First publ. in: FEMS Microbiology Ecology 47 (2004), 2, pp. 179-189

Preferential cultivation of type II methanotrophic bacteria from littoral sediments (Lake Constance)

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Received 5 May 2003; received in revised form 4 August 2003; accepted 29 September 2003

First published online 19 November 2003

Abstract

Most widely used medium for cultivation of methanotrophic bacteria from various environments is that proposed in 1970 by Whittenbury. In order to adapt and optimize medium for culturing of methanotrophs from freshwater sediment, media with varying concentrations of substrates, phosphate, nitrate, and other mineral salts were used to enumerate methanotrophs by the most probable number method. High concentrations (>1 mM) of magnesium and sulfate, and high concentrations of nitrate (>500 μM) significantly reduced the number of cultured methanotrophs, whereas phosphate in the range of 15–1500 μM had no influence. Also oxygen and carbon dioxide influenced the culturing efficiency, with an optimal mixing ratio of 17% O_2 and 3% CO_2; the mixing ratio of methane (6–32%) had no effect. A clone library of *pmoA* genes amplified by PCR from DNA extracted from sediment revealed the presence of both type I and type II methanotrophs. Nonetheless, the cultivation of methanotrophs, also with the improved medium, clearly favored growth of type II methanotrophs of the *Methylosinus*/*Methylocystis* group. Although significantly more methanotrophs could be cultured with the modified medium, their diversity did not mirror the diversity of methanotrophs in the sediment sample detected by molecular biology method.

Keywords: Cultivation; Methanotroph; Terminal restriction fragment length polymorphism; Sediment; Medium composition

1. Introduction

Methanotrophic or methane-oxidizing bacteria (MOB) are a small but important group of bacteria that use CH_4 as their sole source of carbon and electrons. There is an increasing interest in MOB because of their importance in greenhouse gas consumption and their potential application in bioremedial degradation of industrial pollutants such as trichloroethylene [1]. Since the fundamental work on enrichment and isolation of MOB was set by Whittenbury and co-workers [2], the growth medium (with either ammonium or nitrate as N source) designed in their studies has been used with minor modifications for isolation of MOB from a broad range of environments such as tundra soils [3], hot springs [4], peat bog [5], seawater [6], and freshwater sediments [7]. Studies using the original source water for enrichment from the respective study site have led to the isolation of novel types of MOB, which indicates that the commonly used techniques for detection and culturing of MOB remain to be improved [6,8].

Despite major advances in molecular ecology and systematic of MOB [9], only few studies have tried to improve the cultivation conditions. A modified version of Whittenbury's medium with reduced substrate concentrations has been used for enrichment of MOB in counter-gradients of CH_4 and O_2 [10]. The crucial factors of acidic or alkaline environments, like pH and salt concentration, have been adapted in new media for these environments [5,11]. However, no systematic studies on medium optimization for methanotrophs from freshwater lakes have been performed.

MOB include species in the α-*Proteobacteria* (type II MOB) and in the γ-*Proteobacteria* (type I MOB) [9]. The oxidation of methane to methanol is carried out by either a soluble or a particulate membrane-associated form of methane monooxygenase (sMMO and pMMO, respectively) [1]. The pMMO genes are universal in MOB, with a possible exception, that is *Methylocella palustris* [12].

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One gene of this operon, *pmoA*, is evolutionarily strongly conserved and can serve as functional phylogenetic marker for MOB [6].

The aim of the present study was to develop growth medium yielding an increased 'cultivation efficiency', i.e. a medium suitable for the culturing of MOB from freshwater sediments with the highest number and the greatest diversity possible. We systematically modified the concentrations of phosphate, nitrate, and other mineral salts, and the composition of the incubation atmosphere in order to cultivate significantly more methanotrophs than with the standard medium. After extraction of DNA from the sediment and from most probable number (MPN) enrichments, followed by polymerase chain reaction (PCR) amplification of the *pmoA* gene, a clone library and terminal restriction fragment length polymorphism (T-RFLP) analysis were used to compare the selectivity of the different media in relation to the diversity of MOB in sediment samples.

2. Materials and methods

2.1. Study site and sediment sampling

Experiments were carried out with sediment samples from the littoral zone of Lake Constance, Germany. Methane production rates in the littoral sediments are high and show strong seasonal variations [13]. At the study site, CH_4 concentrations in the sediment ranged from 2 to 24 μM directly below the sediment surface, and maximal concentrations of 200 μM were measured at 15 cm depth. Methane concentrations in the sediment (in μmol per liter sediment) were measured by the headspace method [14]. Methane was analyzed with a Carlo Erba gas chromatograph with a flame ionization detector. The sediment consisted mainly of fine sand with a porosity of 0.62. Sediment cores (2.3 cm in diameter) were taken by scuba-diving between June 1999 and August 2002 at 2–5 m water depth near the Limnological Institute of the University of Constance. Sediment samples for the construction of a clone library and T-RFLP analysis were sampled in August 2001 and August 2002, respectively, with a sediment corer as described by Tessenow [15].

2.2. Enumeration and isolation of MOB

Serial dilutions were initiated by adding 2 ml of surface sediment sampled with a cut-off syringe to 18 ml of filter-sterilized lake water, followed by 1:2 dilutions up to 5×10^{-6}. The first two dilution tubes were mixed vigorously for 2 min. After allowing the sediment to settle for 1 min, an aliquot was transferred to the next dilution tube. A series of 12 dilutions in eight replicates were set up in one microtiter plate (Nuclon) filled with the respective medium. Plates were incubated at 16°C in the dark for 8 weeks. After this time no further increase of turbidity was observed. Growth of MOB was analyzed by measuring turbidity every 2 weeks with an Anthos HT II photometer at 595 nm. Wells were scored positive if the turbidity increased steadily over time. Plates incubated under air served as controls for non-methanotrophic growth. MPNs were calculated according to Russek and Colwell [16]. The small dilution steps and the high number of replicates allowed a good statistical resolution of the MPN values. MPN values with a non-overlapping 95% confidence interval were considered significantly different, with $P<0.05$. The influence of the nitrate and ion concentrations on the MPNs was tested with a regression analysis (StatView 4.5, Abacus Concepts). Data were log transformed to obtain a normal distribution. Because multiple regressions were performed the significance level was Bonferroni-adjusted (adjusted significance level = 0.05 divided through the number of analyses) [17]. The influence of the incubation atmosphere on the variability of the MPNs was tested with analysis of variance (ANOVA) (SAS/STAT 6.03, SAS Institute Inc., NC, USA). Data were log transformed to obtain a normal distribution and nested for sampling date. The significance level was set to 0.01%.

The highest positive dilutions were used for isolation of pure cultures. Most of the enriched microorganisms did not grow on agarose plates. Therefore, the enrichment cultures were serially diluted (10-fold) under the conditions described below. Highest positive dilutions were used as inoculum for four to five repetitive dilutions. The difficulty to grow MOB on solid medium has been reported also by Bowman and references therein [9]. Purity of the isolates was checked microscopically and by testing for growth on complex medium (0.05% w/v nutrient broth plus 0.01% yeast extract, Difco [18]).

2.3. Growth conditions and medium modifications

The diluted mineral medium contained the following salts (per liter): 0.1 g NaCl, 0.04 g $MgCl_2{\cdot}6H_2O$, 0.05 g KCl, 0.015 g $CaCl_2{\cdot}2H_2O$, 0.016 g Na_2SO_4, and trace element solution SL 10 (1 ml l^{-1}) [19]. In initial experiments the basic medium with a 10-fold concentration of the salts was used but proved not to be optimal and so the diluted solution was used for all subsequent experiments. K–Na–phosphate buffer (pH 7.2) and KNO_3 were added from 1 M sterile stock solutions. The standard medium contained 150 μM phosphate and 50 μM nitrate, and was buffered to pH 7.2 with 0.01 M HEPES. Microtiter plates were incubated in desiccators under an atmosphere of 17% O_2, 24% CH_4, 2% CO_2, and 57% N_2 (Table 1) unless stated otherwise. Gases were provided by Messer Griesheim, Krefeld, Germany, with analytical grades 2.8, 2.5, 3.5 and 5.0, respectively. This medium was modified further as follows: Buffers used were HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, Sigma), TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic

Table 1
Combinations of methane, oxygen, and carbon dioxide mixing ratios in the medium to test their influence on growth of methanotrophic bacteria

Mixture	Mixing ratio (%)[a]		
	CH_4	O_2	CO_2
A	5	6	0
B	5	13	2.1
C	5	17	8.2
D	5	23	3.5
E	14	6	2.1
F	14	13	3.5
G	14	17	0
H	14	23	8.2
I	24	6	3.5
J	24	13	8.2
K	24[b]	17[b]	2.1[b]
L	24	23	0
M	32	6	8.2
N	32	13	0
O	32	17	3.5
P	32	23	2.1

The equivalent molar concentration in the liquid phase was: methane (97, 227, 396 and 532 μM), oxygen (96, 195, 249 and 345 μM) and carbon dioxide (0, 0.86, 1.43 and 3.37 mM).
[a]The rest was filled up with nitrogen.
[b]Standard mixing ratios used in all other experiments.

acid, Fluka), MOPS [3-(*N*-morpholino)-propanesulfonic acid, Biomol], or phosphate (Na_2HPO_4/KH_2PO_4, Merck). After autoclaving the medium, the filter-sterilized buffers were added to 0.01 M final concentration at pH 7.2.

Phosphate and nitrate concentrations were given in 25 different combinations, ranging from 15 to 1500 μM phosphate and from 50 to 5000 μM nitrate.

In another experiment, different previously described media were compared for cultivation efficiency. The basic medium mentioned above was used also in a two-fold and 10-fold diluted version. Results obtained with the basic medium were compared with those obtained with published media [2,20], with these media modified by buffering with HEPES instead of phosphate, and with aged lake water and Milli-Q water (Millipore). In all media (except for the original medium of Whittenbury and of Heyer), the concentrations of nitrate, phosphate, buffer (HEPES), and trace elements were kept constant. This experiment was repeated twice.

The influence of the partial pressures of CH_4, O_2, and CO_2 on the cultivation efficiency was investigated in a series of experiments employing four levels of each gas, resulting in 16 combinations (Table 1). The accuracy of the gas mixtures was checked by gas chromatography, and the mixture was renewed every 2 weeks. This experiment was carried out in three replicates, and replicate plates were incubated in the same desiccator.

2.4. DNA extraction and PCR amplification

Cultures were centrifuged (10 min at 15 000 × *g*, 4°C) and cells were lysed by adding 800 μl Na–phosphate buffer (0.12 M, pH 8.0) and 260 μl sodium dodecyl sulfate (1%) to the pellet, and incubating for 1 h at 70°C [21]. The suspensions were centrifuged and the pellets were extracted again with the same volumes of phosphate buffer for 1 h at 70°C. The supernatants were combined and stored at 4°C. Thereafter, the procedure followed that of the DNA extraction from sediment as described below.

DNA from sediment (1 g fresh weight) was extracted using a modification of the bead-beating protocol described by Henckel et al. [22]. Modifications comprise protein and debris precipitation with phenol/chloroform/isoamyl alcohol (25:24:1, 0.5 ml) (Sigma) in phase-lock-gel tubes (Eppendorf) for samples analyzed by T-RFLP and DNA purification with polyvinylpolypyrrolidone spin columns (Sigma) [23].

Extracted DNA was used for amplification of *pmoA* gene fragments (531 bp) with the primer pair A189f and A682r [24]. For T-RFLP analysis, primer A189f was labelled with a fluorescent dye (IRD 700, pentamethine-carbocyanin, MWG). PCR reactions were performed in an Eppendorf thermocycler using a touch-down program and a recombinant Taq DNA polymerase (Eppendorf). PCR with sediment DNA was performed with HotStarTaq® DNA polymerase (Qiagen, initial denaturation for 15 min) for the clone library and with the FailSafe™ Enzyme Mix and PreMix C (Epicentre) for the T-RFLP analysis. Touch-down PCR was initiated at 95°C for 4 min, followed by six cycles of 1 min at 92°C, 1 min at 62°C by reducing the temperature in succession 1°C per cycle, and 45 s at 72°C, followed by 25 cycles of 1 min at 92°C, 1 min at 56°C, and 45 s at 72°C (29 cycles for enrichment analysis). The final extension step was at 72°C for 5 min. Aliquots of amplicons were checked by electrophoresis on 1% agarose gel.

2.5. Clone library

The clone library was constructed using the Invitrogen TA Cloning® kit (Invitrogen). Randomly selected clones were subjected to a Tooth-Pick PCR (recombinant Taq polymerase, MBI Fermentas) using the primer pair M13f and M13r. The Tooth-Pick PCR was initiated at 94°C for 3 min, followed by 26 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The final elongation step was at 72°C for 4 min. Amplicons were analyzed by RFLP using the restriction endonuclease *Msp*I (18 μl PCR product, 2 μl Y^+/Tango buffer, and 1.5 U *Msp*I) and clones were grouped according to the restriction pattern of their amplicons.

2.6. Sequencing and phylogenetic analysis

pmoA fragments of the isolated strains and *pmoA* fragments of representatives of different RFLP clone groups were sequenced from both ends at GATC Biotech AG

(Konstanz, Germany). Sequences were checked for chimeras by dividing them into two partial sequences of equal length and performing a Blast search at the NCBI website with the whole sequence and the two partial sequences (http://www.ncbi.nlm.nih.gov/) [25]. If the results of the Blast search differed to a larger extent, sequences were regarded as chimeras. Afterwards, sequence data were analyzed using the ARB software package [version 2.5b (http://www.arb-home.de) [26]]. New sequences were added to a *pmoA*/*amoA* database (constructed from publicly available sequences) and pre-aligned using the Fast Aligner Tool implemented in ARB. Alignments were always corrected manually. Phylogenetic distance dendrograms were constructed using the method of Fitsch and Margoliash as implemented in PHYLIP [27]. The construction of the dendrogram was based on global rearrangement, randomized input order (three jumbles), and 160 derived amino acids.

The sequences of *pmoA* gene fragments have been deposited in GenBank under accession numbers AY355386 to AY355400.

2.7. T-RFLP analysis

pmoA fragments were amplified as described above. Amplicons always resulted in the predicted size. The amplicons were purified with a MiniElute kit (Qiagen) and up to 5 ng DNA was digested with 3 U of the restriction endonuclease *Msp*I (MBI Fermentas). The digestions were carried out in a total volume of 20 µl for 3 h at 37°C. Five µl of the digested amplicons were mixed with 3 µl stop solution (LI-COR), denatured for 3 min at 95°C, and immediately placed on ice. The digestion products were electrophoretically separated on a polyacrylamide gel (5.5%) at 2000 V, 25 mA, 50 W, and 50°C using an automated DNA sequencer (model 4200, LI-COR). The signal gain of the automated sequencer was set to 300-fold. Sizes of terminal restriction fragments (T-RF) were calculated by comparison with molecular size markers (50–700 bp, LI-COR) using the Gelscan Professional software (version 5.02, BioSciTec, Frankfurt, Germany). The accuracy of T-RF length calculation was ±2 bp for fragments up to 400 bp, ±3 bp for fragments between 400 and 530 bp, and ±4 bp for fragments over 530 bp as was experimentally determined (details not shown). Signals representing fragments of 244, 278, 349, 445, 505, and 531 bp could be correlated with their exact length because of the usage of incompletely digested *pmoA* amplicons of the strains Wd-C12 and 2-8e as an internal standard (Fig. 5E,F). The corresponding restriction sites were known from the *pmoA* sequences of strains Wd-C12 and 2-8e.

3. Results

MPN enrichments of MOB were set up in microtiter plates. An increase in turbidity was an indication of methanotrophic growth. Preliminary experiments showed a good concordance of turbidity formation and CH_4 consumption, i.e. the contents of positively scored wells showed also CH_4 consumption when transferred into fresh medium (23 of 28 samples). Negative control cultures free of CH_4 never showed any increase in turbidity.

3.1. Optimization of growth medium composition

In an initial experiment, we tested the influence of the buffering system on the cultivation efficiency. HEPES-buffered medium yielded a significantly higher MPN (712 ± 178 cells ml^{-1}) than phosphate-buffered medium (303 ± 92 cells ml^{-1}), and slightly higher counts than TES-(430 ± 123 cells ml^{-1}) and MOPS-buffered media (480 ± 133 cells ml^{-1}). Therefore, we used HEPES buffer (10 mM) in the subsequent experiments.

The influence of phosphate and nitrate and their combinations on the efficiency of MOB culturing was also investigated. Maximal counts ($23\,510 \pm 5500$ cells ml^{-1}, Fig. 1) were obtained with 50 µM nitrate and 150 µM phosphate, minimal counts with 5 mM nitrate and 150 µM phosphate (62 ± 34 cells ml^{-1}). Phosphate concentration had no influence on the MPNs obtained. However, there was a significant positive correlation between nitrate concentration and the MPNs obtained (Fig. 1, log-transformed data, polynomial regression, $P_{slope} < 0.0001$, $r^2 = 0.90$, $n = 24$).

In order to optimize the mineral composition of the medium, we tested the dilutions of the basic medium and of the published media (Table 2) for their efficiency in cultivating MOB. In the first set of experiments, the basic medium yielded maximal MPNs (712 ± 178 cells $ml^{-1} = 100\%$). Aged lake water and modified Whittenbury medium (HEPES buffered with 150 µM phosphate and 50 µM nitrate) yielded lower MPNs (486 ± 125 and 483 ± 125 cells $ml^{-1} = 68\%$). The original Whittenbury medium (with

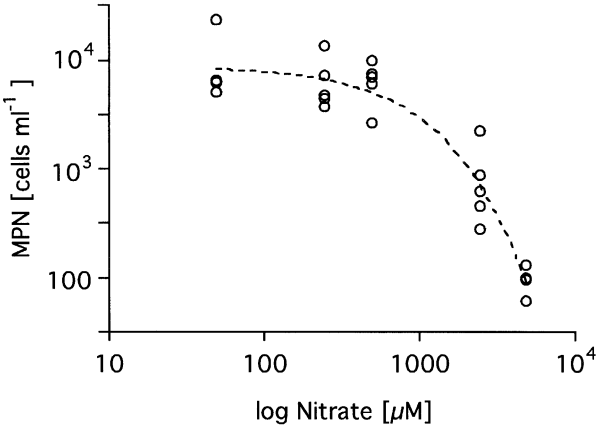


Fig. 1. Correlation between nitrate concentration in the incubation medium and the number of cultivated methanotrophic bacteria. With log-transformed data a polynomial regression was performed ($P_{slope} < 0.0001$, $r^2 = 0.90$, $n = 24$).

Table 2
Media and conditions used by different authors for cultivation of methanotrophic bacteria

Authors	Ion concentration (mM)									Ionic strength	Head-space (%)		pH
	Ca^{2+}	Cl^-	K^+	Mg^{2+}	Na^+	NO_3^-	PO_4^{2-}	SO_4^{2-}	Others	($\times 10^{-3}$ mol l^{-1})	CH_4[a]	CO_2	
This study[b]	0.10	2.67	0.76	0.20	1.92	0.05	0.15	0.11		4.0	5–32	3	7.2
Bowman, 2000 [9]	0.05	0.05	1.02	0.15	12.98	2.00	2.00	0.15	10 CO_3^-	16.7	15–20	3–5	6.8
Dedysh et al., 1998 [5] M2	0.06	0.12	1.14	0.16		0.99	0.15	0.16		2.8	50		3–6
Heyer et al., 1984 [20]	0.05	9.40	2.20	0.41	3.93		6.13	0.41	9.85 NH_4^+	25.1	20	5	7.2–7.4
Khmelenina et al., 1999 [11]	0.14	0.14	9.89	0.81	7.30	9.89	7.3	0.81		35.5	50		6.5 or 9
Roslev and Iversen, 1999 [53]	0.05	0.05	11.90	0.20	3.90	10.00	5.00	1.00		24.6	30		5.8–6.8
Whittenbury et al., 1970 [2]	1.36	1.36	10.10	4.06	0.41	9.89	0.62	4.06		31.6	30	1–2	6.8
Lake water[c]	1.07[d]	0.15[e]	0.12[c,e]	0.30[d]	0.29[c,e]	0.11[c]	0.15[c]	0.32[d]		2.1			
Milli-Q water[c]	0.001[d]	n.d.	0.09[c]	0.0001[d]	0.11[c]	0.05[c]	0.15[c]	n.dt.		0.4			

n.d. not determined; n.dt. not detectable.
[a]Balance: air.
[b]Medium according to Widdel 1988 [19], diluted 1:10, containing in addition KNO_3^- (50 μM) and K-Na-phosphate buffer (pH 7.2, 150 μM).
[c]With added KNO_3^- (50 μM) and K–Na–phosphate buffer (pH 7.2, 150 μM).
[d]Measured by ion chromatography and AAS.
[e]Data from Stabel, 1998 [54].

0.6 mM phosphate and 9.9 mM nitrate) yielded significantly lower MPNs than the basic medium (222 ± 68 cells ml^{-1} = 31%). In a second set of experiments with our basic medium, 5248 ± 1231 cells ml^{-1} were obtained (= 100%). Insignificantly higher counts were obtained with lake water (7298 ± 1787 cells ml^{-1} = 139%), Milli-Q water (7654 ± 1851 cells ml^{-1} = 146%), and 10-fold-diluted basic medium (8041 ± 1933 cells ml^{-1} = 153%). The medium of Heyer (5169 ± 1212 cells ml^{-1} = 98%), the HEPES-buffered Heyer medium (5856 ± 1377 cells ml^{-1} = 112%), and a two-fold-diluted version of our basic medium (5648 ± 1439 cells ml^{-1} = 108%) all yielded cell counts comparable to the basic medium. Again, Whittenbury medium yielded significantly lower MPNs (1290 ± 414 cells ml^{-1} = 25%), as well as the HEPES-buffered Whittenbury medium with 3233 ± 959 cells ml^{-1} (= 62%).

In a second step, the concentrations of each single ion and the total ionic strength were calculated. With a linear regression analysis we tested the influence of each single ion (Ca^{2+}, Cl^-, K^+, Mg^{2+}, Na^+, and SO_4^{2-}) and of the total ionic strength. The total ionic strength did not influence the culturing efficiency. However, the numbers of cultured MOB were significantly correlated with the concentration of magnesium and sulfate in the medium (Fig. 2).

3.2. Effect of the incubation atmosphere

In another experiment, we investigated the influence of CH_4, O_2, and CO_2 partial pressures on culturing efficiency. On the first of three sampling dates MPNs of methanotrophs ranged from 486 ± 117 cells ml^{-1} with gas mixture A (Table 1) to 13 053 ± 3049 cells ml^{-1} with mixture P. On the second sampling date MPNs ranged from 52 ± 27 cells ml^{-1} with mixture A to 6106 ± 1474 cells ml^{-1} with gas mixture O. On the third sampling date, MPNs were overall lower, and smaller differences were observed between the different settings; MPNs ranged from 248 ± 69 to 3076 ± 719 cells ml^{-1} with mixtures G and F.

An ANOVA with log-transformed data (nested within each sampling date) was used to test whether the different mixing ratios of CH_4, O_2, and CO_2 influenced the variability of the MPNs obtained. The variability of the MPNs was significantly influenced by oxygen and carbon dioxide ($P = 0.0051$ and $P = 0.0065$, $n = 46$), whereas methane had no influence on the MPNs (Fig. 3).

3.3. Clone library

A clone library was constructed from *pmoA* fragments amplified from the DNA extract of a sediment sample. Analysis of the clone library documented the presence of both type I MOB and type II MOB in littoral sediments of Lake Constance (Fig. 4). All *pmoA* clones representing type I MOB were distinct from the methanotrophs cultured thus far and formed three distinct clusters. Clones representing type II MOB clustered within the *Methylosinus*/*Methylocystis* group.

3.4. Isolates

The *pmoA* sequences from 13 strains isolated from the highest positive dilutions of different MPN enrichments were compared. All isolates clustered within the phylogenetic radiation of the type II MOB (Fig. 4). Sequencing results indicated that no isolate possessed different copies of the *pmoA* gene. Based on 99% sequence similarity, three groups could be distinguished. The first and second group were affiliated with *pmoA* sequences of the *Methylocystis* cluster: six isolates, represented by strain Wd-C12, were closely affiliated with *Methylocystis echinoides* and more distantly related to clone 11 whereas another six isolates, represented by strain 2-8e, were closely affiliated with clone 64 and *Methylocystis parvus* or *Methylosinus* sp. strain LW2. The third group, which consisted only of

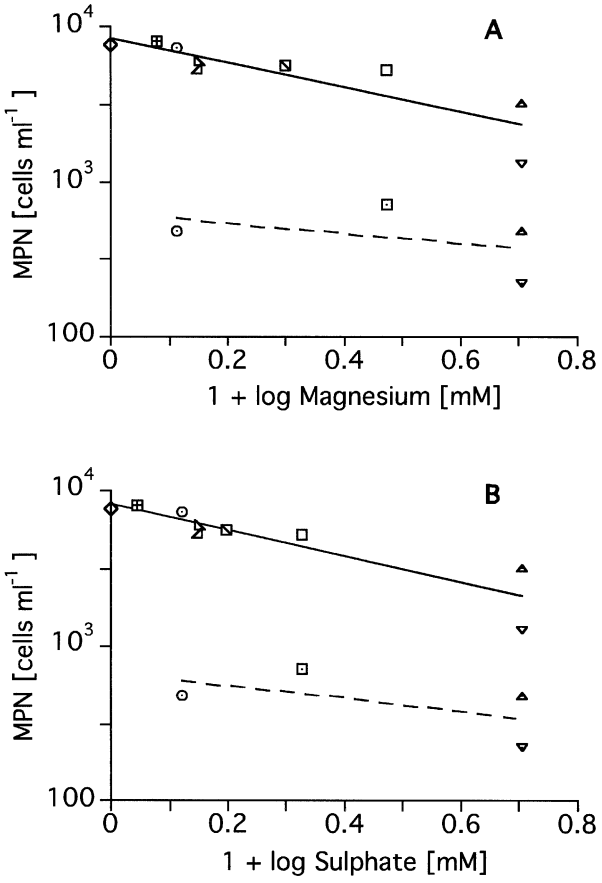


Fig. 2. Dependence of the number of cultivated methanotrophic bacteria on the concentration of (A) magnesium ($P_{slope} = 0.0034$, $r^2 = 0.73$, $n = 9$) and (B) sulfate ($P_{slope} = 0.0032$, $r^2 = 0.79$, $n = 9$) in the medium. Data were log transformed and a value of 1 was added to the ion concentration before log transformation. Data are from two experiments (–, - - -); the latter data were not included in the regression analysis. The different media were: ⊙ lake water, ◇ Milli-Q water, ◿ Heyer medium, ◺ modified Heyer medium, ▽ Whittenbury medium, △ modified Whittenbury medium, □ basic mineral medium, ◪ basic mineral medium diluted 1:1, ⊞ basic mineral medium diluted 1:10.

one single isolate (strain RG), was most closely related to *Methylosinus sporium* strain SE2 and to none of the clones. No isolates represented type I MOB.

3.5. T-RFLP analysis

In two independent experiments performed with inocula from different sediment cores, the number of microorganisms cultivated in Whittenbury medium was lower (7032 ± 1791 cells ml^{-1} = 60% or 11468 ± 2309 cells ml^{-1} = 23%) than that in the diluted basic medium (11717 ± 2773 cells ml^{-1} = 100% and 50797 ± 10664 = 100% cells ml^{-1}, respectively). However, the T-RF patterns of the enrichment cultures grown on Whittenbury medium were virtually identical to those obtained on the diluted basic medium in the respective dilutions (e.g. Fig. 5B,C). Comparison of the T-RF patterns within a dilution series of the same original sample in basal medium gave also quite similar patterns, except that the peak at 278 bp was sometimes lost in the higher dilutions (e.g. Fig. 5D). The same was true for dilution series with Whittenbury medium (details not shown).

The predicted T-RFs of all isolates were identical (244 bp). This was experimentally verified for the representative strains Wd-C12 and 2-8e. The corresponding peak was present in the T-RFLP profiles of all enrichment cultures. However, comparison with the sediment sample indicated that the MOB enriched and isolated on both media constituted only a minor fraction of the natural population of MOB (Fig. 5A). The T-RFLP profiles were recorded at very high sensitivity (signal gain: 300-fold) in order to detect also small MOB populations in the sediment sample; however, the peak representing the T-RF of 244 bp,

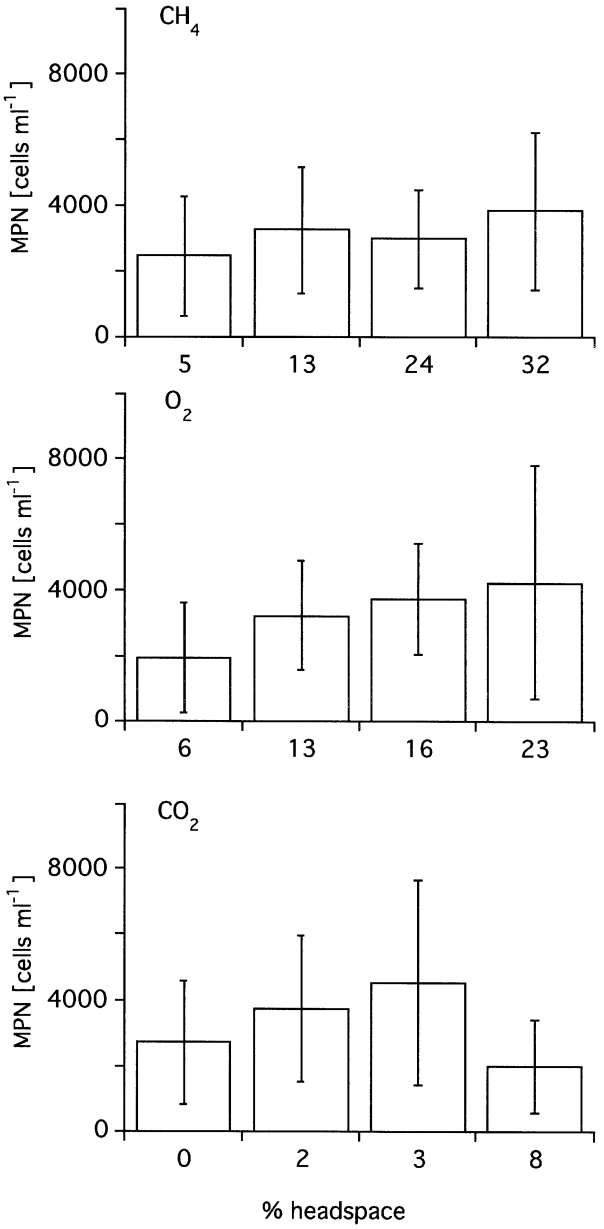


Fig. 3. The influence of incubation atmosphere (CH_4, O_2 and CO_2) on the number of cultivated methanotrophic bacteria.

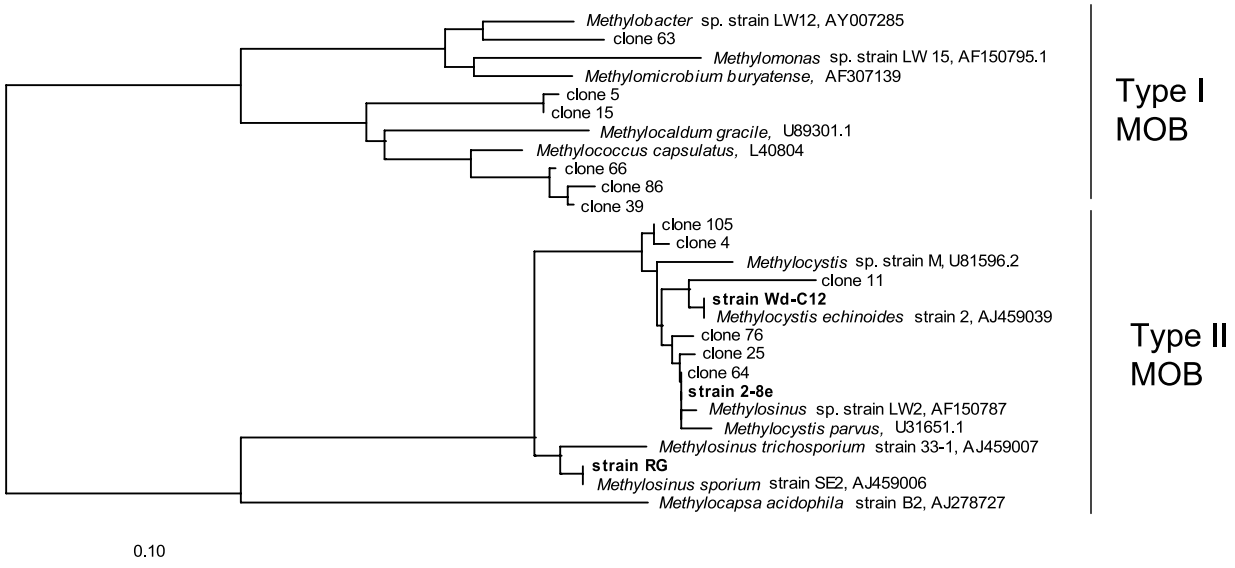
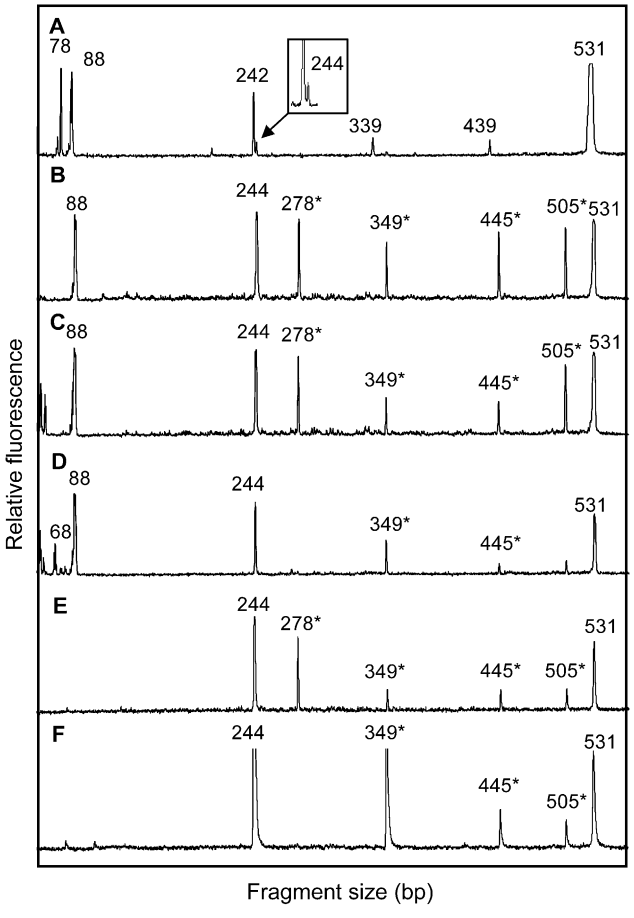


Fig. 4. Unrooted phylogenetic PmoA dendrogram showing the position of MOB isolates (bold) and *pmoA* clones from this study in relation to cultured methane oxidizers. The NCBI accession numbers of the *pmoA* sequences are given together with the names of cultured methane oxidizers. The bar indicates 10% sequence divergence.

characteristic for all isolates obtained from the enrichment cultures, was hardly visible in the sediment profile. By contrast, the T-RF of 88 bp, representing a major peak in the enrichment cultures and in the sediment sample, was



not explained by the *pmoA* sequences of the isolates or clones.

Due to the high sensitivity settings, the T-RFLP profiles of the enrichment cultures showed also a number of peaks apparently caused by low amounts of incompletely digested PCR products (T-RFs at 278, 349, 445, 505, and 531 bp). The corresponding T-RFs represented secondary restriction sites predicted from the *pmoA* sequences of the isolates (Table 3). The formation of such pseudo-T-RFs has been documented [28] and could be confirmed when the strains Wd-C12 and 2-8e were analyzed at the same sensitivity settings (Fig. 5E,F). The large peak heights of the pseudo-T-RFs relative to the T-RF peaks are caused by the oversaturation of the T-RF signal, which was confirmed experimentally by lowering the signal amplification (details not shown).

Although the T-RF at 244 bp was unique for the isolated strains and most of the type II MOB, as revealed by comparison with all publicly available *pmoA* and the closely related *amoA* sequences (data not shown) [24], other T-RFs of the sediment sample, e.g. at 242, 339, 439 and 531 bp, might be 'real' T-RFs, pseudo-T-RFs, or a combination of both (Table 3). Therefore, a further interpreta-

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Fig. 5. pmoA-based T-RFLP analysis of a littoral sediment sample (A), enrichment cultures obtained with Whittenbury medium (B) and with diluted basic medium (C), both at a dilution of 10^{-4}, with diluted basic medium at a dilution of 10^{-6} (D), and of the isolated strains 2-8e (E) and Wd-C12 (F). Numbers indicate the length (bp) of the dominant T-RFs; asterisks indicate peaks that can be explained by secondary restriction sites in the *pmoA* sequences of the isolates (pseudo T-RFs). T-RF lengths were calibrated with external and internal standards; for accuracy and other details, see Section 2. The peak at 531 bp represents undigested PCR product.

tion of the T-RFLP profile of the sediment sample was not done.

4. Discussion

In the present study, we aimed at developing growth medium that allows the culturing of the highest number and the greatest diversity of MOB from freshwater sediments. Maximal MPN counts in Lake Constance littoral sediment obtained with the new medium were 5×10^4 cells ml^{-1}, which corresponds to 4×10^4 cells g^{-1} wet weight or 7×10^4 cells g^{-1} dry weight, and were four- to six-fold higher than those obtained with the conventional Whittenbury medium. Comparisons of our results with MOB counts obtained in other studies are difficult because the environmental and culturing conditions often differ significantly. Nevertheless, numbers of MOB similar to those obtained in this study have been reported for other lake sediments (Stechlinsee, 10^5 cells ml^{-1} [20]; Lake Ontario, 10^4 cells g^{-1} [29]). Slightly higher numbers (10^6 cells g^{-1}) have been cultivated from sediment of Antarctic lakes [30]. However, estimations of methanotroph abundance in Lake Washington sediment on the basis of cultivation-independent methods revealed much higher numbers (10^8–10^9 cells g^{-1}) [31], indicating there is still an enormous gap between the number of culturable MOB and the numbers detected with cultivation-independent methods.

The inocula used in this study were taken from the same study site between June 1999 and August 2002. Since seasonal variations of the pore water chemistry are bound to influence the community of MOB in the sediment we cannot exclude the fluctuations in the structure and composition of the methanotrophic population. Since MOB are especially sensitive to freezing [32], we did not use frozen aliquots of a single sediment sample for all experiments, as it may affect viable counts and introduce a pre-selection for strains resistant to freezing. To minimize the influence of natural variability, we repeated most experiments several times. Because the results of all experiments were relatively similar, we assume that the results are largely independent of the sampling date and of possible qualitative variations in the MOB community.

4.1. Effect of medium constituents

The phosphate and nitrate concentrations used in conventional culturing media are substantially higher (millimolar range) than those found in lake waters (micromolar range). Phosphate usually limits primary productivity in lakes, and bacteria that express high-affinity uptake systems for phosphate [33] might be hampered by a sudden increase in phosphate concentration. We therefore tried to mimic the natural low phosphate concentration and substituted HEPES buffer for the commonly used phosphate buffer (Table 2). However, phosphate at 0.015–1.5 mM did not influence the cultivation efficiency. Despite a proposed high phosphate demand (2–25 mM) of *Methylosinus trichosporium* for growth [34], even concentrations as low as 0.015 mM phosphate proved to be sufficient in our study. This contradiction might be explained by the finding that MOB can rely on intracellular polyphosphates [35], which have been accumulated when the MOB were growing in the sediment.

High nitrate concentrations, however, had a negative influence on the cultivation efficiency, indicating that nitrate concentration of Whittenbury medium (10 mM) is far too high. These results are in contrast to a study on growth requirements of *M. trichosporium*, for which an optimal nitrate concentration of 2–100 mM has been reported [34]. It is possible that MOB that are freshly recovered from their natural environment react differently than pure cultures grown in the laboratory for many generations.

We also compared the newly designed growth medium to the cultivation efficiency obtained with several published media for MOB. Our results showed that media with low ionic strength generally yielded the highest MPNs. However, it appears that magnesium and sulfate rather than the total ion concentration inhibit growth at high concentrations (>1 mM). In similar experiments with MOB from a peat bog, it has been observed that diluting the medium two-fold or 10-fold considerably increased the methane-oxidizing activity [5]. Another study reports that lower ion concentrations tend to enrich for type II MOB [36]. For growth of *M. trichosporium*, a magnesium concentration of 50 μM has been found optimal [34].

4.2. Effect of the incubation atmosphere

In an effort to optimize the incubation atmosphere, we found in our study an optimal mixing ratio of 3.5% carbon dioxide. Other authors have reported that there is no influence of CO_2 on the success rate of enrichments or on the isolation of type I or type II MOB, in the range of 1–5% CO_2 [30,36].

In our study the mixing ratio of O_2 significantly influenced the cultivation efficiency. This influence was not linear but exhibited a maximum at 17% O_2. A detrimental effect of O_2 on growth of most heterotrophic bacteria has been proposed [37], even though this was not the case with planktonic bacteria from Lake Constance [18]. The influence of O_2 on MOB has been investigated at different levels, with quite ambiguous results. On one hand, high O_2 mixing ratios are necessary for good growth; O_2 mixing ratio of 0.1–11% resulted in decreased biomass of *Methylococcus capsulatus* [38]. In rice soils, O_2 availability limits methanotrophic growth [39]. On the other hand, O_2 does not influence the growth or activity of MOB. The success rate of enrichments of MOB is not affected by lowering the O_2 mixing ratio from 21% to 1–3% [30], *M.*

trichosporium and *Methylobacter luteus* oxidize CH_4 at maximum rates with O_2 mixing ratios from 0.45 to 20% [40] and methanotrophic biomass measured indirectly via analyzing biomarker concentration is not controlled by the O_2 concentration [41]. Our samples originated from surface sediment where O_2 concentrations are low. However, in the littoral zone, resuspension of surface sediment is quite common [42], and bacteria might be adapted to frequent exposure to higher O_2 concentrations in the water column.

Methane mixing ratios of 6–23% (97–532 μM) had no influence on the cultivation efficiency. The lowermost concentration was still sufficient to support methanotrophic growth. This is not surprising since the CH_4 concentrations at the study site have been lowered to the in situ values (2–24 μM) by these bacteria and the half saturation concentration of methane for the methane monooxygenase, as well as for natural samples, is in the range of 4–10 μM [43]. Increased methane concentrations lead to higher activities of MOB [1]. Yet, as shown in this study, this increased activity does not result in high cell numbers. Substrate inhibition seems unlikely to be the reason because methane is an inert gas. Probably the influence of methane would reveal only at much lower concentrations.

4.3. Clone library and T-RFLP analysis

In this study we combined a cultivation-based approach and a molecular approach to study the methanotrophic microbial community of freshwater sediment. Cultivation yielded only MOB of the *Methylocystis*/*Methylosinus* group (type II MOB), whereas the clone library of the sediment detected both type I and type II MOB, indicating cultivation bias favoring certain type II MOB. This bias was also apparent in the T-RFLP profiles of the enrichment cultures, where the T-RFs and pseudo-T-RFs of the isolates were among the dominant peaks, although it cannot be ruled out entirely that the peaks of the pseudo-T-RFs were at least partly peaks of T-RFs from other MOB, e.g. the T-RF at 531 bp corresponds to clone 64 (Table 3), whereas the T-RFs at 349 and 505 bp might represent other *pmoA* sequences [44] not present in the clone library. The almost-identical T-RFLP profiles obtained for the enrichments in Whittenbury medium and in diluted basic medium indicate that the diluted basic medium, although yielding higher MPNs, still favors the same populations of MOB.

Another dominant T-RF in the T-RFLP profiles of the enrichment cultures and the sediment sample had a length of 88 bp. This T-RF could neither be explained by the *pmoA* sequences of the isolated strains or clones of this study nor by the *pmoA* sequences of any methanotroph cultured to date. It might represent a novel methanotroph since it was detected in the T-RFLP profiles of the MOB-specific enrichment cultures, which makes amplification of the closely related *amoA* gene or of unspecific DNA unlikely.

The T-RFLP analysis of the sediment sample indicates that the cultured type II MOB represent only a minor fraction of the total MOB community in the littoral sediment of Lake Constance. Similar results have been reported in a study with meadow soil; however, additional kinetic investigations in that study indicate that the *Methylocystis* population is responsible for the observed CH_4 oxidation activity [45]. Also in rice soils, type II MOB seem to be the more abundant and stable part of the methanotrophic community [46]. Contrasting findings have been reported for Lake Washington sediments where cultivation-independent methods indicated a dominance of type I MOB [31], although both types of MOB had been isolated from these sediments in a previous, cultivation-based study [47].

Cultivation-dependent and cultivation-independent methods both have their constraints. Cultivation of MOB favored the *Methylocystis*/*Methylosinus* group, even though the medium composition was adapted to the natural conditions in the sediment (lower ion and nutrient concentrations). Since the application of counter-gradients of CH_4 and O_2 in a different study favored the enrichment of type I MOB [10], further improvements of the cultivation conditions should focus on a better simulation of the in situ distributions of CH_4 and O_2 in the sediment.

On the other hand, biases in DNA extraction [48] and PCR amplification may lead to a preferential recovery of certain sequences [49]. This effect may be caused, for example, by the annealing temperatures used, which might

Table 3
T-RFs and possible pseudo-T-RFs resulting from an in silico restriction analysis with the endonuclease *Msp*I and the *pmoA* sequences of isolates and clones of this study

	Isolates/clones	T-RF (bp)	Pseudo-T-RF (bp)
Type I MOB	clone 5, clone 15	241	337, 349, 373, 437, 505, 531
	clone 39	79	208, 226, 241, 337, 373, 437, 445, 505, 531
	clone 66	79	208, 226, 241, 337, 373, 445, 505, 531
	clone 63	531[a]	–
	clone 86	36	79, 208, 226, 241, 337, 373, 437, 445, 456, 505, 531
Type II MOB	strain Wd-C12 and RG, clones 4, 11, 76, and 105	244	349, 445, 505, 531
	strain 2-8e, clones 25 and 64	244	278, 349, 445, 505, 531

[a]No restriction site.

have resulted in a preferential amplification of certain *pmoA* fragments, and/or by different cell wall properties of type I and type II MOB. *Methylosinus* and *Methylocystis* strains form exospores and lipid cysts, respectively, while most type I MOB may form so-called 'immature *Azotobacter*-type' cysts, with *Methylobacter* spp. being the only exception [9,50,51]. Although we used a bead-beating method for DNA extraction to minimize these effects, we cannot totally exclude that DNA of type I MOB might have been preferentially extracted.

Based on our results certain improvements of culturing conditions for freshwater MOB can be suggested. A diluted mineral medium with low concentrations of magnesium and sulfate (<1 mM), phosphate and nitrate (especially nitrate, <250 μM) under an atmosphere of moderate O_2 pressure (17%) plus 3% CO_2 reproducibly yielded the highest numbers of MOB. Although molecular analysis of DNA from the sediment revealed the presence of type I and type II MOB, only *Methylosinus*/*Methylocystis* types were cultivated with the new medium as well as with Whittenbury medium. Further improvements of the cultivation conditions and novel quantitative approaches (e.g. the microcolony technique combined with fluorescence in situ hybridization [52]) will be necessary to study the numerically predominant group of MOB in sediments.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 454), research funds of the Universität Konstanz, and the Fonds der Chemischen Industrie, Frankfurt/M. We wish to thank P. Merkel and K. Denger for determination of ion concentrations, Dirk Schmitt-Wagner, Ulrich Stingl, and Markus Egert for helpful discussions, and Michael Friedrich for the introduction to the ARB software package.

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