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Isolation and characterization of a facultative methanotroph degrading malodor-causing volatile sulfur compounds

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HIGHLIGHTS

- *Sphingopyxis* sp. MD2 was isolated from a microbial consortium degrading CH_4 and DMS.
- MD2 is phylogenetically distinct from known methanotrophic genera.
- MD2 can maintain methanotrophic activity after facultative growth.
- MD2 possessed a particulate methane monooxygense system.
- MD2 exhibited the strong ability to degrade sulfur compounds such as H_2S, methanethiol and DMS.

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ABSTRACT

Simultaneous removal of methane and malodor-causing volatile sulfur compounds (MVSCs), both emitted from landfills, is a desirable characteristic for methane-mitigation approaches. A methanotrophic bacterium was isolated from a microbial consortium, enriched with methane and dimethyl sulfide (DMS). It grew in the complex nutrient medium R2A without methane, and stably exhibited methanotrophic activity after facultative growth. It was identified as *Sphingopyxis* sp. MD2 by comparison of the 16S rRNA gene. It belongs to *Sphingomonadales*, whose members have not shown methanotrophic activity, phylogenetically distinct from orders of known methanotrophs. The MD2 biomass increased at a growth rate of 1.18 d^{-1} when methane was used as the sole growth substrate. An inhibition test with allylthiourea and PCR/sequencing confirmed the presence of particulate methane monooxygenase in MD2. DMS decreased the methane oxidation rate (2634 ± 146 μmole g DCW^{-1} h^{-1}) by 12%, while H_2S had no effect on the methane oxidation rate. Interestingly, methanethiol (MT) enhanced the methane oxidation rate by more than 50%. MD2 degraded H_2S and MT, regardless of the presence of methane. MD2 also degraded DMS in the presence of methane, indicating co-metabolism. These combined results indicate that MD2 may be a promising biological resource for simultaneous removal of methane and MVSCs.

1. Introduction

Methanotrophs (restricted to aerobic methane-oxidizing bacteria in this study) are a valuable biological resource for the removal of methane and pollutants and biotransformation of organic substrates [1]. Methanotrophs are defined as bacteria capable of utilizing methane as a sole carbon and energy source. They are present in a wide variety of environments and play a key role in global carbon circulation [1–3]. Methanotrophs transform methane to methanol–aldehyde–formate–carbon dioxide. There are two forms of methane monooxygenase (MMO) that convert methane to methanol: soluble and particulate forms. In addition to methane, the low specificities of MMOs to substrates allow MMOs to metabolize a wide variety of hydrocarbons and sulfur compounds such as trichloroethylene and dimethyl sulfide [2,4]. Furthermore, methanotrophs produce commercially valuable compounds such as poly-β-hydroxybutyrate and astaxanthin [1].

A total of 18 methanotrophic genera have been identified to date, and are distributed in the phylums *Proteobacteria* and *Verrucomicrobia* [2,3,5]. However, *Proteobacteria* includes almost all of the known methanotrophs, which belong to the orders *Rhizobiales* (the class *Alpha-proteobacteria*) and *Methylococcales* (the class *Gamma-proteobacteria*), named '*Methylo-*'. In general, methanotrophs are classified into types I and II groups based on their physiological characteristics and phylogenetic affiliation [2]. Most methanotrophs have been known to be obligately methanotrophic. Recently, it has been reported that there are facultative methanotrophs, such as *Methylocella*, *Methylocapsa* and *Methylocystis* (belonging to *Rhizobiales*), that can utilize carbon compounds

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greater than C_1 as sole growth substrates [6]. Facultative growth of methanotrophs is not only scientifically intriguing but may also offer many advantages, including easy cultivation and management. In addition, facultative methanotrophs utilize a broader range of growth substrates than obligate methanotrophs, and in turn, may exhibit greater competitive ability in environmental biotechnological systems to mitigate methane emission, i.e. biofilters and biocovers.

The significant greenhouse gas methane (CH_4) and malodor-causing volatile sulfur compounds (MVSCs) are simultaneously produced from landfills during anaerobic digestion. MVSCs from landfills include hydrogen sulfide (H_2S), dimethylsulfide (DMS, CH_3SCH_3), and methanethiol (MT, CH_3SH) [7,8]. MVSCs have been shown to adversely affect the growth and activity of methanotrophs [9,10]. Thus, simultaneous removal of methane and MVSCs is a desirable characteristic of a biological resource to mitigate methane emission in biotechnological systems. The main objectives of this study were to obtain a facultative methanotroph degrading MVSCs and to characterize the identified facultative methanotroph as a potential biological resource.

2. Experimental

2.1. Methane- and dimethyl sulfide-degrading bacterial consortia

Soil samples were sampled from domestic waste sanitary landfills (located in Gong-ju city, Chungchungnam-do and at Gapyeong-gun, Gyeonggi-do), where landfill gases were continuously emitted, wetland (Gum-river, Chungchungnam-do) and rice paddy (Paju city, Gyeonggi-do) in South Korea. Soil samples were collected at a depth of 10 cm from the surface. Soil samples were sieved through a 2-mm mesh sieve (unsterilized), air-dried and stored at 4 °C for a month prior to use.

Two g of each soil were added to 600-mL serum bottles containing 12 mL of nitrate mineral salt (NMS) medium. NMS medium contains $MgSO_4{\cdot}7H_2O$ 1 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.295 g L^{-1}, KNO_3 1 g L^{-1}, KH_2PO_4 0.26 g L^{-1} and $Na_2HPO_4{\cdot}2H_2O$ 0.41 g L^{-1} [11]. $CuSO_4$ was added to a final concentration of 30 μM. The bottles were sealed with butyl-rubbers and incubated for a day at 30 °C with agitation at 200 rpm. After maintaining the samples under a static condition for 2 h, the supernatants (2 mL) were transferred to new 600-mL serum bottles containing 18 mL of NMS. Each bottle was sealed with a butyl rubber septum and aluminum cap. Methane (99%, Dong-A gas, Seoul, Korea) was added to the bottles to be a final concentration of $50{,}000 \times 10^{-6}$ mol mol^{-1} in headspace (equivalent to 1194.7 μmol $bottle^{-1}$ at 30 °C) using a 50-mL syringe (Korea Vaccine, Seoul, Korea). Dimethyl sulfide solution (99%, Acros Organics, Geel, Belgium) was injected into bottles to be a final concentration of 1200×10^{-6} mol mol^{-1} in headspace using a 10-μL syringe (Hamilton, Nevada, USA). The serum bottles were incubated at 30 °C with agitation (200 rpm). Gases were periodically sampled from the bottles using a 1-mL gas-tight syringe (Hamilton) and analyzed by gas chromatography to measure methane and DMS concentrations. When they were undetected, the serum bottles were aerated for 30 min on a clean bench and then methane and DMS were injected into the bottle as described above. They were incubated under the same conditions. To prevent exhaustion of nitrogen and phosphorous sources in bottles, 1 mL of sterilized concentrated N and P solutions were separately added to the bottles every second injection. The N and P solutions contained KNO_3 20 g L^{-1}, and KH_2PO_4 5.2 g L^{-1} and $Na_2HPO_4{\cdot}12H_2O$ 16.5 g L^{-1}, respectively.

2.2. Isolation of facultative methane/DMS-degrading microorganisms

Two microbial consortia with the ability to degrade both methane and DMS were obtained. The consortia were serially diluted using sterile 0.9% NaCl solution and spread on NMS agar plates (15 g L^{-1}). The plates were incubated in closed aerobic jars (5 L) containing 250 mL of methane (99%) and 16.7 μL of DMS at 30 °C. This procedure was repeated until enough candidates were obtained. The collected colonies were categorized into 7 groups based on their morphological characteristics. They were inoculated on Difco™ R2A agar (BD Diagnostics, Saprks, USA) plates and incubated at 30 °C without methane. R2A medium has been commonly used to determine contamination of heterotrophs in pure methanotroph cultures or confirm facultative growth of methanotrophs since obligate methanotrophs cannot grow in R2A. Pure colonies were obtained by subsequent transfers to new R2A agar plates more than three times. R2A agar medium contains yeast extract 0.5 g L^{-1}, proteose peptone No. 3 0.5 g L^{-1}, casamino acids 0.5 g L^{-1}, dextrose 0.5 g L^{-1}, soluble starch 0.5 g L^{-1}, sodium pyruvate 0.3 g L^{-1}, K_2HPO_4 0.3 g L^{-1}, $MgSO_4$ 0.05 g L^{-1} and agar 15 g L^{-1}.

Pure colonies from R2A plates were inoculated into 120-mL serum bottles containing 4 mL of NMS medium. Each bottle was sealed with a butyl rubber septum and aluminum cap. Methane (99%) was injected into the bottles to be a final concentration of $50{,}000 \times 10^{-6}$ mol mol^{-1} in headspace using a sterile 10-mL syringe (Korea Vaccine). DMS (Acros Organics) was injected into the bottles to be a final concentration of 1200×10^{-6} mol mol^{-1} in headspace using a 10-μL syringe (Hamilton, Nevada, USA). The serum bottles were incubated at 30 °C with agitation (200 rpm). Gases were periodically sampled from the bottles using a 1-mL disposable syringe (Korea Vaccine) and analyzed by GC to measure methane and DMS concentrations. Three isolates exhibited methane/DMS-oxidizing activity. They were streaked on R2A agar plates to confirm their purity. The isolates were inoculated into 120-mL serum bottles containing 4 mL of NMS medium again and incubated with methane only at 30 °C. A strain exhibiting methane-oxidizing activity was selected and designated MD2. MD2 has been deposited to the center of Korean Collection for Type Cultures (http://kctc.kribb.re.kr) under the collection number KCTC 11845BP.

To identify MD2, its genomic DNA was extracted from a pure colony using G-spin Genomic DNA Extraction Kit for Bacteria (iNtRON Biotechnology, Sungnam, Korea) as described in the manufacturer's instructions. The partial sequence of the 16S rRNA gene was amplified using the primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGAC-3′) [12]. A 50-μL PCR mixture contained 5 μL of 10× PCR buffer (Genenmed, Seoul, Korea), 2 μL of each primer (10 μM), 1.25 U of Ace *Taq* polymerase (Genenmed), 4 μL of 2.5 mM dNTPs and 1 μL of template DNA. The reaction was performed in a 2700 GeneAmp® PCR system (Applied Biosystems, Foster, USA). An initial denaturation step at 95 °C for 5 min and 30 cycles of denaturation at 96 °C for 60 s, primer annealing at 58 °C for 30 s and elongation at 72 °C for 105 s were performed, which was followed by a final elongation step at 72 °C for 10 min. The PCR product was subjected to electrophoresis on 1.5% agarose gel. The agarose gel containing the PCR product was excised using a scalpel and then purified using the HiYield™ Gel/PCR DNA Mini Kit (Real Biotech, Taipei, Taiwan), as recommended by the manufacturer. The PCR product was commercially sequenced. The sequence was compared with known DNA sequences using Basic Local Alignment Search Tool (BLAST) analysis (http://blast.ncbi.nlm.nih.gov). MD2 was identified as a *Sphingopyxis* sp. The sequence was deposited into the GenBank (http://www.ncbi.nlm.nih.nov) database under the accession number EF424391. Phylogenetic comparison