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MINI-REVIEW

Novel approaches and reasons to isolate methanotrophic bacteria with biotechnological potentials: recent achievements and perspectives

Miye Kwon[1] · Adrian Ho[2] · Sukhwan Yoon[1]

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Abstract

The recent drop in the price of natural gas has rekindled the interests in methanotrophs, the organisms capable of utilizing methane as the sole electron donor and carbon source, as biocatalysts for various industrial applications. As heterologous expression of the methane monooxygenases in more amenable hosts has been proven to be nearly impossible, future success in methanotroph biotechnology largely depends on securing phylogenetically and phenotypically diverse methanotrophs with relatively high growth rates. For long, isolation of methanotrophs have relied on repeated single colony picking after initial batch enrichment with methane, which is a very rigorous and time-consuming process. In this review, three unconventional isolation methods devised for facilitation of the isolation process, diversification of targeted methanotrophs, and/or screening of rapid growers are summarized. The soil substrate membrane method allowed for isolation of previously elusive methanotrophs and application of high-throughput extinction plating technique facilitated the isolation procedure. Use of a chemostat with gradually increased dilution rates proved effective in screening for the fastest-growing methanotrophs from environmental samples. Development of new isolation technologies incorporating microfluidics and single-cell techniques may lead to discovery of previously unculturable methanotrophs with unexpected metabolic potentials and thus, certainly warrant future investigation.

Keywords Methanotroph · Isolation techniques · Extinction culturing · Soil substrate membrane system · Chemostat screening

Introduction

Aerobic methanotrophs are a specialized group of organisms capable of utilizing methane as the sole source of energy and carbon (Hanson and Hanson 1996; Semrau et al. 2010). Until recently, only proteobacterial methanotrophs, belonging to either *Alpha-* or *Gamma-proteobacteria* class, were known; however, the discovery of verrucomicrobial methanotrophs has expanded the methanotroph diversity (Dunfield et al. 2007; Islam et al. 2008; Sharp et al. 2014). The list of microbial taxa (families) with physiologically confirmed methanotrophs now includes *Methylococcaceae*, *Crenothrichaceae*, *Methylcystaceae*, *Beijerinckiaceae*, and *Methylacidiphilaceae* (Semrau et al. 2010). Despite the broad phylogenetic and physiological diversity, these methanotrophs share the distinguishing physiological capability to catalyze the oxidation of methane (CH_4) to methanol (CH_3OH) using either particulate (pMMO) or soluble methane monooxygenases (sMMO).

These two MMOs have identical metabolic function in methanotrophs, but are structurally and evolutionarily distinct groups of metalloenzymes. The more prevalent form, pMMO, is a membrane-bound enzyme with copper in the active site, while sMMO, found only in selected groups of methanotrophs, is a cytoplasmic enzyme with a di-iron active center (Semrau et al. 2010, 2018). The pMMOs share evolutionary history with ammonia monooxygenases (AMO) and thus, are structurally similar to these cuproenzymes catalyzing ammonia (NH_3) turnover to hydroxylamine (NH_2OH) in ammonia oxidizing bacteria and archaea (Murrell et al. 2000a, b; Culpepper and Rosenzweig 2012; Hatzenpichler 2012). The sMMO are evolutionarily related to soluble di-iron monooxygenases and share modest structural similarity with toluene monooxygenases and phenol hydroxylases (Leahy et al. 2003; Coleman et al. 2006).

✉ Sukhwan Yoon
syoon80@kaist.ac.kr

[1] Department of Civil and Environmental Engineering, Korea Advanced Institute of Science and Technology, Daejeon 34141, South Korea

[2] Institute for Microbiology, Leibniz Universität Hannover, 30419 Hannover, Germany

Methanotrophs harboring both pMMO(s) and sMMO(s) are not rare among cultured methanotrophic isolates; however, methanotrophs with only pMMO are the vast majority among methanotrophic isolates (Osborne and Haritos 2018). Methanotrophs possessing only sMMO fall within the *Beijerinckiaceae* family and recent researches have found these organisms dominating the methanotrophic populations in specialized ecological niches such as natural gas seeps and microbial mats of Movile Cave (Farhan Ul Haque et al. 2018; Kumaresan et al. 2018). Extrapolating the observations from the experiments with the methanotrophs possessing both pMMO and sMMO, it is now widely accepted that availability of bioavailable copper regulates the expression and activity of the MMOs (Semrau et al. 2010, 2018).

The methanotrophs, as the sole possessor of the MMOs, have drawn immense interests for their potential biotechnological applications; however, successful cases of industrial implementation have rarely been reported, due to several bottlenecks, one of them being the limited physiological diversity of isolated strains. In this review, the reasons why novel isolation techniques are essential for successful implementation of methanotroph biotechnology are explained and three novel approaches for methanotroph isolation are summarized.

The value of methanotrophs in biotechnology

The capability to wield the methane monooxygenases for conversion of CH_4 to CH_3OH are restricted exclusively to the aforementioned groups of methanotrophs. Several attempts to express sMMOs in heterologous hosts failed to reproduce the high CH_4 turnover efficiency observed in methanotrophs, and no successful heterologous expression of pMMO has been reported to date (Jahng et al. 1996; Jahng and Wood 1994; Kalyuzhnaya et al. 2015; Murrell et al. 2000a; Strong et al. 2015). Due to insufficient knowledge on how active sMMO and pMMO complexes are formed in vivo, generating an artificial methanotroph from model laboratory organisms more amenable to manipulation (e.g., *Escherichia coli*, and *Methylobacterium extorquens*) remains elusive.

Due to this exclusive ownership of fully active MMOs, the aerobic methanotrophs have for long attracted interests for their potential utility in biotechnological applications. As both sMMO and pMMO are capable of co-oxidizing a broad range of xenobiotic organic compounds, including carcinogenic halogenated compounds and organic micropollutants, methanotrophs have been investigated for their potential application in in situ bioremediation of contaminated soils and groundwater (Benner et al. 2015; Little et al. 1988; Semrau 2011; Semrau et al. 2010). More recently, CH_4 itself is considered as a target for bioremediation due to its high global warming potential (34 times that of CO_2 in a 100-year scale), and various bioreactor configurations have been devised to reduce CH_4 emissions from point sources, e.g., landfills and animal feeding operations, utilizing methanotrophic consortia (Ganendra et al. 2015; Limbri et al. 2014; Yoon et al. 2009). The ability of methanotrophs to mineralize and detoxify methyl-mercury has also been recently investigated, suggesting that methanotrophs may have value in detoxifying such toxic heavy metals (Lu et al. 2017). Production of various value-added chemical compounds using CH_4 has also been a widely investigated research topic, as CH_4 has been an attractive feedstock due to its low price and high availability, especially since the onset of the recent shale gas boom (Haynes and Gonzalez 2014; Strong et al. 2015). Single-cell protein for livestock feeds have been produced with gammaproteobacterial methanotrophs for decades, and utilization of alphaproteobacterial methanotrophs for polyhydroxyalkanoate (PHA) production have been investigated as a biodegradable alternative for plastics (Kalyuzhnaya et al. 2015; Marshall et al. 2013; Pieja et al. 2017; Strong et al. 2016). Lately, genetic modifications of biosynthetic pathways in methanotrophs for conversion of CH_4 to feedstock chemicals, e.g., CH_3OH, organic acids, and fatty acids, or liquid fuels have been rigorously investigated in both academic and industrial sectors (Conrado and Gonzalez 2014; Hwang et al. 2018).

These potential industrial applications of methanotrophs capitalize on a variety of physiological traits that are not commonly shared by different methanotroph groups. For example, the capability to detoxify methyl mercury is limited to the selective group of methanobactin-producing alphaproteobacterial methanotrophs, while only fast-growing gammaproteobacterial strains, e.g., *Methylococcus* spp., proved profitable for single-cell protein production industry. As alpha- and gammaproteobacterial methanotrophs utilize distinct carbon assimilation and biosynthetic pathways, one pathway is favored over the other when a specific chemical is targeted as the product of metabolic engineering (Kalyuzhnaya et al. 2015). The ribulose monophosphate cycle in gammaproteobacterial methanotrophs concentrates the carbon flux to pyruvate synthesis via the Embden-Meyerhof-Parnas (EMP) pathway, thus favoring the production of chemical compounds branching from pyruvate, while the serine cycle in alphaproteobacterial methanotrophs is thought to be favorable for biofuel production due to the higher flux to acetyl-CoA. Theoretically, genetic manipulation would be able to fill such missing gaps in the pathways leading to desired processes or products once fast and efficient genetic tools are established in tractable methanotrophic strains; however, such approach has only been modestly successful (Henard et al. 2016).

Although synthetic biology approaches appear to be unlikely for biotechnological application of methanotrophs in the foreseeable future, the attractiveness of the aerobic methanotrophs as methane-transforming biocatalysts is difficult to ignore. Hence, the more realistic strategy for their biotechnological applications is to utilize methanotrophs without or with minimal genetic modifications. Therefore, having an

inventory of methanotrophs with diverse traits and physiological properties (e.g., ownership of sMMO, resistance to change in pH and/or salinity, high/low carbon conversion efficiency and methanobactin production) and distinct biosynthetic metabolic pathways would be pertinent to meet biotechnological demands. General relevant traits necessary for potential industrial methanotrophs include rapid growth and high CH_4 utilization rate, which would allow for faster population establishment and rapid metabolic turnover of CH_4 and cometabolites, and facilitate genetic manipulation procedures should genetic manipulation be necessary (Jiang et al. 2010; Puri et al. 2015; Rocha-Rios et al. 2011; Rostkowski et al. 2013; Yan et al. 2016). Methanotrophs with relatively high growth rates, with specific growth rates higher than 0.1 h^{-1}, have been reported (Table 1); however, the diversity of methanotrophs with such high growth rates under unrestricted access to methane is limited and most of these fast-growing methanotrophs are phylogenetically positioned within the family *Methylococcaceae* (Ho et al. 2013; Semrau et al. 2010). Therefore, an expansion in the library of fast-growing methanotrophs with a broader phylogenetic and metabolic spectrum would be necessary to facilitate implementation of methanotrophic biotechnology.

Methanotroph isolation techniques

The traditional method for isolating microorganisms, including the methanotrophs, has remained largely unchanged for over 50 years. The majority of publicly available methanotroph cultures were isolated from enrichment of environmental samples in liquid medium supplemented with CH_4 in the headspace gas, followed by repeated single-colony picking on agar plates (Dunfield et al. 2003; Kip et al. 2011; Lidstrom 1988; Wise et al. 1999; Zúñiga et al. 2011). Variation in medium compositions and incubation conditions enabled isolation of several unconventional methanotrophs (Dedysh 2011; Dunfield et al. 1999, 2003, 2007; Islam et al. 2008). The use of diluted media, e.g., diluted nitrate mineral salt medium (DNMS) with five-fold lower concentrations of salts, enabled isolation of acidophilic *Beijerinckiaceae* family of methanotrophs including *Methylocella*, *Methylocapsa*, and *Methyloferula* (Dunfield et al. 2003, 2010; Vorobev et al. 2011). Incubation at elevated temperatures and acidic pH and addition of CO_2 to the headspace enabled isolation of the verrucomicrobial methanotrophs (Dunfield et al. 2007; Islam et al. 2008).

A major drawback of this isolation approach is extended time and rigorous effort needed for isolation, which limit the quantity and diversity of the strains isolated. Methanotrophs, even in cultures enriched with CH_4 as the sole carbon substrate, tend to form aggregates with methylotrophs, as well as other heterotrophs living on the metabolites exuded by the methanotrophs (Ho et al. 2016; Kalyuzhnaya et al. 2013; Kim et al. 2018; Oshkin et al. 2015). In such cases, repetitions of spreading-and-single-colony-picking may prolong the isolation effort to yield cultures devoid of contaminant non-methanotrophic strains. The isolation procedure typically selects for abundant fast-growing methanotrophs, and rarely captures methanotrophs present in relatively low abundances (even if these are fast-growers) or slow-growers in environmental samples (Fig. 1). These inadequacies and isolation biases call for development of novel isolation techniques that can expedite the isolation procedure and target the organisms that were less likely to be isolated with the conventional isolation technique (Hoefman et al. 2012; Kim et al. 2018; Svenning et al. 2003).

Increasing the throughput of the oft-used extinction culturing technique was suggested as a methodology to expedite methanotroph isolation procedures (Hoefman et al. 2012). Extinction culturing (i.e., isolation using serial dilution series) of 46 CH_4-fed enrichments was performed simultaneously with multiple 96-well plates in a high-throughput manner, with 10^{-2}-to-10^{-9} dilution series prepared for each enrichment (Fig. 2A). The most diluted samples exhibiting growth were then evaluated for CH_4 oxidation capability and purity. With a single round of experiment, 14 monocultures of methanotrophs were obtained immediately following the extinction culturing and 8 more isolates were obtained after further purification steps. The initial enrichment step with 20% CH_4 appeared to have selected towards isolation of rapid-growing methanotrophs; however, the methanotrophs targeted by this method were essentially the same as those targeted by

Table 1 The list of fast-growing methanotrophs with reported growth rates higher than 0.1 h^{-1}

Family	Genus	Source	Growth rate (h^{-1})	Reference
Methylococcaceae	*Methylomonas*	River sediment	0.4 ± 0.04	Kim et al. (2018)
	Methylosarcina	Anaerobic digester effluent	0.31 ± 0.02	Kim et al. (2018)
	Methylomarinum	Marine sediment	0.33	Hirayama et al. (2013)
	Methylomicrobium	Soda lake	0.2	Yan et al. (2016)
	Methylothermus	Thermal aquifer	0.3	Hirayama et al. (2011)

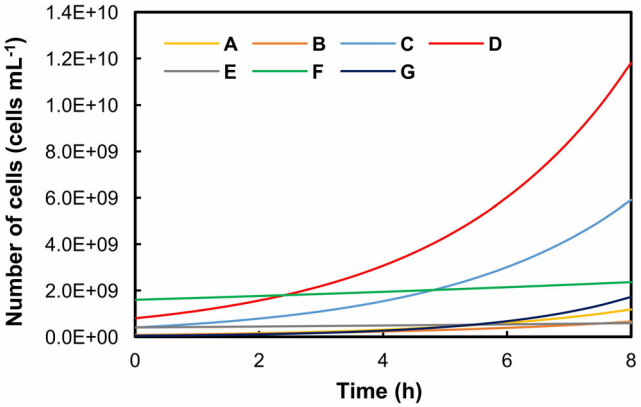

Fig. 1 The simulated growth curves of methanotrophs with varying growth rates and initial abundances, assuming incubation in 50-mL medium in 160-mL serum vial with limited amount of methane and air (20% *v/v* and 80% *v/v*, respectively, in the headspace). An identical growth yield of 0.94 (g dry cell weigh per g CH_4) was assumed for all methanotrophs (Mahmoud 2017). The simulation result illustrates that initial enrichment with methane that precedes conventional isolation procedure selectively enriches only abundant and fast-growing methanotrophs. Neither minor fastest-growers nor slow-growers dominant in the original environmental sample is enriched, and thus, such organisms are likely to be excluded from isolation targets in the downstream procedures. I_o: initial abundance (cells mL^{-1}); G_r: growth rate (h^{-1}). (A) I_o: 8×10^7, G_r: 0.4; (B) I_o: 8×10^7, G_r: 0.3; (C) I_o: 4×10^8, G_r: 0.4; (D) I_o: 8×10^8, G_r: 0.4; (E) I_o: 4×10^8, G_r: 0.05; (F) I_o: 1.6×10^9, G_r: 0.05; (G) I_o: 8×10^6, G_r: 0.6

the conventional isolation technique: fast-growing organisms that are relatively abundant in the environmental samples.

The isolation technique using soil substrate membrane system (SSMS), originally developed and applied for isolating methanotrophs (Svenning et al. 2003), shortened the time needed for isolation and yielded more diverse isolates than the conventional method. The initial processing of environmental samples in the SSMS procedure includes extraction of microbial consortium from an environmental sample and its serial dilution (Fig. 2B). The serially diluted samples were applied initially on 0.4-μm polycarbonate membranes, which were then inserted to petri dishes carrying soil slurry (soil suspended in MilliQ water) providing micronutrients to the microorganisms on the membranes. The colonies developed on the membranes were picked and dotted onto membranes with 0.2-μm pore size. The colonies on the 0.2-μm membrane were serially diluted and the dilutions were incubated on identically prepared soil membrane system. After the second membrane growth of the diluted colonies, the obtained cultures were confirmed against heterotroph contamination by floating the membrane on nutrient-rich medium. The SSMS technique enabled simultaneous isolation of gammaproteobacterial and alphaproteobacterial methanotrophs with remarkable diversity within a few months. The drawback of the method in isolating potential biotechnologically applicable methanotrophs, however, is that additional screening for fast-growers would be necessary as the method does not specifically select for fast-growing microorganisms. Although the SSMS technique was further developed into a more general technique for isolation of bacterial groups resistant to culturing, further improvement or application of this methodology for isolation of methanotrophs has not been reported (Ferrari et al. 2005, 2008).

In contrast to the SSMS method which accentuates the diversity of acquired isolates, the recently demonstrated isolation technique utilizing a continuous cultivation system aimed to select the fastest-growing methanotroph from an environmental sample (Kim et al. 2018). This isolation method was based on the fundamental chemostat principle, that is, only organisms with growth rates higher than the dilution rates would be retained in the chemostat at steady-state (Jannasch 1969). After initial batch incubation of an environmental sample with CH_4 as the sole source of carbon and energy, the enriched methane-oxidizing consortium was incubated in a fed-batch reactor with continuous flow of the gas phase (80:20 mixture of air and CH_4 provided at 1.0 mL min^{-1}) for further enrichment of fast-growing methanotrophs (Fig. 2C). The reactor was eventually converted to a chemostat with the initial dilution rate of 0.1 h^{-1} and the dilution rate was gradually increased with 0.05 h^{-1} increments to screen out methanotrophs with a slower growth rate or contaminating heterotrophs and methylotrophs. The reactor sample collected from the highest dilution rate before washout was selected for further purification by repeated single colony selections. In less than 2 months, the method successfully isolated a methanotrophic strain affiliated to the genus *Methylomonas* (originating from a stream sediment) with a specific growth rate of 0.4 h^{-1}, a remarkably high value for growth on methane (Kim et al. 2018).

The feasibility of this isolation method has only been tested under prototypical incubation conditions for optimal methanotrophic growth, i.e., neutral pH, optimal CH_4:air ratio (20:80), 10 mM NO_3^- as the nitrogen source, and sufficient copper (10 μM). Incubation conditions modified to exert selection pressures towards specific phenotypes, e.g., the absence of copper endowing selective advantage to sMMO-expressing organisms or the absence of dissolved N source selecting for nitrogen-fixing methanotrophs, as well as use of diverse environmental samples as the initial inocula, may allow for diversification of target isolates (Auman et al. 2001; Murrell et al. 2000b). Lanthanides (e.g., cerium, lanthanum, and praseodymium) were found to promote methanol oxidation and growth by the methanotrophs with Xox-type methanol dehydrogenases (Xox-MeDH) and thus, variation of lanthanide compositions in the media may also provide additional selection for methanotrophs relying exclusively on Xox-MeDH (Semrau et al. 2018). This enrichment/screening methodology would also be suitable for enriching methanotrophs capable of utilizing low methane concentrations (e.g., < 100 ppmv). Such methanotrophs have potential applicability in CH_4 removal systems for landfills and cattle barns, as

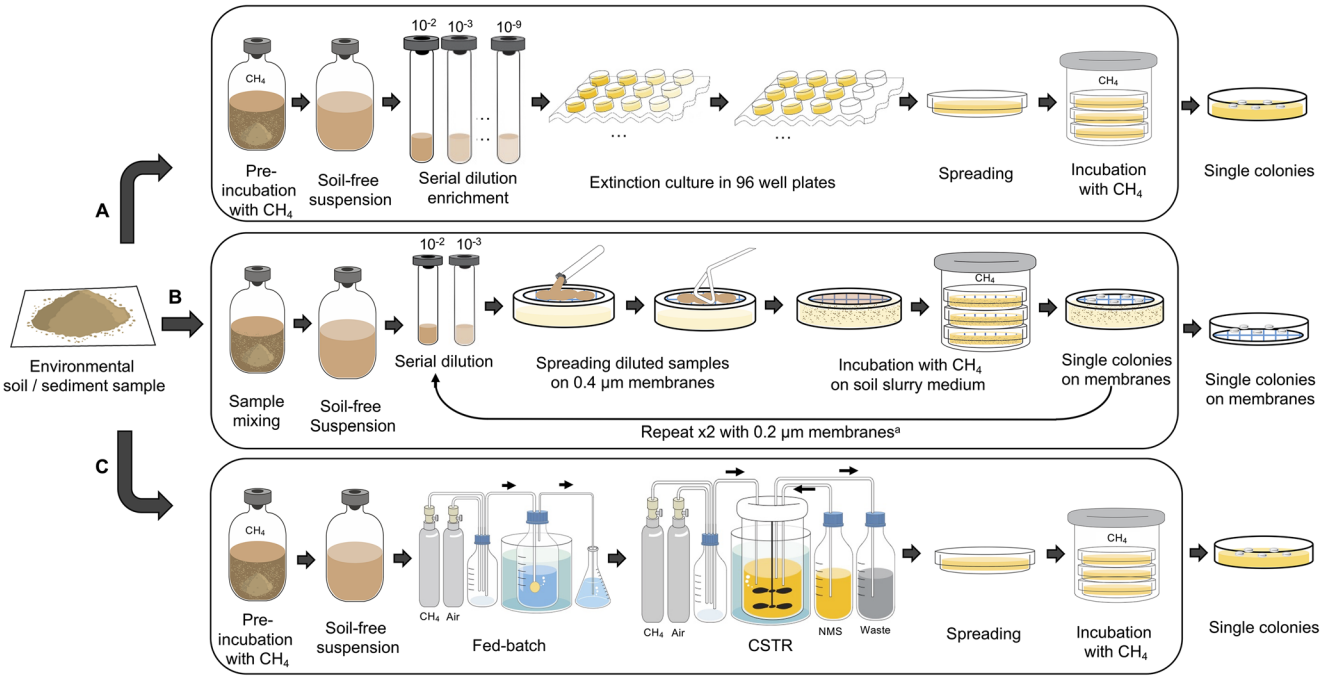

Fig. 2 The schematic overview of the unconventional methods developed for the isolation of methanotrophs. (A) The high-throughput extinction cultivation; (B) the soil substrate membrane system; and (C) the continuously stirred tank reactor (CSTR) screening method for isolation of fast-growing methanotrophs. Further isolation from the resulting single colonies may be necessary to obtain pure cultures. The figures were adapted from the references cited in the text. [a]First with the dotted colonies transferred from 0.4-µm membranes and then with serial dilutions of the single colonies developed on the 0.2-µm membranes after the transfer

CH_4 emitted from such sources rarely exceed 100 ppmv in concentration (Joo et al. 2015; Ngwabie et al. 2009; Yoon et al. 2009).

Conclusion and outlook

Methanotrophs have recently regained immense interests as the biocatalysts for industrial applications. Although securing of fast-growing isolates with diverse metabolic pathways and physiological properties is a prerequisite for successful biotechnological applications of methanotrophs, surprisingly few advances have been made to improve isolation techniques in the past decades. The unique isolation approaches reviewed here, may not be sufficiently optimized, although these methods have proved their potential. Development of novel isolation techniques for methanotrophs may also take advantage of the recent scientific advances in microfluidics, cell imaging, and Raman microspectroscopy techniques (Hol and Dekker 2014; Ishii et al. 2010; Ma et al. 2014; Song et al. 2016). Several cases of successful implementation of microfluidics-based single-cell techniques to isolate as yet "uncultivable" microorganisms have been recently reported (Jiang et al. 2016; Zhang et al. 2014). Applying these state-of-the-art techniques to methanotroph isolation may yield a substantial expansion of the current methanotroph inventory. *Crenothrix* spp., the enigmatic filamentous gammaproteobacterial methanotrophs, recently confirmed as major CH_4 sinks in stratified lakes, have evaded isolation for more than a century since its initial discovery (Oswald et al. 2017; Stoecker et al. 2006). As these methanotrophs were observed to actively incorporate ^{13}C-labeled CH_4 to their biomass, these evasive organisms may be able to be isolated with help of Raman microspectroscopy and microfluidics (and/or optical tweezer) techniques (Wang et al. 2016).

Accumulating evidence suggests a synergistic effect of methanotroph and non-methanotroph interaction in modulating methane oxidation (Ho et al. 2014). To this end, the concerted effort of a methanotroph paired to non-methanotrophic microorganisms have been shown to significantly stimulate methane oxidation rates (Ho et al. 2014). Given that many methanotrophs resist cultivation, but could be highly enriched with specific accompanying community members (Oshkin et al. 2015) indicate an intricate relationship likely driven by metabolic exchange (Oshkin et al. 2015; Krause et al. 2017). This begs the question whether it is necessary to isolate individual methanotrophs, or capitalize on naturally occurring enriched methane-oxidizing consortia for biotechnological purposes. However, previous lessons from successful industrial implementations of microbial biotechnology, e.g., bioremediation of chlorinated ethene-contaminated soils with *Dehalococcoides* consortia, suggest that the discovery of new microorganisms may lead to unexpected breakthroughs, resolving long-standing dilemmas (He et al. 2003; Löffler et al. 2013). Thus, the development of isolation and culturing

techniques targeting elusive methanotrophs warrant continued attention and efforts for successful implementation of methanotroph biotechnology.

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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 (Dunfield et al. 1999)

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