



Rapid isolation of fast-growing methanotrophs from environmental samples using continuous cultivation with gradually increased dilution rates

Jisun Kim¹ · DaeHyun D. Kim¹ · Sukhwan Yoon¹

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Abstract

Methanotrophs have recently gained interest as biocatalysts for mitigation of greenhouse gas emission and conversion of methane to value-added products; however, their slow growth has, at least partially, hindered their industrial application. A rapid isolation technique that specifically screens for the fastest-growing methanotrophs was developed using continuous cultivation with gradually increased dilution rates. Environmental samples collected from methane-rich environments were enriched in continuously stirred tank reactors with unrestricted supply of methane and air. The reactor was started at the dilution rate of 0.1 h^{-1} , and the dilution rates were increased with an increment of 0.05 h^{-1} until the reactor was completely washed out. The shifts in the overall microbial population and methanotrophic community at each step of the isolation procedure were monitored with 16S rRNA amplicon sequencing. The predominant methanotrophic groups recovered after reactor operations were affiliated to the gammaproteobacterial genera *Methylomonas* and *Methylosarcina*. The methanotrophic strains isolated from the reactor samples collected at their respective highest dilution rates exhibited specific growth rates up to 0.40 h^{-1} ; the highest value reported for methanotrophs. The novel isolation method developed in this study significantly shortened the time and efforts needed for isolation of methanotrophs from environmental samples and was capable of screening for the methanotrophs with the fastest growth rates.

Keywords Methane · Methanotrophs · Continuous cultivation · Growth rate · Isolation

Introduction

Methane (CH_4) is a potent greenhouse gas with ~ 28 times higher global warming potential (GWP) than carbon dioxide (CO_2) if released to the atmosphere (Pachauri et al. 2015), while highly concentrated CH_4 collected as biogas and natural gas is a valuable resource utilizable as energy source or feedstock for chemical industry (Conrado and Gonzalez 2014; Fei

et al. 2014; Olah et al. 2009). The recent resurgence in natural gas production due to the global shale gas boom has rekindled worldwide interest in the biological and chemical processes that would maximize value creation from CH_4 (Conrado and Gonzalez 2014). Chemical or biological syntheses of value-added products from methane inevitably involve partial oxidation of methane to methanol as its first step (Olah et al. 2009). Aerobic methanotrophs, the unique groups of bacteria utilizing CH_4 as their sole carbon and energy source, cope with this difficult task by expressing and utilizing two distinct forms of methane monooxygenases, i.e., membrane-bound particulate methane monooxygenases (pMMO) and/or cytoplasmic soluble methane monooxygenases (sMMO) (Semrau et al. 2010). Thus, methanotrophs have recently attracted significant scientific attention as potential biocatalysts for methane transformation (Haynes and Gonzalez 2014; Kalyuzhnaya et al. 2013; Strong et al. 2015; Strong et al. 2016).

The majority of aerobic methanotrophs belong to either of two phylogenetic groups, *Alpha*- or *Gammaproteobacteria*

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✉ Sukhwan Yoon
syoon80@kaist.ac.kr

¹ Department of Civil and Environmental Engineering, Korea Advanced Institute of Science and Technology, Daejeon 350-701, South Korea

(Semrau et al. 2010). The two groups of methanotrophs both utilize pMMO or sMMO for initial oxidation of CH₄ to methanol (CH₃OH), but have dissimilar carbon assimilation pathways. Gammaproteobacterial methanotrophs utilize ribulose monophosphate (RuMP) pathway, while alphaproteobacterial methanotrophs utilize the serine cycle for assimilation of C₁ compounds resulting from oxidation of CH₄. Utilization of more efficient RuMP pathway may explain higher growth rates, carbon conversion efficiencies, and methane utilization rates generally observed for the Gammaproteobacterial methanotrophs, which are often viewed to be more attractive for industrial applications (Hoefman et al. 2012; Kalyuzhnaya et al. 2013); however, alphaproteobacterial methanotrophs certainly have their own advantages, as their metabolic diversity would allow for production of broader range of chemical products (Crombie and Murrell 2014; Semrau et al. 2011; Strong et al. 2015). The number of available methanotrophic isolates is too limited and physiological studies have been too biased to a selected group of laboratory strains. Thus, expansion of the methanotroph inventory is warranted to enhance understanding of their physiological diversity and stretch their industrial potential.

One of the bottlenecks in biotechnological applications of methanotrophs is their slow generation time. Dated literatures report specific growth rates as high as ~0.4 h⁻¹ for moderately thermophilic *Methylococcus* genus; however, such high specific growth rate was not reproduced in recent studies and the highest specific growth rate reported since was 0.33 h⁻¹ of *Methylomarinum vadi* and *Methylomarinovum caldicuralii* isolated from marine environments (Hirayama et al. 2014; Hirayama et al. 2013). As genetic manipulations are necessary for redirection of metabolic pathways towards production of value-added chemicals, the slow growth is a definite drawback in developing methanotrophs as microbial refineries (Yan et al. 2016). The failure in finding methanotrophs with faster growth may be due to the reliance of the isolation technique on the half-a-century-old traditional method—batch enrichment of environmental samples with CH₄ and O₂ followed by repetitions of streaking and single-colony-picking on agar plates or dilution-to-extinction (Hoefman et al. 2012; Whittenbury et al. 1970; Wise et al. 1999). These traditional isolation procedures are rigorous and time-taking and lack the capability to specifically select for methanotrophs with the highest growth rates if the fastest-growing methanotrophs exist as unseen minority and/or have a long lag period.

Continuous cultivation has been traditionally used for selective enrichment or isolation of microorganisms. As chemostats can provide natural growth conditions that cannot be simulated with batch cultivation (e.g., consistent supply of the limiting substrate), chemostats have been employed to select for organisms that thrive under substrate limitations (Hanke et al. 2014; van den Berg et al. 2015). Chemostats were also used to enrich organisms that utilize cytotoxic

compounds as their growth substrates or generate suicidal compounds, as the concentration of the toxic but necessary substrates can be maintained below the toxicity threshold concentrations (Spain and Nishino 1987). A chemostat reactor would be an ideal tool also for screening of the fastest-growing organism in an environmental sample, as the fundamental property of a chemostat (the specific growth of a steady-state chemostat culture equals the dilution rate) implies that the organisms with specific growth rates lower than the dilution rate of the chemostat would eventually be washed out; however, such utilization of a chemostat has yet to be explored. In this study, a novel isolation method for specific selection of the fastest-growing methanotrophs in an environmental sample was proposed applying the chemostat principle. A chemostat reactor supplied with a continuous stream of gas mixture (80% air and 20% CH₄) was operated with gradually increased aqueous phase dilution rates until complete washout was observed. The shift in methanotrophic population through each enrichment process was monitored with 16S rRNA gene amplicon sequencing using MiSeq sequencing technology. The cell suspension sample extracted from the chemostat at the highest dilution rate (before washout) was subjected to the subsequent isolation procedure, which was completed within 2 weeks. The entire isolation procedure was completed within a month. The isolated strains, both *Gammaproteobacteria*, exhibited the fastest specific growth rates ever reported for mesophilic methanotrophs with specific growth rates measured up to 0.4 h⁻¹ at 30 °C.

Materials and methods

Sampling sites description

The stream sediment (SS) sample used in this study was collected from the north bank of Gapcheon stream and the anaerobic digester effluent (AD) from Daejeon municipal wastewater treatment plant (Daejeon, Korea; 36° 21' 37.1" N 127° 21' 15.1" E and 36° 23' 03.1" N 127° 24' 34.7" E, respectively). As the coexistence of methane and oxygen is essential for methanotrophic metabolism and growth, the SS sample was collected from the sediment ~20 cm below the ground surface, and the AD sample was collected from the water surface to target the oxic-anoxic interface. Plant biomass was carefully removed from the SS sample. The samples were stored in sealed sterile Mason jars at 4 °C before use. The pH of both SS and AD samples were circumneutral, with the values ranging from 6.8 to 7.2.

Growth conditions for methanotrophs

A typical nitrate mineral salts (NMS) medium pre-defined for cultivation of methanotrophs was used for all experiments

performed in this study (Whittenbury et al. 1970). The NMS medium contained, per liter, 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (4.06 mM), 1 g KNO_3 (9.89 mM), 0.2 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.36 mM), 0.1 mL of 3.8% (w/v) Fe-EDTA solution, 0.5 mL of 0.1% (w/v) $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ solution, and 1 mL of the trace element solution. To avoid precipitation, a phosphate buffer stock solution adjusted to pH 6.8 was prepared separately and added to the medium to the final concentration of 5 mM after autoclaving. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ was added from a stock solution to the final concentration of 10 μM . The $\times 200$ vitamin stock solution was prepared aseptically as previously defined, and 5.0 mL of the stock solution was added per liter of culture medium (Whittenbury et al. 1970). All culture bottles were incubated at 30 °C and CH_4 was provided as the sole electron donor and carbon source in all experiments performed in this study.

Batch enrichment

Duplicate batch enrichment cultures were prepared by adding 10 g (wet weight) slurries of the SS sample or 1-mL aliquots of the AD sample into 50 mL of NMS medium dispensed in 160-mL serum bottles (Wheaton, Millville, NJ). The serum bottles were sealed with butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK) and aluminum crimp seals. After sealing, 20% of the headspace (22 of 110 mL headspace) was replaced with 99.999% CH_4 (Deokyang Co., Ulsan, Korea) using a sterile syringe connected to a 0.2- μm syringe filter (Advantec INC, Tokyo, Japan). Samples were placed horizontally in a shaking thermostat incubator and incubated in dark with shaking at 140 rpm. The mixing ratio of CH_4 in the headspace was monitored until it dropped to 10% (v/v). One milliliter of each enrichment culture was transferred to a fresh NMS medium and incubated identically to the initial enrichment. After CH_4 consumption was verified, 5 mL of the enrichment culture was extracted from one of the duplicate bottles and used as the inoculum for the chemostat reactor.

Selection of fast-growing methanotrophs using a chemostat reactor

A continuously stirred tank reactor (CSTR) was constructed with a 500-mL Duran® pyrex glass bottle (DWK Life Sciences GmbH, Wertheim am Main, Germany) sealed with a GLS 80® cap and a Masterflex® dual-channel peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL) (Fig. 1). The volume of NMS medium in the CSTR was maintained at 250 mL, as the rate of inflow of fresh NMS medium was the same as the rate of outflow of waste medium from the reactor. The mixed gas (~20% CH_4 and ~80% air) supplied to the CSTR was prepared by passing separate streams of compressed air (Deokyang Co., Ulsan, Korea) and 99.999% CH_4 through an empty 500-mL pyrex bottle. The flowrates of the

compressed air and CH_4 were maintained at 0.8 and 0.2 mL min^{-1} , respectively. The mixed gas exiting the pyrex bottle was diffused into the liquid phase of CSTR through a ball-shaped gas diffuser (Thermo Fisher Scientific, Waltham, MA). Neither CH_4 nor O_2 was likely the limiting substrate for growth of methanotrophs in the chemostat reactor under any examined culturing condition, as the CH_4 mixing ratio of the gas effluent was > 15% at all times. The CSTR was submerged in a water bath maintained at 30 °C.

After inoculation, the chemostat was operated in a fed-batch mode with the peristaltic pump turned off and the gas stream continuously feeding CH_4 and O_2 into the reactor. The increase in cell density was monitored by measuring OD_{600} values with 1-mL samples extracted from the CSTR. After growth halted, the peristaltic pump was turned on and the reactor was operated as a chemostat with the dilution rate initially set to 0.1 h^{-1} . The dilution rate was gradually increased with an increment of 0.05 h^{-1} after passing through five reactor volumes of fresh NMS medium at each dilution rate. The maximum dilution rates at which steady states were established were 0.3 h^{-1} for the SS sample and 0.45 h^{-1} for the AD sample, as higher dilution rates resulted in washout. At each dilution rate, 15 mL of the reactor effluent was collected and stored at 4 °C for isolation of methanotrophs and another 15-mL aliquot was stored at -20 °C for analysis of the microbial community.

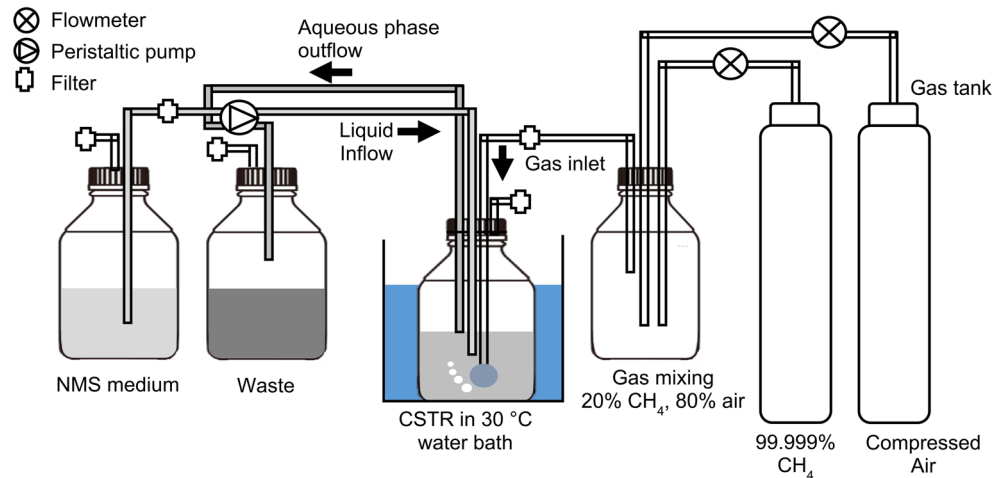
Analytical methods

The methane concentration in the headspace was measured with a gas chromatograph equipped with GC-Alumina column (30 m length \times 0.53 mm inner diameter, 1.5- μm film thickness) and a flame ionization detector (YL instrument, Gyeonggi, Korea). Injector, oven, and detector temperatures were set to 250, 100, and 275 °C, respectively. Helium was used as the carrier gas and a gas split ratio of 50:1 was used. For each sampling event, 150 μL of headspace was sampled using a 1700-series gas-tight syringe (Hamilton Company, Reno, NV) and 100 μL was manually injected into the gas chromatograph. The syringe was flushed three times with sterile pressurized N_2 gas before use to remove residual CH_4 in the syringe.

DNA extraction and microbial community analysis

Each enrichment process was monitored with 16S rRNA amplicon sequencing of the samples collected at different enrichment stages. The DNA extracted from the original environmental sample, the batch enrichment after initial incubation with CH_4 and O_2 , the reactor culture after fed-batch incubation, and the steady-state chemostat enrichment at different dilution rates were analyzed. DNA extraction was performed with 0.25 g (wet weight) of a sediment sample or 1.5 mL of an aqueous sample. Power Soil DNA Isolation Kit (MoBio Laboratories, Hilden, Germany) was used for DNA extraction, according to the

Fig. 1 A schematic diagram of the CSTR used for isolation of fast-growing methanotrophs



protocol provided by the manufacturer. The extracted DNA was stored at $-20\text{ }^{\circ}\text{C}$ before use. The hypervariable V6–V8 region of 16S rRNA gene was amplified with the 926F (5'-AAACTYAAAKGAATTGRCGG-3')/1392R (5'-ACGGGCGGTGTGTRC-3') primer pair (with barcodes attached to the 5' end of 926F) and the amplicons were sequenced by Macrogen (Seoul, Korea) using MiSeq sequencing platform (Illumina, Inc., San Diego, CA) (Matsuki et al. 2002). The raw sequence data supporting the conclusions of this article have been deposited in the NCBI SRA database, <https://www.ncbi.nlm.nih.gov/sra> (accession numbers: SRP115081 and SRP115083 for the SS and AD samples, respectively).

Non-16S rRNA sequences were screened from the raw sequence datasets using SortMeRNA v2.0 (Kopylova et al. 2012). The resulting datasets were processed using the QIIME pipeline v 1.9.1 with options set to default values (Caporaso et al. 2010). After demultiplexing of the raw reads, ambiguous reads or reads with quality scores lower than the default cutoff value (Q20) were filtered out and the chimeric reads were removed. The filtered reads were then clustered into operational taxonomic units (OTUs) with USEARCH algorithm against the Greengenes v13.8 database and de novo clustering (for sequences with no matching sequence in the database) using 97% as the cutoff value (Schloss et al. 2009). The OTUs were assigned to taxa using the RDP classifier against the Greengenes V13.8 database. The reads that remained unclassified after assignment were discarded.

Isolation and determination of the growth rates

The samples extracted from the CSTRs operated at the highest dilution rates were used as the starting points for isolation of the fastest-growing methanotrophs. One milliliter of the reactor effluent sample stored at $4\text{ }^{\circ}\text{C}$ was inoculated into fresh NMS medium prepared as described above. After the culture was grown to an OD_{600} value of ~ 0.4 with CH_4 as the sole carbon source, $100\text{ }\mu\text{L}$ of the enrichment culture was spread onto NMS

agar plates, which were then incubated in a BBL® GasPak® 150 anaerobic jar (Becton Dickinson, Franklin Lakes, NJ) filled with 80% air and 20% CH_4 . The jar was placed in a dark thermostat incubator set to $30\text{ }^{\circ}\text{C}$. Single colonies from the agar plates were transferred to fresh agar plates. The process was repeated three times and the single colonies from the fourth transfer culture were taken and suspended into fresh NMS medium and grown with CH_4 . For each isolate, 16S rRNA gene and *pmoA* gene were amplified with 27f/1492r and A189f/mb661r primer sets, respectively, and cloned using TOPO TA cloning kit (Invitrogen, San Diego, CA). Ten 16S rRNA clones and ten *pmoA* clones were sequenced for confirmation of purity and identification of each isolate.

The growth rates of the isolated methanotrophs and a reference strain of methanotroph (*Methylosinus trichosporium* strain OB3b) were determined by monitoring the OD_{600} values of the batch cultures incubated with CH_4 . Five milliliters of the preculture at exponential phase ($\text{OD}_{600} \sim 0.25$) grown with 80% air and 20% CH_4 in the headspace was inoculated to 45 mL of fresh NMS medium in a 160-mL serum bottle. The serum bottles were incubated at $30\text{ }^{\circ}\text{C}$ with shaking at 250 rpm. At each sampling time point, 1 mL of the culture was extracted to measure the OD_{600} value. The experiment was performed in triplicates.

Results

Characterization of the overall microbial communities and methanotrophic populations in the environmental samples

The indigenous methanotrophic population was identified in the environmental samples prior to enrichment, using MiSeq amplicon sequencing targeting the V6–V8 hypervariable region of 16S rRNA genes (Table S1). The samples collected from the Gapcheon stream sediment and the anaerobic

digester effluent varied greatly in the compositions of microbial communities and indigenous methanotrophic populations (Table 1, Fig. 2, Table S1). The most abundant OTUs in the SS and AD samples were assigned to the uncultured DA101 genus of *Chthoniobacteraceae* family and the uncultured T78 genus of *Anaerolinaceae* family, respectively. In both SS and AD samples, the bacterial OTUs assigned to genera previously identified as alpha- and gammaproteobacterial methanotrophs constituted minor populations. The OTU assigned to genus *Crenothrix* was the most abundant methanotrophic population identified in the SS sample before enrichment (0.246% of the total reads). Other methanotrophs identified in the SS sample included the OTUs assigned to the genera *Methylosinus* (0.072%), *Methylocaldum* (0.030%), *Methylomonas* (0.016%), *Methylomicrobium* (0.002%), and *Methylococcus* (0.002%) and unidentified genera (or unable to be unambiguously assigned to a specific genus) in the *Methylocystaceae* and *Methylococcaceae* families (0.025

and 0.011%, respectively). The most abundant methanotrophic OTU in the AD sample was assigned to the genus *Methylosinus* (0.210%). Other methanotrophs included the OTUs affiliated to the genera *Methylosarcina* (0.019%), *Methylocaldum* (0.012%), *Methylomonas* (0.002%), *Crenothrix* (0.001%), and *Methylocella* (0.001%) and an unidentified genus in the *Methylocystaceae* family (0.019%). The fractions of indigenous methanotrophic populations in the SS and AD samples were 0.42 and 0.27% of the total microbial populations, respectively.

Monitoring of the population shifts at different stages of enrichment

The shift in microbial community was monitored at different enrichment stages using MiSeq 16S rRNA amplicon sequencing (Table S1). Although methanotrophs were significantly enriched after the initial batch enrichment, the proportions of

Table 1 The 16S rRNA amplicon sequencing analyses of (a) SS and (b) AD enrichment samples

Samples ¹	ES	IB	FB	HD
(a) The 16S rRNA amplicon sequencing analyses of SS enrichment samples				
Total number of reads after quality trimming	123,050	155,257	91,239	76,778
Reads assigned to bacterial 16S rRNA genes	123,030	155,245	91,238	76,778
Reads assigned to Methanotrophic 16S rRNA genes	521	4060	17,050	50,255
Bacterial groups with highest relative abundances ²	<i>g_DA101</i> (6.3) <i>o_iii1-15</i> (3.4) <i>g_Rhodoplanes</i> (2.6) <i>f_Gaiellaceae</i> (2.4) <i>g_Bacteroides</i> (2.1)	<i>g_DA101</i> (6.6) <i>o_iii1-15</i> (3.9) <i>g_Rhodoplanes</i> (2.7) <i>f_Gaiellaceae</i> (2.7) <i>o_0319-7L14</i> (2.2)	<i>g_Rhodobacter</i> (21.8) <i>g_Methylomonas</i> (18.3) <i>g_Flavobacterium</i> (8.0) <i>g_Methyloversatilis</i> (7.6) <i>f_Chitinophagaceae</i> (4.7)	<i>g_Methylomonas</i> (65.2) <i>g_Flavobacterium</i> (11.6) <i>g_Methylotenera</i> (7.6) <i>f_Methylophilaceae</i> (5.0) <i>g_Methylobacillus</i> (3.0)
(b) The 16S rRNA amplicon sequencing analyses of AD enrichment samples				
Total number of reads after quality trimming	108,317	131,393	152,713	159,353
Reads assigned to bacterial 16S rRNA genes	108,317	131,391	152,713	159,353
Reads assigned to Methanotrophic 16S rRNA genes	287	16,494	25,215	62,257
Bacterial groups with high relative abundances ²	<i>g_T78</i> (10.6) <i>g_Mycobacterium</i> (5.3) <i>g_Clostridium</i> (3.7) <i>o_Actinomycetales</i> (3.1) <i>p_OD1</i> (2.8)	<i>g_Methylomonas</i> (8.9) <i>g_Novosphingobium</i> (8.2) <i>f_Sphingomonadaceae</i> (5.0) <i>g_T78</i> (4.1) <i>g_W22</i> (4.0)	<i>g_Flavobacterium</i> (24.6) <i>g_Methylomonas</i> (10.0) <i>o_GMD14H09</i> (8.6) <i>f_Flavobacteriaceae</i> (8.1) <i>g_Flavihumibacter</i> (6.1)	<i>g_Methylosarcina</i> (36.8) <i>g_Leadbetterella</i> (14.8) <i>g_Hyphomicrobium</i> (12.0) <i>o_Rickettsiales</i> (2.6) <i>g_Pleomorphomonas</i> (2.4)

¹ ES: the environmental samples; IB: the samples collected after initial batch cultivation; FB: the samples collected after fed-batch cultivation; and HD: the samples collected from the chemostats operated at the highest viable dilution rates (0.30 h⁻¹ for the SS sample and 0.45 h⁻¹ for the AD sample)

² The numbers in brackets are the relative abundances (%) of the listed groups of bacteria. The prefixes g, f, o, and p denote assignment at genus, family, order, and phylum level, respectively. Five most abundant OTUs are presented for each sample

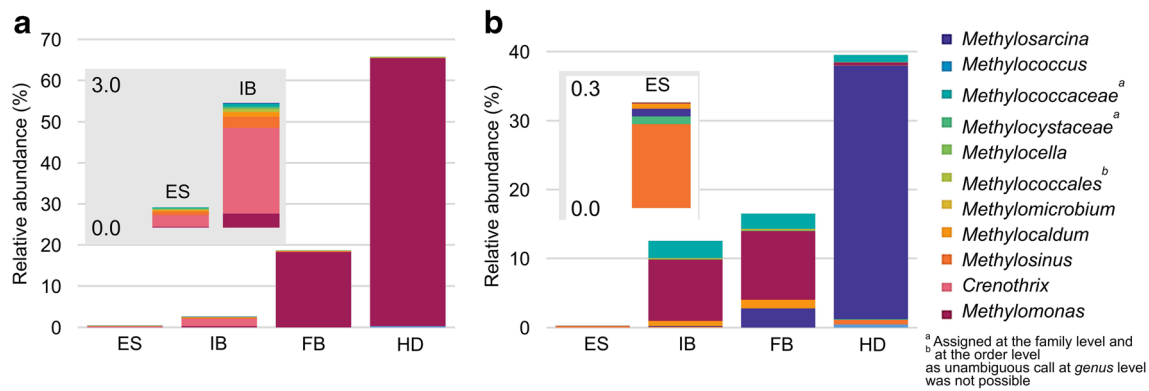


Fig. 2 The shift in the methanotrophic communities of **a** SS and **b** AD samples upon enrichment as monitored with 16S amplicon sequencing. The relative abundances of methanotrophic genera at different stages of the enrichment procedures are presented. The inserted figures are blowups of the data with the same label(s) in the main Figs. EB: the

the methanotrophic populations remained at 2.61 and 12.6% of the total microbial population in the batch enrichments of the SS and AD samples, respectively. The OTUs affiliated to the genus *Crenothrix* spp. and *Methyломonas* spp. were the most abundant methanotrophs in the SS and AD enrichments (68.9 and 70.7% of the methanotrophic population), respectively.

Extended period of incubation with unrestricted supply of CH₄ and O₂ in the reactor operated at a fed-batch mode specifically favored enrichment of gammaproteobacterial methanotrophs. In the reactor inoculated with SS batch enrichment, the OTUs assigned to the genus *Methyломonas* constituted 97.7% of the methanotrophic population and 18.3% of the overall microbial population. The OTUs assigned to the genus *Methyломonas* was also the dominant population in the reactor inoculated with AD batch enrichment (60.2% of the methanotrophic population and 9.95% of the overall microbial population). In the reactor fed with the SS sample, the wash-out of the chemostat culture occurred at the dilution rate of 0.35 h⁻¹. *Methyломonas* spp. remained as the dominant microbial group that constituted 53.8% of the total bacterial/archaeal population and 99.2% of the methanotrophic population. As for the AD chemostat, the microbial community of the suspension collected at the highest dilution rate, 0.45 h⁻¹, contained *Methylosarcina* as the dominant group of microorganism (36.8% of the bacterial/archaeal population and 94.1% of the total methanotrophic population). Notably, the dominating methanotrophs at the fed-batch mode, *Methyломonas* spp. was nearly washed out (0.402% of the total microbial population) upon chemostat cultivation. In both reactor operations, the methanotrophic OTUs observed to be the most abundant in the original environmental sample and initial batch enrichments, e.g., *Clenothrix* spp. and *Methylosinus* spp., were recovered only at very low relative abundances.

The shift in the composition of non-methanotrophic population was evident in both SS and AD samples at different

environmental samples; IB: the samples collected after initial batch cultivation; FB: the samples collected after fed-batch cultivation; and HD: the samples collected from the chemostats operated at the highest viable dilution rates (0.30 h⁻¹ for the SS sample and 0.45 h⁻¹ for the AD sample)

stages of enrichment (Table 1, Table S1). The OTUs affiliated to the genera or families under the *Flavobacteriales* order of the *Bacteroidetes* phylum (the genera *Flavobacterium* and *Leadbetterella* or the *Flavobacteriaceae* family) were commonly enriched in both chemostats (> 10% relative abundances). The composition of the enriched non-methanotrophic organisms outside *Flavobacteriaceae* family varied greatly between the two chemostats, likely due to the differences in the quantities and/or compositions of organic compounds released by the major methanotrophic populations. The obligate methylotrophs (genera *Methyлотenera* and *Methylobacillus* and *Methylophilaceae* family) constituted a substantial fraction of the microbial population in the SS reactor but were negligible in the AD reactor, suggesting that the composition of the exuded organic compounds from the *Methyломonas* strain enriched in the SS chemostat and the *Methylosarcina* strain enriched in the AD chemostat may differ substantially.

Isolation and initial characterization of fast-growing methanotrophs

In order to isolate and obtain pure cultures of fast-growing methanotrophs, the sample from the chemostat operated at the highest dilution rate was enriched and spread onto NMS agar plates. Single colonies appeared within 2 days of spreading and were transferred to fresh NMS agar plates on the third day. For both SS and AD chemostat enrichments, nine single colonies were picked after four transfers and subjected to confirmation of methane oxidation activities. Seven of the nine SS enrichment cultures turned turbid and depleted 20% CH₄ in the headspace within 2 days of inoculation (with an average final OD₆₀₀ value of ~0.4). Three of the seven microbial cultures were randomly selected and their purity and identity were confirmed by sequencing ten 16S rRNA gene clones and *pmoA* clones each. The consensus 16S rRNA gene amplicon sequence was identical to

the amplicon sequence of the dominant *Methylomonas*-affiliated OTU, and unexpectedly, the amplicon sequences of 16S rRNA gene and *pmoA* were both 100% identical at nucleotide sequence level to *Methylomonas* sp. LW13 (GenBank accession number AF150792.1 and AF150793.1), previously isolated from Lake Washington (Auman et al. 2000). Two of nine single colonies from AD enrichment chemostat grew on CH₄ and O₂ and were identified as pure cultures of *Methylosarcina* spp., based on amplicon sequencing of 16S rRNA and *pmoA* genes. The 16S rRNA and *pmoA* gene sequences of the isolate were both > 99% identical to those of *Methylosarcina fibrata* (GenBank accession number NR_025039.1 and WP_020564881.1) at nucleotide sequence level. These results were rather unexpected, as neither *Methylomonas* sp. LW13 nor strains belonging to *Methylosarcina fibrata* had been previously recognized for high growth rates. The exponential growth rates of the isolated strains affiliated to *Methylosarcina* and *Methylomonas* were measured to be $0.31 \pm 0.02 \text{ h}^{-1}$ and $0.40 \pm 0.04 \text{ h}^{-1}$, respectively, and were significantly higher than the exponential growth rates of the reference methanotrophic strain *M. trichosporium* strain OB3b ($0.16 \pm 0.01 \text{ h}^{-1}$) determined using the identical method (Fig. 3).

Discussion

Methanotrophs have great potential as environmentally friendly biocatalysts in the chemical industry, mainly due to their capability to transform CH₄, a potent greenhouse gas, to various value-added organic chemical products via CH₃OH. Such biochemical transformations have been found to be feasible in laboratory-scale experiments (Demidenko et al. 2017; Dong et al. 2017; Henard et al. 2016; Pieja et al. 2012). Success of methanotroph biotechnology depends primarily on rapid isolation of metabolically diverse fast-growing methanotrophs that are amenable to rapid genetic modifications. The novel isolation technique proposed in this study was able to significantly shorten the time required for isolation

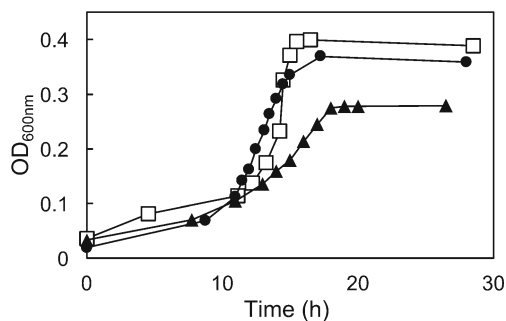


Fig. 3 The growth curves of the methanotrophs isolated from the SS sample (open squares), the AD sample (closed circles), and a reference strain, *M. trichosporium* strain OB3b (closed triangles). The data points are the averages of triplicate measurements (error bars are not presented, as the standard deviations of the triplicate OD_{600nm} measurements were negligible)

of methanotrophs to within a month of sample collection. This novel technique screened out most of the slow-growing methanotrophs before plating, saving rigorous efforts necessary for screening arbitrary isolates for high specific growth rates. The fast-growing methanotrophs isolated using this method will still require extensive characterization before being adopted as platform organisms; nevertheless, our novel technique would significantly enhance the throughput of the upstream screening process without the necessity for any sophisticated equipment or procedure.

Initial batch cultivation of the environmental samples with limited amounts of CH₄ and O₂ enriched multiple phylogenetic groups of methanotrophs, which included both alpha- and gammaproteobacterial methanotrophs; however, further enrichment with continuous supply of CH₄ exclusively enriched gammaproteobacterial methanotrophs of the genera *Methylomonas* and *Methylosarcina*. The genera *Crenothrix* and *Methylosinus*, with large abundance in the original environmental samples and the initial batch enrichments, were outcompeted and washed out upon chemostat cultivation. These results were in support of the general perception that the gammaproteobacterial methanotrophs have higher specific growth rates than alphaproteobacterial or verrucomicrobial methanotrophs under CH₄- and O₂-rich growth conditions (Demidenko et al. 2017; Hoefman et al. 2012). That *Methylosarcina* spp. outcompeted *Methylomonas* spp. in the AD chemostat, while *Methylomonas* was the dominating genera in the SS chemostat suggested fast growth is not a genus- or species-specific characteristic. In fact, the fastest growth rate recorded for any methanotroph prior to this study was observed with *Methylomicrobium buryantense* strain 5GB1, and none of previously isolated *Methylomonas* or *Methylosarcina* strains exhibited growth rates exceeding 0.20 h^{-1} (or, in other words, doubling times lower than ~ 3.5 h). Although 16S rRNA and *pmoA* sequences of the fast-growing isolates were nearly identical to *Methylomonas* sp. LW13 and *Methylosarcina fibrata*, the specific growth rates of the *Methylomonas* and *Methylosarcina* isolates were at least 1.5-fold higher than the reported growth rates of any isolates affiliated to these genera. Genome sequencing and annotation of the *Methylomonas* and *Methylosarcina* isolates are currently in progress and the search for specific genomic features that allow for this extraordinarily fast growth may be an interesting topic for future research. Identification of such genomic features may provide insights necessary to resolve the long-standing enigma that methanotrophic *Proteobacteria* have surprisingly low growth rates compared to heterotrophic *Proteobacteria* even when the system is not limited by mass transfer of CH₄ or O₂.

Isolation of methanotrophs is often complicated by methylotrophs and heterotrophs attached to the methanotrophs in what appears as single colonies (Hoefman et al. 2012). Even when CH₄ is provided as the sole source of carbon and electron

that can be utilized only by organisms possessing pMMO or sMMO (in oxic condition), methanotrophs are known to exude one-carbon, e.g., CH₃OH and formate (HCOOH), and multi-carbon organic compounds (e.g., fatty acids and exopolymeric substances) utilizable to non-methanotrophic methylotrophs and heterotrophs (Hilger et al. 2000; Oshkin et al. 2014; Sheets et al. 2016). The chemostat cultures, even at the highest dilution rates, were not completely devoid of the methylotrophs and heterotrophs; however, the proportions of the non-methylotrophic organisms were notably lower in the fully operational chemostat than in the initial batch enrichment or the reactor operated in the fed-batch mode. The removal of methylotrophs and heterotrophs from the enrichment culture significantly facilitated the downstream isolation procedures, so that axenic cultures could be obtained without difficulty after three or four rounds of plating and single-colony picking.

Several unexpected phylogenetic groups of microorganisms were enriched in the chemostat reactors. In the chemostat inoculated with the SS sample, *Legionella* spp., a group of organisms that cause Legionnaires' disease in mammals, were enriched as one of the organismal groups with the highest relative abundances. *Legionella* spp. are facultative intracellular bacteria rarely found to replicate in the free-living form due to their unique nutrient requirement (Feeley et al. 1979; Pine et al. 1979). In the chemostat reactor inoculated with the AD sample, an OTU affiliated to the *Opitutaceae* family, a *Verrucomicrobia* group known to be notoriously difficult to enrich or isolate, was one of the organismal groups with greatly increased relative abundance upon chemostat operation (Chin et al. 2001). *Verrucomicrobia*, including *Opitutaceae* family, have evaded cultivation or enrichment although microbial community analyses have suggested their abundance in agricultural soils and presumed importance in the biogeochemical cycles (Hugenholtz et al. 1998; Joseph et al. 2003). Several recent studies investigated non-methanotropic microbial communities developing around methanotrophs; however, neither of these culture-resistant organismal groups was previously identified to associate with gammaproteobacterial methanotrophs (Ho et al. 2016; Oshkin et al. 2014).

Cross-feeding is the most intuitive mechanism of association between methanotrophs and non-methanotrophs, as methanotrophs often release intermediates of CH₄ metabolism, e.g., CH₃OH and organic acids, attracting non-methanotrophic methylotrophs, and heterotrophs (Kalyuzhnaya et al. 2013; Oshkin et al. 2014). The enriched *Opitutacea* and *Legionella* could have certainly benefitted from such organic exudates from the fast-growing methanotrophs; however, this rationale is not sufficient to explain their enrichment in the chemostat, which require the cells to grow at a rate higher than 0.30 h⁻¹ for survival. The fast-growing methanotrophs may have produced and exuded micronutrients essential for these culture-resistant organisms that are absent in synthetic culture media. Such commensal association was previously observed between cobalamin-

producing organisms and vitamin B₁₂ auxotrophs (Yan et al. 2013). An interesting future research would involve identification of such micronutrients in the spent media of the isolated gammaproteobacterial methanotrophs that may facilitate enrichment and isolation of these culture-resistant organisms with potential clinical or environmental significance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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