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Characterization of Methane Oxidation by a Methanotroph Isolated from a Landfill Cover Soil, South Korea

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A methane-oxidizing bacterium was isolated from the enriched culture of a landfill cover soil. The closest relative of the isolate, designated M6, is *Methylocystis* sp. Based on a kinetic analysis, the maximum specific methane oxidation rate and saturation constant were 4.93 mmol·g-dry cell weight^{-1}·h^{-1} and 23 μM, respectively. This was the first time a kinetic analysis was performed using pure methanotrophic culture. The methane oxidation by M6 was investigated in the presence of aromatic (*m*- and *p*-xylene and ethylbenzene) or sulfur (hydrogen sulfide, dimethyl sulfide, methanthiol) compounds. The methane oxidation was inhibited by the presence of aromatic or sulfur compounds.

Keywords: *Methylocystis* sp., methane oxidation, aromatic compound, sulfur compounds

Methanotrophs play an important role in the biological methane oxidation in soils [21]. Although methane is mainly emitted *via* the anaerobic degradation of organic compounds by methanogens, alternative hydrocarbons [such as volatile organic compounds (VOCs)], and sulfur compounds [such as hydrogen sulfide (H_2S), dimethylsulfide (DMS), and methanthiol (MT)] are also simultaneously produced at landfill sites [20, 24]. Some hydrocarbons and sulfur compounds are known to have toxic effects toward methanotrophs [5]. To date, most studies have focused on comparing the methane oxidation in different types of landfill soils and on analyzing the community structure of methanotrophs in landfill soils [1, 2, 7, 9, 13, 18], and their kinetic studies have been performed in mixed culture or environmental samples [18, 21]. The structure and function of methane monooxygenase (MMO) and its pathway have previously been investigated [8, 16, 23]. However, there are only a few reports evaluating the effects of alternative hydrocarbons and sulfur compounds on the methane oxidation. In this paper, a pure methanotroph, M6, was isolated from a landfill cover soil. The methane oxidation behavior of M6 was characterized and the methane oxidation rate calculated using the Michaelis–Menten model. The effects of aromatic and sulfur compounds on the methane oxidation were also investigated.

The wet landfill cover soil (8 g), sampled from a domestic waste sanitary landfill at Gong-ju, Chungchungnam-do, South Korea [15], was added to a 600 ml serum bottle to prepare an enriched microbial consortium, with 20 ml of nitrate mineral salt (NMS) medium. NMS medium contains $MgSO_4·7H_2O$ (1 g/l), $CaCl_2·2H_2O$ (0.295 g/l), KNO_3 (1 g/l), KH_2PO_4 (0.26 g/l), and $Na_2HPO_4·2H_2O$ (0.41 g/l). The bottle was sealed with a butyl rubber and an aluminum cap, and methane from a 99% methane cylinder using a 50 ml syringe was injected to be a final concentration of 5% (v/v) methane. The serum bottle was incubated at 30°C with shaking (180 rpm). The methane-oxidizing consortium culture broth was obtained by enrichment culture using the same method as described in our previous study [15]. The culture broth was diluted with 0.9% NaCl solution, spread on NMS-agar plates, and allowed to grow in an aerobic jar containing 20% (v/v) methane, at 30°C for 10 days. Twenty colonies on the NMS-agar plates were chosen, with each colony inoculated into 4 ml of NMS medium, with 5% (v/v) methane, in a 120 ml serum bottle. A strain exhibiting methane-oxidizing activity was selected and designated M6. For the identification of M6, its genomic DNA was extracted using a BIO101 FastDNA SPIN Kit for soil (MP Biomedicals LLC, Solon, USA). Fragments of the 16S rDNA were PCR-amplified using the primers 27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492r (5'-TAC GGY TAC CTT GTT ACG AC-3') [4]. The tree building tool of molecular Evolutionary Genetics Analysis (MEGA) software ver. 4.0 [22] was used for a phylogenetic analysis. A partial 16S rDNA sequencing analysis has been deposited in the NCBI under the accession number GQ255542.

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M6 was pregrown in six 600 ml serum bottles, containing 20 ml of NMS medium and 5% (v/v) methane, for 7 days. To characterize the methane oxidation, methane was injected to be a final concentration of 1, 5, 10, 15, or 20% (v/v) in the headspace of each bottle (the total amount of methane in each bottle corresponded to 240, 1,200, 2,400, 3,600, or 4,800 mmol/bottle, respectively). Using Henry's constant (30°C, 1 atm) for methane [13], each methane concentration in the liquid is calculated as 14, 69, 139, 208, and 277 μM, respectively. The rates of methane oxidation were calculated from the slopes of plots of methane concentration versus time. The maximum specific methane oxidation rate (q_{max}) and saturation constant (K_m) were determined using a Lineweaver−Burk plot [11]. Cell concentrations were determined using the relationship between the optical density measured at 600 nm and gram dry cell weight (DCW).

M6 cells were collected and resuspended in fresh NMS medium. Twenty ml of resuspended cells was transferred to each 600 ml serum bottle. The bottles (prepared as above) were sealed with butyl rubbers and aluminum caps, and methane was injected to be 5% (v/v). Then, 1 μl each of ethylbenzene, *m*-xylene, or *p*-xylene, and 3 μl of DMS were added into the bottle. MT or H_2S from each gas cylinder was injected to a final concentration of 100 and 200 ppm in the bottle's headspace, respectively. The serum bottles were incubated at 30°C, with shaking (180 rpm). The methane was analyzed using gas chromatography (GC; Agilent Technologies Inc., USA), equipped with a flame ionization detector and a wax column (30 m × 0.32 mm; 0.25 μm film thickness; Supelco, USA). The following chemicals were used for this study: MT (99.5%; Sigma-Aldrich, USA), *m*-xylene (98.5%; Junsei Chemical Co., Ltd, Japan), *p*-xylene (98.5%; Junsei Chemical Co., Ltd., Japan), DMS (99%; Acros Organics, USA), ethylbenzene (98%; Junsei Chemical Co., Ltd, Japan), H_2S (10%; Dong-A Gases, Korea) in balance nitrogen, and methane (99%; Dong-A Gases, Korea).

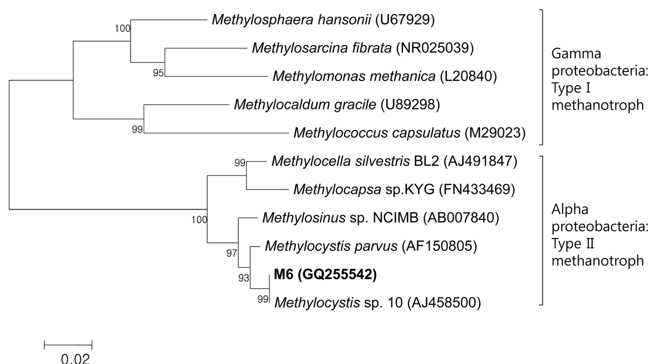

Fig. 1. Neighbor-joining phylogenetic tree of the 16S rDNA sequences of M6 and known methanotrophs.

Bootstrap values are shown; the bar represents 0.02 substitution per nucleotide position.

According to the 16S rDNA sequences analysis, M6 is most closely related to *Methylocystis* sp., a type II methanotroph (Fig. 1). Fig. 2A shows the removal of

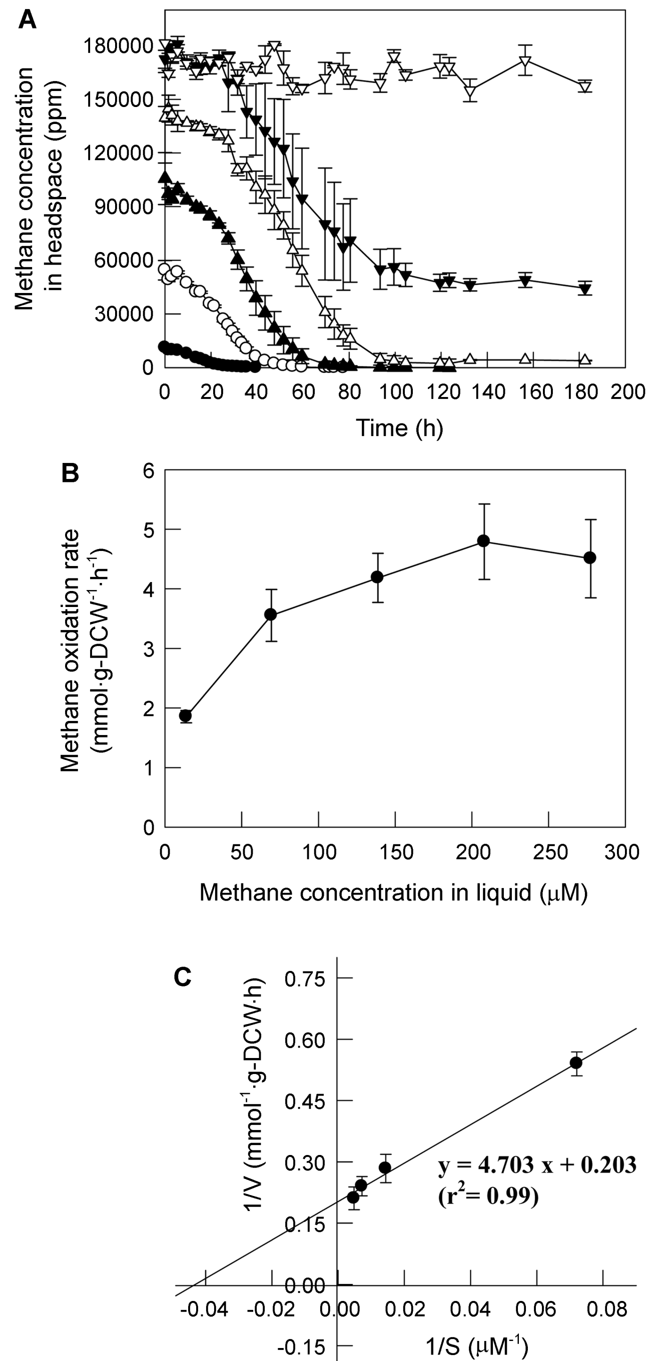

Fig. 2. Effect of methane concentration on methane oxidation.

A. Methane oxidation by strain M6 in liquid medium containing different concentrations of methane. Methane concentration in the headspace of the bottle (%, v/v) : ●, 1; ○, 5; ▲, 10; △, 15; ▼, 20; ▽, control. **B**. Relationship between methane concentrations and specific methane oxidation rate. **C**. Lineweaver−Burk plot for calculating the maximum specific methane oxidation rate (q_{max}) and saturation constant (K_m).

methane at various initial concentrations of 1~20% (v/v) methane in the headspace. The results showed that methane concentrations of 1%, 5%, and 10% (v/v) were completely removed within 1.5, 3.5, and 5 days, respectively. However, M6 could not completely oxidize methane at 15% and 20% (v/v) in the headspace. At a low methane concentration, no lag phase was observed, but with increasing initial methane concentration the lag phase became elongated.

In this study, a kinetic analysis on the methane oxidation, using M6, was first performed. From the slope in Fig. 2A. the specific methane oxidation rates were 1.86 ± 0.10, 3.56 ± 0.44, 4.18 ± 0.41, and 4.79 ± 0.63 mmol·g-DCW^{-1}·h^{-1} at the methane concentration of 14, 69, 139, and 208 μM in the liquid, respectively. Although the specific methane oxidation rates increased with increasing initial methane concentration, the rate at 277 μM methane in the liquid decreased to 4.51 ± 0.66 mmol·g-DCW^{-1}·h^{-1}. The methane oxidation by M6 followed a Michaelis–Menten mechanism. Based on the Lineweaver–Burk plot (Fig. 2C), q_{max} and K_m were calculated as 4.93 mmol·g-DCW^{-1}·h^{-1} and 23 μM, respectively. At 208 μM methane in the liquid [15% (v/v) methane in the headspace], the specific methane oxidation rate was close to the q_{max}. It was expected that at the methane concentration of 277 μM [20% (v/v) methane in the headspace] the rate would not increase, but should be slightly decreased. Possible explanations are that the methane oxidation was inhibited by the high methane concentration; that is, M6 lost the capability to oxidize methane because of reversible or irreversible intermediate inhibition, and that physiological change inside the cell occurred, after which M6 lost the capability of methane oxidation [6].

As shown in Fig. 3, ethylbenzene and *m*-xylene also slightly inhibited the methane oxidation compared with the sample containing methane alone. However, *p*-xylene greatly inhibited the methane oxidation compared with ethylbenzene and *m*-xylene (Fig. 3A and 3C). This indicates that inhibition effects exist between methane as a growth substrate and the cometabolic substances. Scheutz *et al.* [19] and Lee *et al.* [15] reported a similar phenomenon; VOCs lowered the methane oxidation rate, which was possibly caused by uncompetitive inhibitor blocking the binding between the enzyme and substrates. In addition, as shown in Fig. 3B, M6 was able to degrade methane with the co-existence of sulfur compounds. An inhibitory effect of sulfur compounds was observed on the oxidation of methane (Fig. 3B and 3C), and a similar phenomenon was observed in another study [17]. In contrast to aromatic hydrocarbons, the inhibition by sulfur compounds is known to be competitive inhibition. Borjesson [3] reported that the inhibition of methane oxidation by sulfur compounds such as MT and carbon disulfide followed a competitive inhibition model. Genes encoding particulate MMO and ammonia monooxygenase (AMO) share high sequence identity, with both exhibiting very similar characteristics

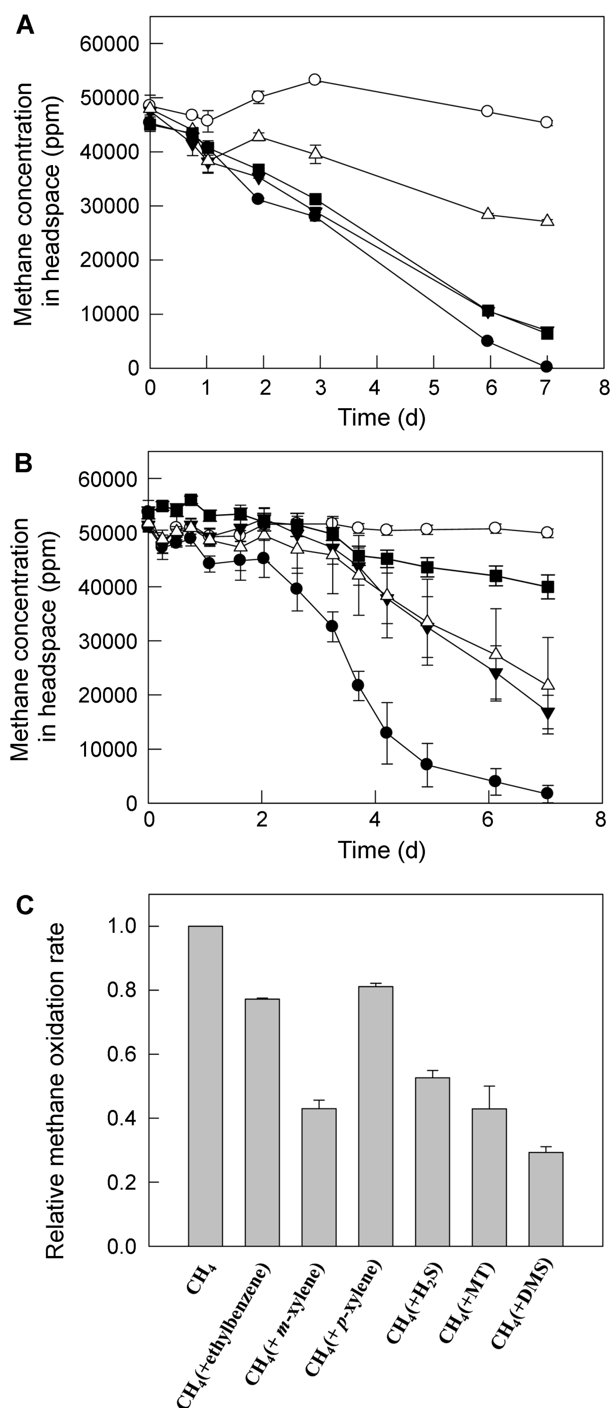

Fig. 3. Effects of aromatic and sulfur compounds on methane oxidation.
A. Utilization of ethylbenzene, *m*-xylene, *p*-xylene, and methane by strain M6; ●, methane only; ○, control; ▼, ethylbenzene+methane; △, *m*-xylene+methane; ■, *p*-xylene+ methane. **B**. Methane oxidation by strain M6 in the presence of sulfur compounds. ●, methane only; ○, control; ▼, H_2S+ methane; △, MT+methane; ■, DMS+methane. **C**. Relative methane oxidation rate.

[10]. Juliette *et al.* [12] showed that inhibition of AMO in cell suspensions of *Nitrosomonas europeae* was caused

by DMS, which was related to competitive inhibition. Consequently, the results in this study showed the effects of organic compounds produced from landfill sites on methane oxidation and the kinetics of methane removal by M6. It is helpful to efficiently design landfill cover soil and control the methane emissions.

Acknowledgments

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