0.01 ^b	Loam

Means sharing the same letters are statistically non-significant at p<0.05

Table II. Sources of isolates and comparison of growth rate, sMMO activity and emission of CH₄.

Isolates	Origin	Isolation method	Growth rate μ_{max} (h ⁻¹)	sMMO activity (U/mL)	Emission of CH ₄ (%)
MT-1	Faisalabad	Nitrate mineral salt media	0.065±0.25	13.59±0.25	ND*
MT-2	Sargodha	Nitrate mineral salt media	0.059±0.00	11.32±0.50	ND*
MG-1	Faisalabad	Basal media	0.048±0.05	1.22±0.25	48.8±1.50
MG-2	Sargodha	Basal media	0.041±0.00	0.89±0.00	27.6±0.50

*ND, not detected.

The results of Gram staining indicated that both bacteria were gram-negative rods as they showed pink color on staining and microscopic examination. The staining procedure also revealed the overall cell morphology of bacteria that can be further labeled as rods (Moyes *et al.*, 2009). The concentration of bacterial cells at different time intervals is presented in Table III.

Table III. The concentration of bacteria per ml of broth media.

Sr. No.	Sample	The concentration of bacterial cells/ml		
		24h	48h	72h
1.	MG-1	2.02x10 ^{8j}	4.08x10 ^{8f}	6.06x10 ^{8b}
2.	MG-2	1.55x10 ^{8k}	3.1x10 ^{8h}	4.67x10 ^{8e}
3.	MT-1	2.76x10 ⁸ⁱ	5.52x10 ^{8d}	8.29x10 ^{8a}
4.	MT-2	1.96x10 ^{8j}	3.93x10 ^{8g}	5.9x10 ^{8c}

** Means sharing the same letters are statistically non-significant at p<0.05.

When bacterial slides were examined under a microscope, they showed mixed cultures of bacteria of different shapes and sizes that were not able to differentiate because of higher cell density in the liquid broth sub-culture. The proportion of bacteria detected in soil A was greater than the proportion of bacteria in soil B. The methanogenic and methanotrophic bacteria varied highly significantly regarding the region in soil A and B. The concentration of MG-1 (6.06x10⁸) and MT-1 (8.29x10⁸) was significantly higher in soil A as compared to MG-2 and MT-2 in soil B.

Similarly, methanogenic and methanotrophic bacteria varied highly significantly regarding the region in soil A and B. The concentration of MG-1 and MT-1 was significantly higher in soil A as compared to MG-2 and MT-2 in soil B. Overall Soil A was resulted more suitable for the growth of methanogens and methanotrophs than soil B according to the soil conditions required for the microbial growth.

Phylogenetic status of the bacterial isolates

Representative sequences were submitted to Genbank

under accession numbers OM371078 (MG-1), OM371079 (MG-2), OM371080 (MT-1), OM371081 (MT-2), respectively.

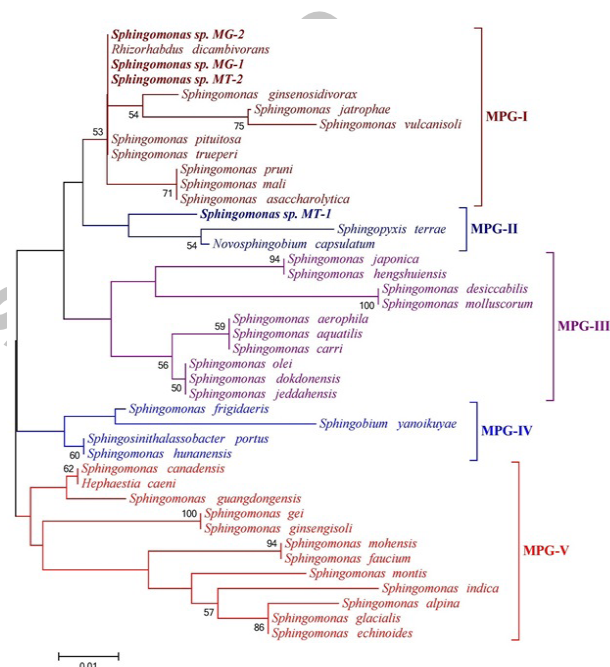


Fig. 1. Evolutionary relationships of isolated *Spingomonas* species.

To predict the evolutionary relationships, a phylogenetic tree of isolated *Spingomonas* species with other bacterial species was reconstructed (Fig. 1). The evolutionary history was inferred using the Neighbor-Joining method (Saitou, 1987). The optimal tree with the sum of branch length = 0.44062500 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units

of the number of base differences per site (Nei and Kumar, 2000). The analysis involved 41 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 85 positions in the final dataset.

The phylogram has been divided into five different monophyletic groups (MPG-I to MPG-V). The three isolated *Sphingomonas* species (i.e., *Sphingomonas* sp. MG-2, *Sphingomonas* sp. MG-1 and *Sphingomonas* sp. MT-2) appeared in MPG-I and showed their close evolutionary relationships. Eight different *Sphingomonas* species also joined this clade. *Rhizorhabdus dicambivorans* also appeared in MPG-I that is showing a close evolutionary relationship with various *Sphingomonas* species. The fourth isolated species (i.e., *Sphingomonas* sp. MT-1) appeared in MPG-II and showed strong evolutionary relationships with *Sphingopyxis terrae* and *Novosphingobium capsulatum*. The appearance of *Sphingomonas* sp. MT-1 in a different clade is evidence of its distant evolutionary relationships with the other three isolated strains of *Sphingomonas* sp. (MG-2, MG-1 and MT-2).

Spearman's correlation analysis was conducted to determine the correlations among methane-producing capacity (MPC) and dominated genera. The results were showing a positive correlation between MPC and bacterial genera related to *Sphingomonas*, *Syntrophomonas*, *Geobacter* and *Opitutus* ($P < 0.05$), indicating potential contributions of these functional taxa in the methanogenic process (Lam *et al.*, 2021).

DISCUSSION

Results of the present study showed that the concentration of available K, P, organic matter and EC were at moderate level in the soil samples collected at the depths of 0-15 cm while comparatively lower in the samples collected at the soil depths of 15-30 cm. Also, Methylobacter-relevant sequences display a relationship with soil profile as oxidation of methane decreases with the depth of soil (Szafranek-Nakonieczna *et al.*, 2019). Rath *et al.* (2019) reported a positive correlation between bacterium and EC values. Saline soil is rich in Na and Cl, and both these components are highly correlated with EC. Slightly saline soils possessed lesser MBI (MOB) communities and showed a rarer type of Methylocystis II phylotypes. In present work soil, Faisalabad and Sargodha have salinity values most suitable for the growth of methanogens and methanotrophs. The high proportions of methanotrophic bacteria provided evidence that under different field conditions methanotrophs are phylogenetically distinct by environmental selection (Shiau *et al.*, 2018). Methanogenic population size has been

reported to decrease in the following order; flowering> ripening> tillering> pre-plantation and postharvest stage (Singh and Dubey, 2012). Similar to our findings Qiu *et al.* (2014) identified the methanogenic strain NM7 isolated from rice paddy soils, with non-motile cells, short rods and pink color was shown on gram staining (Qiu *et al.*, 2014).

The results of the carbohydrate fermentation test were positive for both methanogenic and methanotrophic bacteria. Results revealed that the final pH of prepared fermentative broth media was maintained at 7-7.5 to obtain correct results. Methanogenic activity is sensitive to high pH and methanogenic activity is decreased if pH decreases lesser than 4.5 (Ye *et al.*, 2012). In the glucose, lactose and sucrose fermentation test, the color change and gas production indicated positive results by producing acids and gas. The results of the oxidase test for both bacterial samples indicated that MB are oxidase negative as they are anaerobic bacteria and do not contain cytochrome oxidase system while methanotrophic bacteria are aerobic and consist of cytochrome oxidase enzyme as part of their respiratory chain and turned the reagent color from white to blue on filter paper as reported previously (Deutzmann *et al.*, 2014). In a study, it was also reported that novel *Sphingomonas* sp. can remove odor-producing compounds and methane. Plant-pathogenic strain *Sphingomonas melonis*, alphaproteobacterium was found to be methanol degrading methylotroph, but not for any other methyl compounds (Boden *et al.*, 2008). Isotope probing showed *Sphingomonas* as a methylotroph in the natural habitat (Nercessian *et al.*, 2005).

CONCLUSION

The novel methanotrophic strains were isolated using paddy rice soil samples from Faisalabad and Sargodha regions. 16S rRNA gene sequence analysis specified them as distinct from previously identified species of *Sphingomonas*. Phylogram indicated isolated *Sphingomonas* sp. MT-1 appeared in MPG-II and resulted in a distant evolutionary relationship with other isolated strains including *Sphingomonas* sp. MG-2, *Sphingomonas* sp. MG-1 and *Sphingomonas* sp. MT-2, which were in a close evolutionary relationship together residing MPG-I out of five monophyletic groups (MPG-I to MPG-V). The present study demonstrates some fundamental differences in the abundance of methanogenic archaea and sMMO containing methanotrophs in both strata of soil in the Sargodha and Faisalabad regions of Punjab, Pakistan. Results indicate a significant variation in soil pH, EC, organic matter, phosphorous and potassium among both samples. However, these parameters were found moderate in the upper stratum (0-15cm) and lower stratum (15-

30cm) of soil.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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