

Characterization of Methanotrophic Communities in Soils from Regions with Different Environmental Settings

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Methanotrophic communities from freshwater wetland (FW), seawater wetland (SW), forest (FS), and landfill soils (LS) around Seoul of South Korea, were characterized using comparative sequence analyses of clone libraries. Proportions of *Methylocaldum*, *Methylococcus* and *Methylosinus* were found to be greater in FW and SW, while *Methylobacter* and *Methylomonas* were more notable in FS and *Methylocystis* and *Methylomicrobium* more prominent in LS. Lag periods behind the initiation of methane oxidation significantly varied amongst the soils. Methane oxidation rates were greater in $FW \geq LS \geq SW > FS$ ($p < 0.05$). Thus, the environmental setting is a significant factor influencing the communities and capabilities of methanotrophs.

Keywords: Methanotrophs, clone library, environmental setting, methane oxidation potential

There are diverse methanotrophs in natural environments where they utilize methane as their sole source of carbon and energy. Environmental characteristics such as climatic, physiographic, geologic, biotic, and land-use features can determine growth and activity of methanotrophs. For instance, methane concentration is a key determinant for methanotrophic community composition as well as characteristics of methane oxidation [6, 9, 11, 13]. Methanotrophs in well-aerated upland soils show a high enzymatic affinity for methane since they grow on atmospheric methane [1, 9]. In contrast, methanotrophs in wetland or landfill soils may have lower enzymatic affinity levels since they grow on methane produced in the soils or emitted from the landfills, respectively. The main objective in the present study was to test hypotheses that environmental setting can determine whether a specific type of methanotrophs is present, and that it also can determine methane-oxidation potential of methanotrophs.

We defined methanotrophic communities and characterized CH_4 oxidation of four different soil environments. Four regions with different environmental settings in the suburbs

of Seoul, South Korea, were selected for soil sampling: landfill (Gapyeong, Gyeonggi province), freshwater wetland (Namyangju, Gyeonggi), seawater wetland (Yeongjong island, Incheon) and forest (Ungil mountain, Gyeonggi) in Korea. Five subsamples were collected at a depth of 10 cm from each soil site. Subsamples were thoroughly mixed and sieved using a 2 mm-mesh. Soil pH in soil/water (2:1) solution was measured using 420A pH meter (Thermo Orion, Beverly, USA). Ten g of each wet soil was dried at a 105°C oven for overnight to measure moisture content. Each dried soil was burned at 550°C in an electric muffle furnace (Dae Han Scientific, Seoul, Korea) for 3 h, and its organic matter content was measured. Soil texture was analyzed [5, 8].

To identify methanotrophs present in the samples, a culture-independent PCR-cloning technique was performed. DNA was extracted from soil samples using the UltraClean Soil Kit (Mo Bio, Carlsbad, USA). DNA was quantified using an ASP-2680 spectrophotometer (ACTGene, Piscataway, USA). An alpha-subunit of the particulate methane mono-oxygenase gene (*pmoA*) was amplified with the A189f-mb661r primer set (*ca.* 510 bp) [12]. PCR was performed in 50- μ L volumes, consisting of 5 μ L of 10 \times Ex Taq Buffer, 0.2 M dNTPs, 0.4 μ M A189f and mb661r, 0.01 μ g of bovine serum albumin, 100 ng of template DNA, and 0.75 U of Taq polymerase (TaKaRa, Otsu, Japan). Initial

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Table 1. Characteristics of the 4 environmental samples with different settings.

Sample name	Setting	Sample site	pH	MC ^a (%)	LIC ^b (%)	Soil Texture
FS	Forest	37°33' N 127°18' E	4.71 ± 0.08	21.03 ± 0.02	4.66 ± 0.06	Loam
SW	Seawater sediment	37°25' N 126°25' E	7.30 ± 0.25	39.08 ± 0.23	3.16 ± 0.03	Silt loam
FW	Freshwater sediment	37°32' N 127°19' E	5.73 ± 0.30	47.77 ± 0.49	4.84 ± 0.14	Sandy loam
LS	Landfill cover	37°48' N 127°21' E	7.39 ± 0.03	13.09 ± 0.05	2.63 ± 0.12	Sandy loam

^aMoisture content^bLoss-on-ignition content (Soil organic matter content)

denaturation was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 40 sec, 58°C for 40 sec and 72°C for 30 sec, with a final elongation step at 72°C for 10 min. *pmoA* amplicons were purified with the QIAquick Gel extraction Kit (Qiagen, Valencia, USA) after electrophoresis, then cloned into TOTO TA Cloning Kit (Invitrogen, Carlsbad, USA) and transformed into TOPO10 *E. coli* competent cells (Invitrogen, Carlsbad, USA). Individual white colonies were picked up and suspended in 25 µl of distilled water and boiled for 30 min, followed by spin-down at 13,000 rpm for 10 min. One-µL supernatants were applied to PCR mixtures as described above with the primers M13F and M13R to re-amplify the insert sequences. PCR products were commercially sequenced.

DNA sequences were compared to known nucleic acid or amino acid sequences using BLASTN and BLASTX (www.ncbi.nlm.nih.gov/BLAST). They were identified at the genus level by a threshold level of 90% sequence identity as suggested by Pester *et al.* [15]. Unidentified sequences, not closely related to any partial *pmoA* gene sequences of cultured methanotrophs, were excluded for further data analyses. Shannon-Weaver diversity (*H*) and evenness (*E*) of methanotrophic communities were calculated as following: $H = -\sum p_i \log(p_i)$ and $E = H/\log(n)$, where p_i and n are proportional abundance of the *i*th object and the total number of species, respectively [17]. Principle coordinate analysis (PCoA) and canonical correspondence analysis (CCA) were performed for analyzing relation of the methanotrophic composition and environments and relation of the methanotroph composition and environmental variables (pH, MC and MIC in Table 1), respectively using Canoco version 4.5 software (Microcomputer Power, Ithaca, USA) [18].

Soil (2 g) was placed in a 600-mL serum bottle with

Table 2. Methanotrophic communities in 4 different environments

Genus	FS ^a	SW ^b	FW ^c	LS ^d
Type I				
<i>Methylococcus</i>	5	3	-	2
<i>Methylocaldum</i>	1	-	-	-
<i>Methylomonas</i>	6	5	21	8
<i>Methylomicrobium</i>	-	-	1	2
<i>Methylobacter</i>	1	-	8	4
Type II				
<i>Methylocystis</i>	3	3	-	21
<i>Methylosinus</i>	3	2	-	-
Diversity	1.61	1.33	0.72	1.21
Evenness ^c	0.90	0.96	0.65	0.75
Unidentified	10	4	2	4

^aForest soil^bSeawater wetland soil^cFreshwater wetland soil^dLandfill soil

18 mL nitrate mineral salts medium (n=3 for each) [19]. Serum bottles were sealed with a butyl-rubber stopper and methane was injected to be a final concentration of 5%. They were incubated at 25°C with agitation (200 rpm). Methane concentrations in the headspace were monitored using gas chromatography [14].

Forest, seawater wetland, freshwater wetland and landfill cover soils were designated by FS, SW, FW and LS, respectively. Their characteristics are listed in Table 1. All soils were loams, and FW and LS were coarser than the others. FS was more acidic and had higher moisture and organic matter contents in the dry land soils, while did FW in the wetland soils. Table 2 shows the methanotrophic communities in the sampling sites. The FS community was most diverse, followed by the SW, LS and FW communities, while community evenness was greater in SW>FS>LS>FW. The FS and SW communities may have a greater functioning stability due to their greater diversity and evenness levels [20]. *Methylomonas* was present in all environments,

indicating that it may be the most common methanotrophic genus in area near Seoul. Since it is possible that unidentified sequences can represent unknown methanotrophs, FS might exhibit a higher potential to own new methanotrophs. Consistently, new types of methanotrophs have been often found in forest soils [7]. Methanotrophs are classified into types I and II by the following characteristics: membrane structures, carbon assimilation pathways, fatty acid compositions, and phylogenetic affiliation [16]. It has been considered that type I methanotrophs dominate environments with below 1% methane, while type II methanotrophs dominate environments with methane concentrations higher than 1% [6, 13]. Type II methanotrophs were not found in FW while types I and II existed together in the others. Proportions of type I members were greater in FS and SW, but not in LS. The results were consistent with previous observations. For instance, Costello *et al.* [3] reported that type I methanotrophs strongly dominated Lake Washington sediments. Both types I and II were observed, and however type I was more abundant in rice soil paddies [13]. Both types I and II were found in forest soils, with *Methylocaldum*, *Methylosinus*, and *Methylocystis* as common members [7, 11].

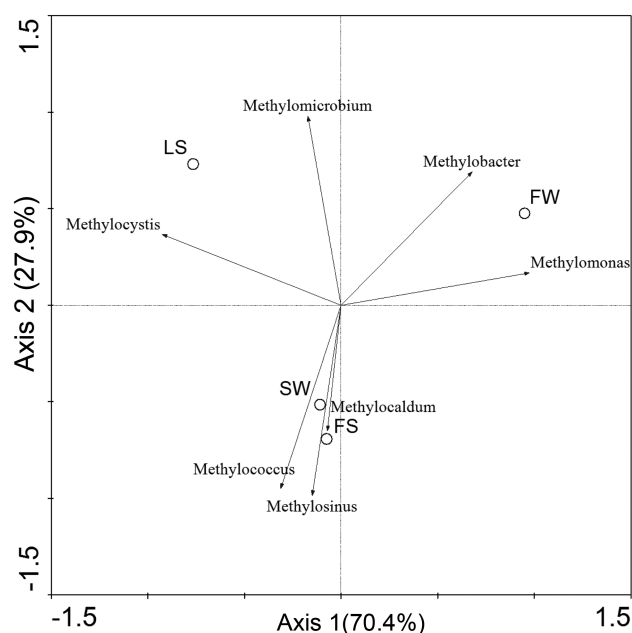


Fig. 1. A principle coordinate analysis biplot of the methanotrophic community composition and environments. Circles represent the environments and arrows indicate the direction of increase for the members. Percentage variance of bacterial genera-environment relation explained by each axis is shown in parentheses. FS, forest soil (FS); SW, seawater wetland soil; FW, freshwater wetland soil; LS, landfill soil.

In contrast, type II methanotrophs were primarily detected from landfill soils [2, 4]. *Methylocystis* and *Methylosinus* in type II and *Methylobacter* and *Methylococcus* in type I were predominant from a landfill cover soil [4].

The PCoA produced 4 axes of which the first three axes accounted for 100% of the total variance (70.4, 27.9 and 1.7%, respectively) in abundances of methanotrophic genera among the 4 soils. Each axis explains the percentage variance of general-environment relation. Fig. 1 shows a PCoA diagram with the first axis vs. second axis, explaining more than 98% of the total variance of genera-environment relation. Interestingly, the PCoA result indicated that the FS and SW communities were similar. Proportions of *Methylocaldum*, *Methylococcus* and *Methylosinus* were greater in FS and SW, while those of *Methylobacter* and *Methylomonas* in FW and those of *Methylocystis* and *Methylomicrobium* in LS. The CCA results revealed no relation between the methanotrophic composition and environmental variables (data not shown). Thus, the soil characteristics could not be a determinant whether a particular type of methanotrophs is present or abundant in this study.

CH₄ oxidation potentials of the 4 different soils were estimated. Significant lag periods behind the initiation of CH₄ oxidation were observed (182.5, 69, 69 and 13.7 h for FS, SW, FW and LS, respectively). Once the CH₄ oxidation initiated, CH₄ was completely removed within 3-4 days. Methane oxidation rates were greater in FW ≥ LS ≥ SW > FS ($p < 0.05$) (Fig. 2). FW had the highest potential for CH₄ oxidation, while FS had the lowest potential. FW was predominated by type I members that were probably

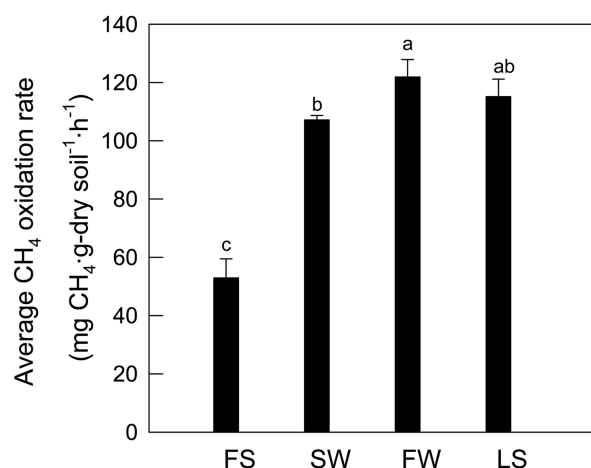


Fig. 2. Methane oxidation rates of forest soil (FS), seawater wetland soil (SW), freshwater wetland soil (FW), and landfill soil (LS) samples. Different letters mean significant difference ($p < 0.05$).

responsible for the majority of methane oxidation in this experiment, which is consistent with a conclusion by Knief *et al.* [10] that type I methanotrophs become active when methane is produced in periodically water-saturated gleyic soils, while type II members may be responsible for atmospheric methane uptake. It has been suggested that type I populations are prone to change, compared to type II populations [6]. The greatest lag period indicated that the initial methanotroph density of FS was considerably less than those of the other samples. In addition, methanotrophs from forest soils probably exhibit slow growth rates since they are considered oligotrophs with a high enzymatic affinity for methane and capability to grow on atmospheric methane [1, 9]. To sum up, the results indicated that environmental setting is important factor to influence community and capability of methanotrophs.

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국문초록

다양한 환경조건을 가진 토양의 메탄산화세균 군집 특성

김태관 · 박현정 · 이상현 · 김평화 · 문경은 · 조경숙*

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서울 근교의 민물 습지(FW), 해수습지(SW), 산림 토양(FS) 그리고 매립지 복토(LS)의 메탄산화세균 군집을 clone library/sequencing 기법을 이용하여 분석하였다. 메탄산화세균인 *Methylocaldum*, *Methylococcus*과 *Methylosinus*는 FS와 SW에서 풍부하였으며, *Methylobacter*와 *Methylomonas*는 FW에서 풍부하였고, *Methylocystis*와 *Methylomicrobium*은 LS에서 우점하였다. 메탄 산화가 관찰되기 전까지 필요한 lag phase는 각 토양별로 유의적으로 차이가 있었고, 메탄 산화속도는 $FW \geq LS \geq SW > FS$ 순이었다. 이러한 결과들은 토양의 환경조건은 메탄산화세균의 군집과 메탄산화능에 영향을 미치는 중요한 인자임을 시사한다.