Journal of Bioscience and Bioengineering
VOL. 120 No. 6, 670–676, 2015

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Inhibitory effects of sulfur compounds on methane oxidation by a methane-oxidizing consortium

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Received 26 December 2014; accepted 8 April 2015
Available online 2 July 2015

Kinetic and enzymatic inhibition experiments were performed to investigate the effects of methanethiol (MT) and hydrogen sulfide (H_2S) on methane oxidation by a methane-oxidizing consortium. In the coexistence of MT and H_2S, the oxidation of methane was delayed until MT and H_2S were completely degraded. MT and H_2S could be degraded, both with and without methane. The kinetic analysis revealed that the methane-oxidizing consortium showed a maximum methane oxidation rate (V_{max}) of 3.7 mmol g-dry cell weight (DCW)$^{-1}$ h^{-1} and a saturation constant (K_m) of 184.1 μM. MT and H_2S show competitive inhibition on methane oxidation, with inhibition values (K_i) of 1504.8 and 359.8 μM, respectively. MT was primary removed by particulate methane monooxygenases (pMMO) of the consortium, while H_2S was degraded by the other microorganisms or enzymes in the consortium. DNA and mRNA transcript levels of the *pmoA* gene expressions were decreased to ~10^6 and 10^3 *pmoA* gene copy number g-DCW^{-1} after MT and H_2S degradation, respectively; however, both the amount of the DNA and mRNA transcript recovered their initial levels of ~10^7 and 10^5 *pmoA* gene copy number g-DCW^{-1} after methane oxidation, respectively. The gene expression results indicate that the *pmoA* gene could be rapidly reproducible after methane oxidation. This study provides comprehensive information of kinetic interactions between methane and sulfur compounds.

[**Key words:** Methane; Particulate methane monooxygenases; Methanethiol; Hydrogen sulfide; Interaction]

Methane (CH_4) is one of the most important global warming gases, with a global warming potential that is 20 times greater than that of carbon dioxide (CO_2) (1). Methane is primarily generated by anaerobic degradation *via* methanogenesis and is produced in large amounts in landfills. Landfills have historically been the largest source of global methane emissions from the waste sector (1).

Volatile sulfur compounds are emitted simultaneously with methane by the anaerobic digestion of sulfur-containing organic compounds (2,3). Methanethiol (CH_3SH, MT) and hydrogen sulfide (H_2S) are predominant landfill gases, strongly contributing to its malodor (2–4). For instance, Gendebien et al. reported a concentration of MT up to 87 mg m^{-3} at British landfills (5). The United States Department of Energy summarized that H_2S comprises <1% of the total landfill gases while methane is 40–60% (6). Thus, the simultaneous emissions of methane and volatile sulfur compounds induces kinetics and interactions in landfills.

Biological methane sink is recognized as being globally important, accounting for approximately 30 ± 15 Tg y^{-1} of global methane consumption (7). In particular, methane-oxidizing bacteria, or methanotrophs, play an important role in atmospheric methane mitigation from soils in wetlands, forests, and landfills. Methane monooxygenases (MMO) present in methanotrophs are involved in methane oxidation, showing relatively wide substrate specificity. This leads that methanotrophs can cometabolize non-CH_4 compounds such as volatile sulfur compounds as well as methane, resulting in the fortuitous metabolism of a very large number of compounds (8).

The MMO exists in two distinct forms, a cytoplasmic or soluble form (sMMO) and a membrane-bound or particulate form (pMMO). The pMMO is present in all methanotrophs, except for the genus *Methylocella* and *Methyloferula*, while only some methanotrophs are able to express sMMO (9–12). On the basis of previous studies, pMMO is more suitable for *in situ* bioremediation, since only a few methanotrophs can produce sMMO. In addition, sMMO expression and activity are repressed at Cu-to-biomass ratios of >5.64 μmol Cu g-protein^{-1} in the environment, as in landfills, forests, and wetlands (13–15).

To date, most of the previous researches have focused on the cometabolic kinetics of volatile sulfur compounds by sMMO or by pure methanotrophic cultures (16–18). However, these studies have been unable to provide sufficient information of the kinetic interactions, since sMMO or pure cultures could not represent methanotrophic activity or community in environmental samples. In addition, earlier studies have only focused on the kinetic values, such as the maximum oxidation rates, saturation constants, or inhibition constants. This has led to a lack of information and limitations on our understanding of the underlying kinetic mechanisms. Accordingly, enzymatic and genetic approaches are required for a better understanding as well as kinetic analysis.

In the present study, kinetic, enzymatic, and genetic approaches were employed to investigate the effects of MT and H_2S on methane oxidation using a methane-oxidizing consortium. In order to

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.04.006

examine the effects of the sulfur compounds, the methane-oxidizing consortium was obtained from landfill soil. The consortium was cultivated to express pMMO exclusively. Batch-scale experiments were employed to analyze the kinetic interactions between the sulfur compounds and the methane. Enzymatic assays were performed to evaluate the effects of MT and H_2S on methane oxidation using the MMO of the methane-oxidizing consortium. The DNA level of *pmoA* gene (encoding the α subunit of pMMO) was measured using quantitative real-time PCR (q-PCR) and mRNA transcription level was analyzed using reverse transcriptase q-PCR The effects of MT and H_2S on methane oxidation were comprehensively discussed at the kinetic, enzymatic, and genetic levels.

MATERIALS AND METHODS

Enrichment of a methane-oxidizing consortium In order to obtain a methane-oxidizing consortium, a landfill soil was sampled at Gongju-si, Chungcheongnam-do, South Korea. Two grams (wet weight) of the soil was added into a 600-ml serum bottle containing 18 ml of a nitrate mineral salt (NMS) medium with 0.03 mM of $CuSO_4$. The bottle was sealed with a butyl rubber stopper and an aluminum cap, and methane gas was injected into the bottle at 5% (v/v) of the final concentration from a methane gas cylinder (99%, Seoul Special Gases, Seoul, Korea). The serum bottle was incubated at 30°C with an agitation of 200 rpm. The methane concentration was periodically monitored in the headspace, and the culture was transferred into the fresh NMS medium after the complete oxidation of methane. This process was repeated twenty times. The NMS medium consists of $MgSO_4 \cdot 7H_2O$ (1 g), $CaCl_2 \cdot 2H_2O$ (0.295 g), KNO_3 (1 g), KH_2PO_4 (0.26 g), and $Na_2HPO_4 \cdot 2H_2O$ (0.41 g per 1-L); plus trace elements of $FeSO_4 \cdot 7H_2O$ (500 μg), $ZnSO_4 \cdot 7H_2O$ (400 μg), $MnCl_2 \cdot 4H_2O$ (20 μg), H_3BO_4 (15 μg), $CoCl_2 \cdot 6H_2O$ (50 μg), $NiCl_2 \cdot 6H_2O$ (10 μg), and $Na_2MoO_4 \cdot 2H_2O$ (250 μg) (19). The sampling site and physico-chemical properties of the soil were described in a previous report (20).

The gram-dry cell weight (g-DCW) was measured to determine the cell concentration of the consortium. One ml of the culture was collected into the 1.5 ml tubes with a centrifugation at 13,000 rpm for 5 min, and dried at 80°C overnight. The g-DCW was determined from the residue in the tubes. All of the experiments were performed in triplicate.

A bacterial community analysis of a methane-oxidizing consortium One milliliter of the culture was collected from the methane-oxidizing consortium. Genomic DNA was extracted in duplicate using a NucleoSpin Soil Kit (Macherey–Nagel GmbH & Co. KG, Düren, Germany) with a modification as previously described (21). DNA was eluted in 100 μL of the elution buffer and stored at −20°C prior to use. DNA concentration was measured using a spectrophotometer (ASP-2680, ACTGene Inc., Piscataway, NJ, USA).

In order to analyze the bacterial community of the methane-oxidizing consortium, the pyrosequencing assay was performed in duplicate. For PCR, a primer set of 340F and 805R was used in order to amplify the 16S rRNA gene. Six different composite primer sets were made, based on the primer set of 340F and 805R, for multiplex pyrosequencing (21). This procedure has been previously described in detail by Kim et al. (21). The purified DNA concentrations were quantified using a spectrophotometer. Equal amounts of the purified DNAs were combined in a single tube and run on a Genome Sequencer 454 FLX Titanium system (Roche Diagnostics Inc., Mannheim, Germany). For high-quality sequences, the primer sites of the sequences were trimmed and the low-quality and chimera sequences were removed. The primer sites and low-quality sequences (length $<$ 400 nt, average quality score $<$ 25, and with an ambiguity) were excluded using the ribosomal database project (RDP) pyrosequencing pipeline (22). Any possible chimeras were removed using Black Box Chimera Check software with the default settings (23). The RDP pyrosequencing pipeline was employed in order to analyze the pyrosequencing data, as previously described by Kim et al. (21). The sequences were identified at the species level, when the similarity was greater than 99%. The pyrosequencing reads obtained in this study were deposited into the DNA DataBank of Japan (DDBJ) Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra) under the accession no. DRA002267.

Kinetic analysis for sulfur compounds effects on methane oxidation Kinetic analysis was performed to investigate the effects of sulfur compounds on methane oxidation by the methane-oxidizing consortium. Twenty milliliters of the enriched methane-oxidizing consortium was added into 600-ml serum bottles, and the bottles were sealed with butyl rubber stoppers and aluminum caps. Control tests were also performed without inoculation of the consortium. Methane, MT (99%, Sigma–Aldrich, St. Louis, MO, USA), and H_2S (99%, Seoul special gas, Seoul, Korea) gases were added to the bottles, for final concentrations of 27.2–204, 163.5–654, and 98.1–392.4 μmol L^{-1}, respectively. The liquid concentrations of methane, MT, and H_2S were calculated using dimensionless Henry's law constants of 29, 0.123, and 0.41, respectively (24,25). The serum bottles were incubated at 30°C with 200 rpm of an agitation. The methane, MT, and H_2S concentrations were periodically measured in the headspace using a gas-tight syringe.

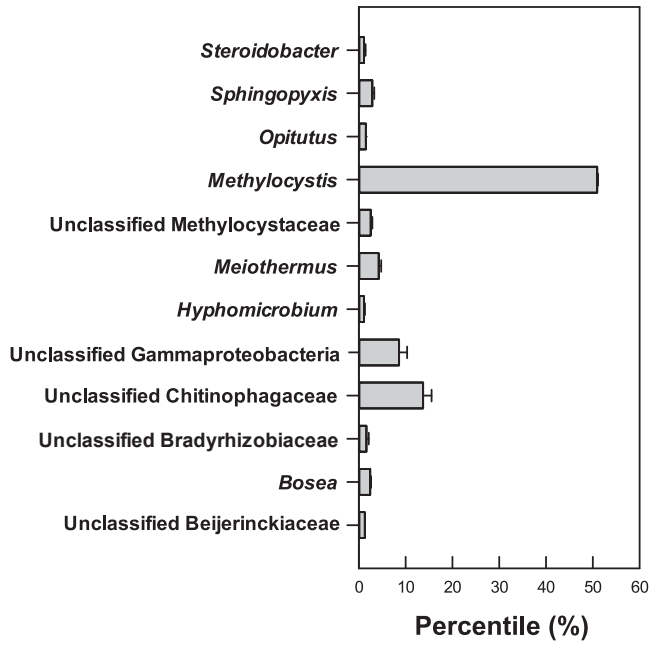

FIG. 1. A bacterial community structure of a methane-oxidizing consortium. The bacterial genera with assigned read numbers of ≤1% of the sequencing effort were excluded.

The methane, MT, and H_2S degradation rates were calculated from the slopes of concentration plotted versus time. The maximum methane oxidation rate (V_{max}) and saturation constant (K_m) were determined from the Lineweaver–Burk equation below (26):

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where V represents the methane oxidation rate and $[S]$ represents the methane concentration.

The inhibition constants (K_i) of MT and H_2S on methane oxidation were calculated using the equation for competitive inhibition, given by Williams et al. (27):

$$\frac{1}{V} = \frac{K_m}{V_{max}}\left(1 + \frac{[i]}{K_i}\right) \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

where $[i]$ represents the MT or H_2S concentration. All of the experiments were performed in triplicate.

Inhibitory effects of methanethiol and hydrogen sulfide on methane oxidation by MMO pMMO was exclusively induced for the methane oxidation of the consortium by cultivating the culture in the presence of Cu. The bottles were prepared, as described above, with the addition of allylthiourea (Sigma–Aldrich). Allylthiourea has been widely used as an inhibitor for pMMO (28,29). Allylthiourea was supplemented into the bottles at 50 mM of the final concentration. Methane, MT, and H_2S gases were added to the bottles, for final concentrations of 68, 327, and 196.2 μmol L^{-1} of liquid, respectively. The serum bottles were incubated at 30°C with 200 rpm of an agitation. The concentrations of methane, MT, and H_2S in the headspace were monitored every 4 or 5 h using a gas-tight syringe. All of the experiments were performed in triplicate.

Quantitative analysis of the *pmoA* gene expression One milliliter of each culture was collected from the serum bottles of the kinetic experiments earlier at 48, 12, and 5 experimental hours, which marked the end of the degradation periods for CH_4, MT, and H_2S, respectively (Figs. 2 and 3). The samples were immediately frozen at −70°C prior to analysis. Genomic DNA was extracted from the collected samples in duplicate using a NucleoSpin Soil Kit (Macherey–Nagel GmbH & Co. KG) as described above. The DNA was eluted in 100 μL of the elution buffer and stored at −20°C.

RNA was extracted from the collected samples in duplicate. For RNA extraction, 1 ml of the culture was added to 2-mL microcentrifuge tubes containing 0.1-mm glass beads and 1-mm zirconia/silica beads (0.5 g each). This procedure was described in detail by Kim et al. (21). RNA pellets were suspended in 50 μL of 0.1% DEPC-treated water. RNA was immediately purified using a Qiagen RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) with a RNase-Free DNase set (Qiagen Inc.), as specified by the manufacturer. RNA extracts were quantified using a