Microbial Biotechnology (2012) **5**(3), 368–378 doi:10.1111/j.1751-7915.2011.00314.x

Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria

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Summary

Methane-oxidizing bacteria (MOB) have a large potential as a microbial sink for the greenhouse gas methane as well as for biotechnological purposes. However, their application in biotechnology has so far been hampered, in part due to the relative slow growth rate of the available strains. To enable the availability of novel strains, this study compares the isolation of MOB by conventional dilution plating with miniaturized extinction culturing, both performed after an initial enrichment step. The extinction approach rendered 22 MOB isolates from four environmental samples, while no MOB could be isolated by plating. In most cases, extinction culturing immediately yielded MOB monocultures making laborious purification redundant. Both type I (*Methylomonas* spp.) and type II (*Methylosinus* sp.) MOB were isolated. The isolated methanotrophic diversity represented at least 11 different strains and several novel species based on 16S rRNA gene sequence dissimilarity. These strains possessed the particulate (100%) and soluble (64%) methane monooxygenase gene. Also, 73% of the strains could be linked to a highly active fast-growing mixed MOB community. In conclusion, miniaturized extinction culturing was more efficient in rapidly isolating numerous MOB requiring little effort and fewer materials, compared with the more widely applied plating procedure. This miniaturized approach allowed straightforward isolation and could be very useful for subsequent screening of desired characteristics, in view of their future biotechnological potential.

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Introduction

Next to carbon dioxide, methane is the second most important greenhouse gas contributing to one-fifth of global warming (Dalal and Allen, 2008). Yearly, 30 Tg of atmospheric methane is removed through oxidation by aerobic methane-oxidizing bacteria (MOB), a unique group of bacteria capable of utilizing methane as their sole carbon and energy source (Trotsenko and Murrell, 2008). The key enzyme of these microorganisms, particulate or soluble methane monooxygenase (pMMO and sMMO), is remarkable in its broad substrate specificity (Semrau *et al.*, 2010). To date, MOB have shown potential in three fields of industrial biotechnology, namely (i) geoengineering the climate through assimilation of methane and thus mitigating greenhouse effects (Scheutz *et al.*, 2009), (ii) bioremediation of pollutants via co-metabolism by MMOs (Wendlandt *et al.*, 2010), and (iii) production of commercially relevant metabolites (Zhang *et al.*, 2008; Jiang *et al.*, 2010). Despite the multifunctional potential of these bacteria, there are still several factors limiting large-scale applicability in industrial processes, which are mostly related to the MOB themselves, such as slow growth rates and low substrate affinity (Jiang *et al.*, 2010). In order to achieve the full potential of MOB and communities for such biotechnological applications, a straightforward isolation technique is of high value, as it allows the optimization of the growth conditions of the MOB of interest. However, their cultivation is still laborious (Bowman, 2006). To date, only few MOB have been studied and examined thoroughly for further biotechnological applications (Jiang *et al.*, 2010). The examined cultures were often not selected for their optimal use in these bioprocesses but were just the only available methanotrophic cultures at the time.

The first MOB were isolated by Söhngen (1906), but it was not until 1970 that Whittenbury and co-workers established a successful procedure to isolate and characterize methane-oxidizing bacteria by plating on nitrate- or ammonium mineral salts (NMS/AMS) medium (Whittenbury *et al*., 1970). Since then, novel research on MOB mainly encompassed exploring new environments and using cultivation conditions adapted to the corresponding environment (Dedysh *et al*., 1998a; Wise *et al*., 1999; Svenning *et al*., 2003; Bussmann *et al*., 2004). Most of the methanotrophic diversity thus obtained could be phylogenetically positioned within the *Alphaproteobacteria* [such as *Methylosinus* (Type II)] and the *Gammaproteobacteria* [such as *Methylomonas* (Type Ia) and *Methylococcus* (Type Ib)] (Dedysh, 2009). Since the recognition of 'The Great Anomaly' (Staley and Konopka, 1985), alternative ways to increase the general cultivability of the microbial diversity were explored, for example by application of growth conditions that closely mimic the natural environment (Dedysh *et al*., 1998a), prolonged incubation at low temperatures (Song *et al*., 2009) or the use of alternative gelling agents replacing agar (Janssen *et al*., 2002; Dedysh *et al*., 2007; Stott *et al*., 2008; Tamaki *et al*., 2009). Another approach was extinction culturing by diluting a sample to the point of extinction and thereby purifying to a less complex sample containing only one or a few organisms (Button *et al*., 1993; Schut *et al*., 1993), which was further optimized by the development of high-throughput culturing methods (Connon and Giovannoni, 2002; Rappe *et al*., 2002; Stingl *et al*., 2007). To date, MOB are still mostly isolated via plate methods, whereby one or several liquid enrichment steps are followed by a serial dilution onto plates (Dunfield *et al*., 2003; Dedysh *et al*., 2004; Heyer *et al*., 2005; Tsubota *et al*., 2005; Wartiainen *et al*., 2006). However, these procedures are very laborious and almost always require elaborate purification.

The main objective of this study was to combine and optimize several of the above-mentioned cultivation approaches for the specific isolation of methane-oxidizing bacteria, and to compare the isolation efficiency of the resulting two-step liquid isolation procedure, consisting of an initial prolonged enrichment and subsequent miniaturized extinction culturing, with conventional dilution plating. Initial enrichment followed by extinction culturing greatly simplified purification procedures and easily rendered novel methanotrophic strains, which were further characterized by sequence analysis of the 16S rRNA gene, repetitive element sequence based PCR fingerprinting (rep-PCR), *pmoA* and *mmoX* gene amplification (encoding for pMMO and sMMO respectively) as well as sMMO activity assays. Most of the strains were traced back to highly active and fast-growing methanotrophic communities through denaturing gradient gel electrophoresis (DGGE) targeting the *pmoA* gene.

Results

Isolation of fast-growing MOB

Four environmental samples (wastewater treatment plant, wetland, biofilter and slurry pit) were serially diluted and enriched, while monitoring methane and oxygen consumption and carbon dioxide production. During the first four weeks, higher dilutions were gradually found positive with each subsequent measurement, stabilizing by the fifth week (Fig. S1). These data were used to calculate the abundance of cultivable MOB under given growth conditions (MPN g^{-1} expressed as MPN index [95% confidence]): cultivable MOB were most abundant in the wastewater treatment plant (4.3×10^4 MOB g^{-1} [9.0×10^3 to 1.8×10^5 MOB g^{-1}]), followed by wetland (4.3×10^3 MOB g^{-1} [9.0×10^2 to 1.8×10^4 MOB g^{-1}]) and then the slurry pit and biofilter samples (both 2.3×10^2 MOB g^{-1} [4.6×10^1 to 9.4×10^2 MOB g^{-1}]). After 5 weeks of incubation at 20°C, 23 dilutions were found positive for methane oxidation and were used further for MOB isolation.

The extinction culturing procedure was performed in duplicate to isolate MOB from these enrichment cultures, resulting in 46 extinction series, of which only four did not show growth after two weeks of incubation. The highest dilutions of the remaining 42 series were transferred to gas-tight vials and diluted nitrate mineral salts (dNMS) medium solidified with gellan gum for confirmation of methane consumption and purity check respectively. Methane oxidation was observed in 27 series, of which 14 were immediately monocultures. Seven methane-oxidizing cultures consisted of an MOB in co-culture with a non-methanotrophic bacterium (identified as a member of *Nocardioides* with 16S rRNA gene sequence analysis) forming a distinct colony morphology surrounding the methanotrophic colonies. From the remaining six methane-oxidizing cultures, several different colony morphologies were found upon plating. Subsequent purification of these MOB was achieved after a maximum of three sub-cultivation steps. One dilution series resulted in a monoculture (identified as *Ancylobacter* with 16S rRNA gene sequence analysis), but did not oxidize CH_4. Fourteen dilutions were discarded since these were not able to oxidize CH_4 and did not result in a pure culture upon plating.

Dilution plating was performed in parallel. The 23 initial methane-oxidizing enrichments were diluted, plated on dNMS (solidified with gellan gum) and incubated under atmospheric conditions supplemented with CH_4. Randomly, 200 colonies were picked up and purified. Almost all purified isolates (197 out of 200) showed heterotrophic growth on diluted TSA without CH_4 and were therefore not considered as potential MOB. The three remaining isolates, not able to grow on diluted TSA, also failed to grow on solid dNMS with or without CH_4 added to the

headspace. To confirm these results, eight randomly selected isolates obtained via dilution plating were identified to the genus level through 16S rRNA gene sequence analysis. These sequences were affiliated with *Nocardioides*, *Zoogloea*, *Rhizobium*, *Pseudomonas*, *Polaromonas*, *Rhodobacter* and *Enterobacter*, genera not harbouring known MOB.

Identification and characterization of MOB isolates

In total, 22 purified MOB isolates were retrieved from four different samples (Table 1). Dereplication with rep-PCR fingerprint analysis grouped the isolates into 11 distinct clusters, representing 11 unique strains (Fig. 1). Randomly chosen representatives of each strain were further identified to the genus level with 16S rRNA gene sequence analysis. Ten out of 11 methanotrophic strains were assigned to the gammaproteobacterial genus *Methylomonas*, while the remaining strain was assigned to the alphaproteobacterial genus *Methylosinus* (Table 1). Pairwise comparisons of the 16S rRNA gene sequences of the 10 newly isolated strains with the type strains of all species of *Methylomonas* suggested that representatives of potentially novel *Methylomonas* species were isolated from all four environments, with 16S rRNA gene sequence similarities below 98% (Stackebrandt and Ebers, 2006). The 11 reference strains contained the *pmoA* gene, seven of which also harboured the *mmoX* gene and showed sMMO activity (Table 1): six *Methylomonas* strains isolated from the WWTP sample as well as the *Methylosinus* sp. strain.

Detection of the isolated MOB in fast-growing methanotrophic communities

Sequence batch enrichments from the original samples were set up in parallel with the dilution series enrichments used for MOB isolation, with the same cultivation conditions.

To evaluate the presence of the isolates, under conditions selecting for highly active fast-growing MOB in a mixed community, a sequence batch set-up with a relatively low sludge retention time of 90 h was chosen. All sequence batch enrichments showed methane oxidation activity, although the moment that a significant methane oxidation (a drop below the initial average methane concentration subtracted by five times the standard deviation) was observed differed between samples: 72 h for the WWTP cultures, 96 h for the wetland cultures and 144 h for the cultures inoculated with samples from the slurry pit or the biofilter material (Fig. S2). A steep rise in the methane oxidation rate (MOR) over time was observed, with a maximum after 144 h of 169 $\pm$ 28 mg CH_4 l^{-1} day^{-1} and 184 $\pm$ 24 mg CH_4 l^{-1} day^{-1} for the WWTP and wetland

Table 1. Genus assignment of 11 representative MOB strains (rep-PCR; Fig. 1) based on 16S rRNA gene sequence analysis (> 1400 bp).

				Type strain with highest 16S rRNA gene sequence similarity to query sequence			
Sample	Representative Strain	sMMO & *mmoX*	Genus identification	Species name	Strain number	Sequence similarity	Accession number
WWTP	D1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.5%	AF304196
	C1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.4%	AF304196
	G1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	E1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	H1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	K1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.3%	AF304196
	B1	No	*Methylomonas*	*Methylomonas scandinavica*	SR5^T	97.5%	AJ131369
Slurry Pit	I1	No	*Methylomonas*	*Methylomonas scandinavica*	SR5^T	97.5%	AJ131369
Wetland	A6[a]	No	*Methylomonas*	*Methylomonas fodinarum*	ACM 3268^T	96.2%	X72778
	J1	No	*Methylomonas*	*Methylomonas fodinarum*	ACM 3268^T	95.6%	X72778
	F1	Yes	*Methylosinus*	*Methylosinus sporium*	NCIMB 11126^T	98.9%	Y18946
Biofilter	A6[a]	No	*Methylomonas*	*Methylomonas fodinarum*	ACM 3268^T	96.2%	X72778

a. Five wetland and two biofilter isolates clustered together according to rep-PCR, wetland isolate A6 was randomly selected as representative strain.
Similarity values of 16S rRNA gene sequence to closest type strain, origin of the strains and results of naphthalene oxidation assay and *mmoX* gene amplification are given.

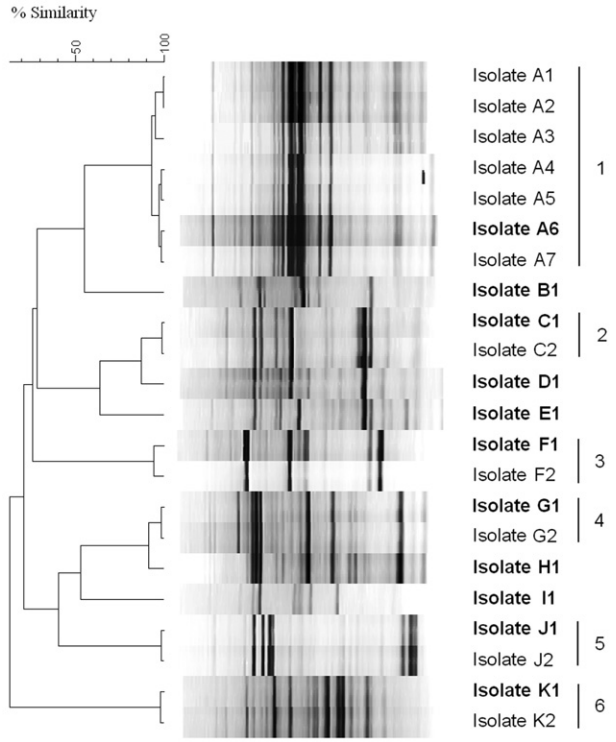

Fig. 1. GTG_5 rep-PCR fingerprinting using Pearson product moment correlation coefficient and UPGMA. MOB isolates were divided into 11 groups: 5 isolates showing unique profiles (B1, D1, E1, H1 and I2) and 6 groups of isolates (1–6) showing identical profiles. F1 and F2 isolates belonged to the genus *Methylosinus*, all the other isolates were identified as members of *Methylomonas* (Table 1). A representative of each cluster was selected randomly (indicated in bold) for further characterization.

cultures respectively. The maximal MOR was lower for the two other cultures, with 83 ± 3 mg CH_4 l^{-1} day^{-1} after 192 h and 39 ± 45 mg CH_4 l^{-1} day^{-1} after 216 h for the slurry pit cultures and the biofilter cultures respectively. When the oxygen concentration in the reactors became limiting, a decrease in the MOR was observed and a second cycle was started. The observed daily MOR for the second cycle was of the same order for all four inocula with a minimum of 108 ± 16 (biofilter) and a maximum of 266 ± 7 (wetland) mg CH_4 l^{-1} day^{-1} (Table S1).

PmoA DGGE analyses (Fig. 2) were performed on the 11 representative strains selected following rep-PCR fingerprinting (Fig. 1) and SBR enrichments after the first cycle of each environmental sample. DGGE profiles of WWTP and wetland were more diverse than those of the biofilter and slurry pit. From the seven *Methylomonas* strains isolated from the WTTP only one (represented by isolate K1) could not be traced back to the complete profile of the SBR enrichment, while this was the case for the other six strains, suggesting that these were dominantly present. It is however clear that different strains (isolates C1, D1, G1, E1 and H1), as proven by rep analysis, did show a *pmoA* band at a similar height, indicating that one band of the complete profile from the SBR enrichment covered a diversity of different methanotrophic strains. From the wetland, the two retrieved *Methylomonas* strains (represented by isolate A6 and J1) could be traced back to the SBR enrichment, while this was not the case for the single *Methylosinus* strain (isolate F1). The two isolates retrieved from the biofilter formed a single stable rep cluster with isolates from wetland, represented by isolate A6. The *pmoA* band of this strain was also observed in the DGGE profile of the biofilter, again suggesting the dominant presence in the SBR enrichment of this sample. From the slurry pit, only one strain was isolated for which the matching *pmoA* band could not be observed in the DGGE profile of the SBR enrichment. However, the *pmoA* band of this strain

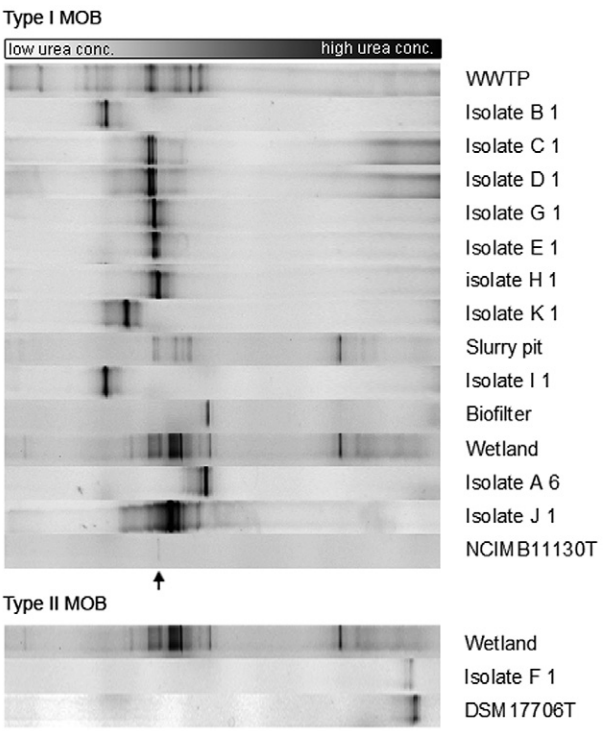

Fig. 2. DGGE analysis, based on the *pmoA* primer set A189fGC/mb661r for type I and type II MOB respectively. The community in the active enrichment from one sequence batch reactor is shown after the first cycle for each of the four samples, i.e. a sample from the wastewater treatment plant (WWTP), a slurry pit, biofilter material and a wetland. The 11 representative strains selected based on rep-PCR fingerprinting (Fig. 1) are shown in relation to their corresponding active enrichment. Strains $NCIMB11130^T$ (*Methylomonas methanica*, band indicated by arrow) and $DSM17706^T$ (*Methylosinus sporium*) are shown as reference. Based on the band position of the strains, eight out of 11 representative strains could be linked to their active community, demonstrating the abundance of these strains in a mixed community selecting for fast-growing methane-oxidizing bacteria.

had a similar GC content as isolate B1 from WWTP, of which rep analysis already showed that they represented different strains.

Discussion

Methane-oxidizing bacteria can serve as important sinks for the greenhouse gas methane or as key players in different biotechnological industries (Semrau *et al*., 2010). Currently, their applicability is limited by the number of suitable strains readily available, which were not specifically isolated for this purpose, and the lack of the necessary properties for efficient use in large-scale industrial applications (Jiang *et al*., 2010). Therefore, novel methanotrophic strains need to be easily obtained in culture and characterized. However, the isolation of MOB with the conventional plating approach is laborious and time-consuming, requiring one or more liquid enrichment steps followed by serial dilution plating and extensive purification (Whittenbury *et al*., 1970; Bowman *et al*., 1993; Bodrossy *et al*., 1995; Dedysh *et al*., 1998b; Iguchi *et al*., 2010). Therefore, in this study, a simple and miniaturized isolation protocol was applied to efficiently isolate MOB by a combination of several recently developed cultivation procedures: a prolonged initial enrichment at low temperatures (Song *et al*., 2009) and adaptation of incubation conditions for desired MOB (Dedysh *et al*., 1998a), followed by high-throughput extinction culturing (Button *et al*., 1993; Wise *et al*., 1999; Connon and Giovannoni, 2002; Bodelier *et al*., 2005) and subsequent purification using gellan gum plates (Janssen *et al*., 2002; Dedysh *et al*., 2007). Other high-throughput culturing approaches were designed to favour the isolation of abundant bacteria *in situ* (Connon and Giovannoni, 2002; Rappe *et al*., 2002; Stingl *et al*., 2007; Song *et al*., 2009), while our protocol specifically targeted a certain sub-population of a specific functional group with a custom-made prolonged enrichment. This made the organisms of interest, in this case fast-growing MOB, abundant *ex situ* before extinction culturing. This approach resulted in immediate pure cultures, avoiding elaborate purification, which is known to be problematic for the isolation of MOB (Bowman, 2006). Without this approach, no immediate MOB pure culture would be obtained since heterotrophic bacteria are more abundantly present in environmental samples (Wise *et al*., 1999). In perspective of biotechnological applications, varying the cultivation parameters of the initial enrichment in combination with miniaturization in 96-well plates has the potential to select for MOB with specific desired characteristics. As such, these wanted MOB can become abundant, even if non-abundant *in situ*, and can then be rapidly isolated via miniaturized extinction culturing. For example, the cultivation conditions chosen in this study, a diluted NMS medium under a high concentration of CH_4 and a relatively low hydraulic retention time, are known to select for highly active fast-growing MOB, a characteristic which is important for industrial use of bacteria (Begonja and Hrsak, 2001; Schrader *et al*., 2009; Wendlandt *et al*., 2010). Indeed, the mixed SBR communities showed a high methane removal rate from 108 ± 16 to 266 ± 7 mg CH_4 l^{-1} day^{-1} respectively, which is in the range of reported highly active methane-oxidizing communities (Melse and Van der Werf, 2005; Nikiema *et al*., 2005; Gebert and Grongroft, 2006; Scheutz *et al*., 2009). By band position analysis of *pmoA*-targeted DGGE, most isolated MOB could be traced back to this active community, demonstrating that the isolated strains were able to rapidly oxidize methane and grow to higher densities in a competitive setting. Most isolated MOB were indeed closely related to *Methylomonas* (Type Ia), known to harbour fast-growing MOB with a short generation time of 3.5 h (Whittenbury *et al*., 1970). Fast-growing Type Ib MOB (i.e. *Methylococcus*) with similar doubling times (Whittenbury *et al*., 1970) were not expected to be retrieved because they require higher isolation temperatures, while type II MOB are generally known to grow slower, with generation times ranging from 5 h up to several days (Whittenbury *et al*., 1970; Dedysh *et al*., 2000; 2002; Vorobev *et al*., 2010). Since DGGE patterns mainly represent the major constituents of a community (Muyzer and Smalla, 1998), the few strains that could not be traced back by band position analysis probably were expected to have longer generation times and as such were not abundantly present in the highly active methane-oxidizing communities. Not all *pmoA* bands in the mixed communities had isolated representatives, therefore an up-scaling of the isolation campaign, with more extinction cultures per enrichment, would detect more novel MOB cultivable under the set conditions. This was confirmed as extinction series from initial enrichments did lead to the isolation of two different MOB strains (strains E1 & H1 and strains B1 & D1).

In total, 22 MOB isolates belonging to 11 distinct strains were obtained, which is a relatively large number compared with other studies (Dianou and Adachi, 1999; Auman *et al*., 2000; Bussmann *et al*., 2004; Miller *et al*., 2004). Members of *Methylomonas* (type I MOB) were isolated from all four samples, while *Methylosinus* representatives (type II MOB) were only obtained from the wetland sample. Despite that isolates were assigned to known genera, they represented at least 10 different *Methylomonas* strains, several of which belonging to novel species within the genus based on 16S rRNA gene sequence dissimilarity with *Methylomonas* type strains. Currently, four species have been validly described, although only the type strain of *Methylomonas methanica* (NCIMB 11130^T) is still accessible for the scientific community. This issue on availability of fastidious microorgan-

isms such as MOB is a widely recognized problem and greatly hampers the in-depth investigation of their biotechnological potential, especially because properties such as substrate affinity, growth rate, substrate range or degradation of xenobiotics are strain-dependent features. For example, six different *Methylomonas* strains isolated in this study possessed sMMO, in addition to pMMO, while the type strain of the genus does not possess sMMO (Koh *et al*., 1993). Both sMMO and pMMO are known to degrade pollutants, such as chlorinated hydrocarbons (Bowman *et al*., 1993; Jiang *et al*., 2010): sMMO has a broader substrate range and is known to rapidly degrade pollutants, pMMO degrades compounds at slower rates but over an extended time frame (Semrau *et al*., 2010). While common in type II & Ib MOB (Hanson and Hanson, 1996), few type Ia MOB such as *Methylomonas* contain sMMO although it has been reported (Koh *et al*., 1993; Shen *et al*., 1997; Auman *et al*., 2000; Bussmann *et al*., 2006). Since type I MOB have a higher efficiency in carbon conversion (Scheutz *et al*., 2009), these *Methylomonas* isolates, which possess both sMMO and pMMO, could particularly be of interest to screen for degradation of recalcitrant compounds.

In this study, the isolation efficiency for the retrieval of fast-growing MOB between miniaturized extinction culturing and conventional dilution plating was compared. To our knowledge, such a comparison has not been previously reported. The plating approach, which is the most applied method for isolation of MOB (Dunfield *et al*., 2003; Dedysh *et al*., 2004; Heyer *et al*., 2005; Tsubota *et al*., 2005; Wartiainen *et al*., 2006), did not render any MOB in this study when performed in parallel with extinction culturing from the same initial enrichments. However, the applied methodology should allow their isolation since MOB isolates obtained from extinction culturing and reference strains from public bacteria collections were cultivated successfully on solid medium in the same manner. Therefore, it is likely that additional plating trials investigating more colonies would allow the isolation of MOB. However, even then miniaturized extinction culturing from initial enrichments will be more time and labour efficient than conventional plating in retrieving numerous methane-oxidizing bacteria with specific desired characteristics.

Experimental procedures

Isolation of methane-oxidizing bacteria

Samples were taken from (i) the top layer of a denitrification tank of a wastewater treatment plant (WWTP, Ossemeersen, Gent, Belgium), (ii) a covered but aerobic slurry pit of a cow stable (Melle, Belgium), (iii) the top litter layer of a wetland (Bourgoyen, Gent, Belgium), and (iv) the biofilter of an anaerobic digester (DRANCO-process, Brecht, Belgium).

On the day of sampling, 3 g of each sample was homogenized in 27 ml of a 5 times dNMS medium (Dunfield *et al*., 2003), with a modified copper concentration (0.8 μM Cu^{2+}) and a 2 mM Na_2HPO_4/KH_2PO_4 buffer. The pH of the medium was adjusted to the pH of each sample (biofilter material: pH 7.8; other samples: pH 6.8). Dilution series of samples (10^{-2} up to 10^{-11}) were prepared in dNMS medium in triplicate. The resulting 120 vials were sealed and 20% (v/v) CH_4 was added to the headspace. The cultures were incubated for 5 weeks at 20°C while shaken (90 r.p.m.). Weekly, concentrations of CH_4, O_2, CO_2 and N_2O in the headspace were analysed with a Compact GC (Global Analyser Solutions, Belgium). Dilutions were considered positive for methanotrophic growth when CH_4 and O_2 were consumed (drop below initial average subtracted by five times the standard deviation), with subsequent rise in CO_2 levels, and observation of turbidity. Available in triplicate for each dilution, this information was used to estimate the abundance of cultivable MOB using Most Probable Number (MPN) tables (Anonymous, 2008).

To retrieve MOB from the enrichment cultures showing methanotrophic activity, dilution plating and extinction culturing were carried out in parallel. A schematic overview of the followed methodology is shown in Fig. 3.

Dilution plating was performed by inoculation of the enrichment cultures (10^{-2}, 10^{-4} and 10^{-6}; 100 μl per plate) on dNMS medium with 0.9% gellan gum and 1% $MgSO_4 \cdot 7H_2O$. After incubation for 2 weeks at 20°C in gas-tight jars under a CH_4 : air (1:1) atmosphere, 200 colonies were randomly selected and subcultured to purity. The isolates were checked for methanotrophy by (i) growth on solid dNMS with methane in the headspace, (ii) absence of growth on solid dNMS under air, and (iii) absence of growth on 1/10 Trypticase Soy Agar (TSA) under air.

Extinction culturing was performed in duplicate by serially diluting the enrichment cultures (10^{-2} to 10^{-9}) with liquid dNMS medium (dilution-to-extinction) in sterile 96-well microtitre plates. After incubation for 2 weeks at 20°C under a CH_4 : air (1:1) atmosphere, turbidity indicative of growth was checked visually and by measuring the optical density at 600 nm. For each dilution series, the highest dilution showing growth was (i) confirmed for methane oxidation by GC analysis (see above), and (ii) plated on solid dNMS medium (with gellan gum) and subcultured to purity if necessary. MOB purity was evaluated by (i) colony morphology, (ii) phase-contrast microscopy, and (iii) absence of growth on 1/10 TSA and dNMS plates supplemented with 0.1% glucose, 0.1% fructose and 0.1% yeast extract under air. The isolates were confirmed for methane oxidation by GC analysis.

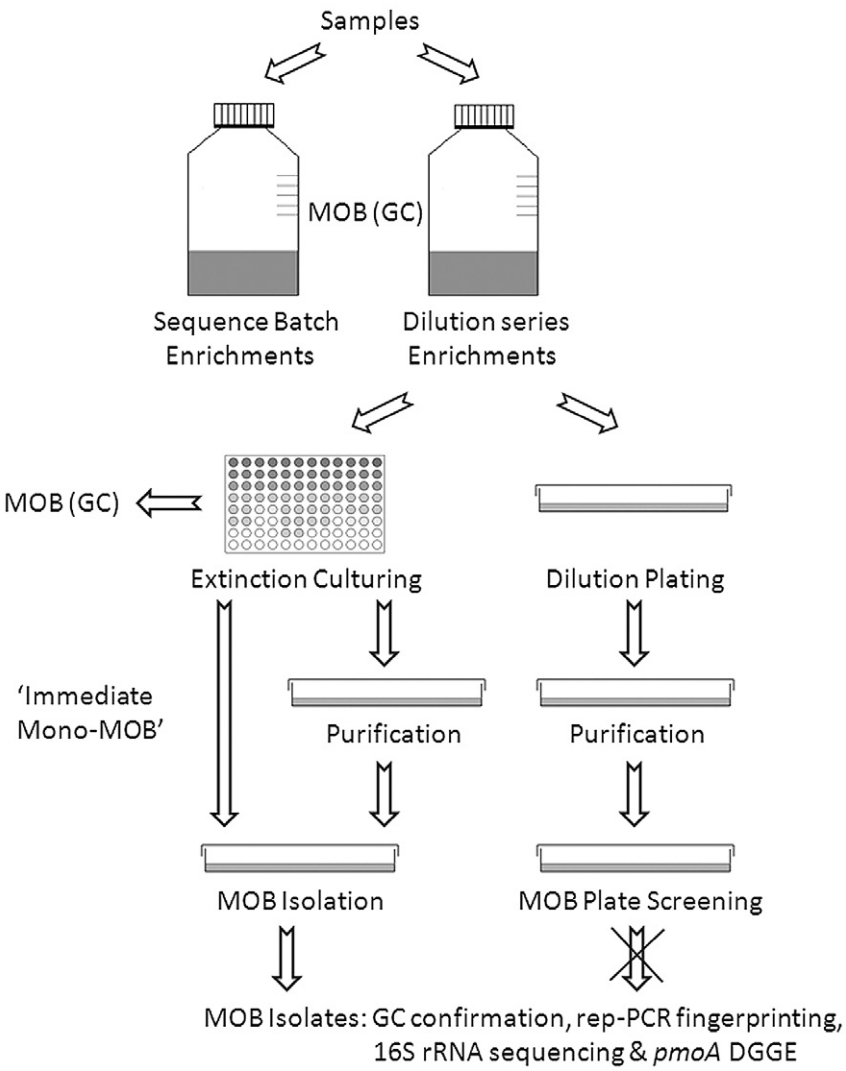

Fig. 3. Flow chart of the followed methodology.

Methane oxidation rate of sequence batch enrichments

In parallel with the dilution series enrichments used for MOB isolation, sequence batch enrichments using the same cultivation conditions were set up in triplicate on the day of sampling for each of the four original samples, allowing the estimation of MORs of the methanotrophic communities cultivable under set conditions. Gastight Schott bottles with a total volume of 1150 ml were filled with 200 ml dNMS medium. Inoculation was performed with 2 ml of the original sample. For the sample of biofilter material, 0.5 g of inoculum was added. After sealing the reactors, 20% (v/v) CH_4 was added to the headspace (950 ml). The cultures were placed on a shaker (100 r.p.m.) at 20°C and GC analysis of the headspace was performed daily. When the activity dropped to almost zero, reactors were opened under non-sterile conditions after which 160 ml of liquid phase was removed for physicochemical analysis or stored at −20°C for *pmoA* gene DGGE analysis (see further). The remainder of the liquid phase (40 ml) of the triplicate sequence batch enrichments was merged together and subsequently distributed equally over the three reactors. Freshly made dNMS medium was added to a total volume of 200 ml after which 20% (v/v) CH_4 was again added to the headspace. In total, this cycle was repeated two times. The hydraulic retention time and sludge retention time for each cycle were 90 h.

Identification and characterization of MOB isolates

Dereplication of all isolates was performed to assess genetic heterogeneity and group isolates with identical genomic fingerprints, further referred to as strains. Rep-PCR was performed as described by Ghyselinck and colleagues (2011) with a $(GTG)_5$-primer (Versalovic *et al.*, 1994). The clustering method was supported by visual inspection: isolates were considered as genomically iden-

tical when they demonstrated identical fingerprints, which led to a cut-off value of 93%. For each group, a representative strain was selected randomly and deposited in the BCCM/LMG Culture Collection (LMG 26258–26263 & LMG 26612–26616).

Each strain was identified to the genus level through 16S rRNA gene sequence analysis. PCR amplification and sequencing of the 16S rRNA gene was performed as described by Heyrman and Swings (2001). The sequences were analysed using a 3130 XL Genetic Analyzer (Applied Biosystems, USA) and assembled with BioNumerics 5.1 software (Applied Maths, Belgium). A reliable genus identification was obtained in two steps: (i) query in the 'Classifier' program of the Ribosomal Database Project II (Cole *et al*., 2005) of the 16S rRNA gene sequence of each new strain; and (ii) all type strains of all species of all genera mentioned in the Classifier report were compared in an exhaustive pairwise manner with the query sequence of each new strain in BioNumerics 5.1. Strains were provisionally assigned to the genus of their closest type strain based on the obtained 16S rRNA gene sequence.

A slightly modified version of the naphthalene oxidation assay of Brusseau and colleagues (1990) was used to measure sMMO activity of the MOB strains. A crystal of naphthalene was added to 5 ml freshly grown culture, in dNMS without copper addition, and incubated at 28°C on a shaker (150 r.p.m.) for 2 h. After incubation, 20 μl of freshly prepared tetrazotized-o-dianisidine solution (2.68 g l^{-1}) was added to 180 μl of each cell suspension in duplicate in microtitre plates, and the formation of a coloured diazo-dye was immediately monitored by recording the absorbance at a wavelength of 525 nm via spectrophotometry. The assay was validated using four MOB reference type strains (Table S2) that possess sMMO (DSM 17706^T, DSM 15673^T, DSM 18500^T, NCIMB 11131^T) and four that only possess pMMO (DSM 13736^T, DSM 17261^T, NCIMB 11914^T, NCIMB 11130^T). Primers described in literature for amplification of the *mmoX* gene were also tested using these type strains (Table S2). Primers described by Hutchens and colleagues (2004) were selected for *mmoX* gene amplification of the isolates positive for the sMMO activity assay. Amplification was confirmed by subsequent sequencing of the *mmoX* gene.

pmoA *gene DGGE analysis*

Primers described in literature were tested for suitability for DGGE analysis targeting either 16S rRNA or *pmoA*, since most known MOB, except for members of *Methylocella* and *Methyloferula* (Dedysh *et al*., 2005; Vorobev *et al*., 2010), as well as all novel strains from this study contained *pmoA*. Thirteen type strains (six type I and seven type II MOB) were used as positive controls for the evaluation. These strains, their main properties, the primers tested and results obtained are listed in Table S2. The PCR mix and temperature-time profiles from the original description were tested, as well as the PCR mix used for 16S rRNA gene amplification (Heyrman and Swings, 2001). Only one *pmoA* primer set (A189f/mb661r) could correctly detect the pMMO gene in all tested strains. The 16S rRNA gene could be amplified in all strains with both the Type IF/Type IR and Type IIF/Type IIR sets of Chen and colleagues (2007); however, amplicons were too long for DGGE analysis. The *pmoA* set A189f/mb661r (Costello and Lidstrom, 1999) with GC clamp 5′-CGC CCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGG-3′ was selected for further DGGE analysis.

The DNA extraction procedure was adapted from Gevers and colleagues (2001) and El Fantroussi and colleagues (1999). DGGE analysis of the PCR amplicons was performed with an INGENY phorU2X2 DGGE-system (Goes, The Netherlands). A 6.5% (w/v) polyacrylamide gel with a 30–80% denaturing gradient [a 100% denaturant solution contains 7 M urea and 40% (w/v) formamide] was applied. Gels were run in 1× TAE buffer for 16 h at 150 V and stained afterwards with SYBR Green I nucleic acid gel stain. The resulting DGGE patterns were processed using BioNumerics 5.1. Band position analysis was used to track the obtained isolates back to the sequence batch cultures and the dilution series enrichments used for MOB isolation and was performed by visual comparison of band location in the gel.

Nucleotide sequence accession numbers

The 16S rRNA gene sequence data generated in this study has been deposited in GenBank/EMBL/DDBJ with accession numbers FR798952 to FR798973.

Acknowledgements

This work was funded by the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005) and a PhD grant for David van der Ha from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, SB-83259). The authors gratefully thank Willy Verstraete, Suzanne Read and Jonas Ghyselinck for proof reading the manuscript. Tim Lacoere and Joke Buyse are greatly acknowledged for their help with the molecular analyses.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Average methane (A, D, G, J), oxygen (B, E, H, K) and carbon dioxide (C, F, I, L) levels (%) during 5 weeks incubation in dNMS at 20°C (90 r.p.m.) under a CH_4 : air (1:4) atmosphere for the WWTP (A, B, C) slurry pit (D, E, F), wetland (G, H, I) and biofilter sample (J, K, L) for the dilution series 10^{-2} (white square), 10^{-3} (white triangle), 10^{-4} (black triangle) 10^{-5} (black square) and 10^{-6} (black diamond) in triplicate (error bars not shown for clarity).

Fig. S2. The average methane oxidation rate ($n = 3$, mg CH_4 $l^{-1}_{liquid\ phase}$ day^{-1}) of sequence batch enrichments of the original samples during the first cycle, for the sample of the wastewater treatment plant (black circle), slurry pit (white circle), wetland (black triangle) and biofilter material (white triangle). One-sided error bars are shown for clarity, with the same line type.

Table S1. Overview of activity parameters of enriched cultures during the second cycle in sequence batch reactors: the methane oxidation rate (mg CH_4 l^{-1} liquid day^{-1}) during the first and second day of the second cycle for the four samples, the ratio of produced CO_2 over consumed CH_4 (mg CO_2-C mg^{-1} CH_4-C), the ratio of produced volatile suspended solids over consumed CH_4 (mg VSS mg^{-1} CH_4-C) and the ratio of consumed CH_4 over consumed NO_3^- (mg CH_4-C mg^{-1} NO_3^-N).

Table S2. 16S rRNA, *pmoA* and *mmoX* gene primer set evaluation using 13 MOB reference type strains.

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