New DGGE strategies for the analyses of methanotrophic microbial communities using different combinations of existing 16S rRNA-based primers

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Abstract

Methane-oxidising microbial communities are studied intensively because of their importance for global methane cycling. A suite of molecular microbial techniques has been applied to the study of these communities. Denaturing gradient gel electrophoresis (DGGE) is a diversity screening tool combining high sample throughput with phylogenetic information of high resolution. The existing 16S rRNA-based DGGE assays available for methane-oxidising bacteria suffer from low-specificity, low phylogentic information due to the length of the amplified fragments and/or from lack of resolving power. In the present study we developed new combinations of existing primers and applied these on methane-oxidising microbial communities in a freshwater wetland marsh. The designed strategies comprised nested as well as direct amplification of environmental DNA. Successful application of direct amplification using combinations of universal and specific primers circumvents the nested designs currently used. All developed assays resulted in identical community profiles in wetland soil cores with *Methylobacter* sp. and *Methylocystis* sp.-related sequences. Changes in the occurrence of *Methylobacter*-related sequences with depth in the soil profile may be related to the decrease in methane-oxidizing activity.

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1. Introduction

Methane is considered as the most potent greenhouse gas after carbon dioxide [1]. Because the CH_4 concentration in the atmosphere has more than doubled in the post-industrial era, much research effort has been expended to identify sources and sinks of methane, and the organisms involved. The balance between the production of methane by methanogenic bacteria under an-

oxic conditions and the consumption of methane by methanotrophic bacteria (MOB) under oxic conditions determines whether a particular environment acts as a source or a sink for atmospheric methane. Submerged wetland soils (e.g., swamps, bogs, rice paddies) are regarded as the most important source of atmospheric methane while non-flooded upland soils (e.g., forests, grassland, arable) are regarded to be the only biological sink of atmospheric methane [2]. In both wetland and upland soils, obligate aerobic methane-oxidising bacteria (MOB) use molecular oxygen to oxidise methane to CO_2 and cell carbon [3]. In wetland soils these bacteria are active in the surface soil layers and in the

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rhizosphere of oxygen-releasing plants (c.f. [4]), thereby substantially reducing the potential amount of methane emitted.

The crucial role MOB play in the global carbon cycle has evoked numerous studies to elucidate their distribution and diversity in various habitats. Molecular biological techniques have been widely used in the past decade to serve this purpose. MOB are ideal microorganisms for molecular biological assessment due to the strong link between the phylogeny and their eco-physiology. All known 11 genera are classified in two groups within the gamma- and alphaproteobacteria [5] and are referred to as type I and II MOB, respectively. The latter classification is also based on corresponding differences in morphology and physiology [3]. With respect to the ecology of both types of MOB, the available information is still far from complete. However, information with respect to distribution and abundance has been obtained using FISH (fluorescent in situ hybridisation) [6,7], PLFA (phospholipid-derived fatty acids) [8,9] and very recently real-time PCR [10]. Diversity has been assessed using cloning (e.g., [11]), T-RFLP (terminal restriction fragment length polymorphism) [12], DGGE (denaturing gradient gel electrophoresis) (e.g., [13,8]) and even microarray [14] approaches based on the analyses of the 16S rRNA gene or the genes coding for the polypeptide of the enzyme methane monooxygenase. DGGE is a widely used tool for screening microbial community dynamics in large numbers of samples and environments yielding diversity patterns as well as phylogenetic information [15]. In contrast to techniques like FISH, PLFA and also real-time PCR, DGGE yields phylogenetic information with high resolution for a complete target community. The only comparable technique at the moment is T-RFLP. However, interpretation of T-RFLP data requires a clone library or isolated organism from the environment under study.

Henckel and co-workers [16] published the first study describing DGGE assays for MOB community analyses. One of the approaches described in that study was based on the functional gene coding for the polypeptide containing the active site of the enzyme particulate methanemonooxygenase (*pmoA*), the latter catalysing the oxidation of methane to methanol. The described PCR-*pmoA*-DGGE assay has been since widely applied in various environments (e.g., [13,17,18]). However, in comparison to 16S-based DGGE approaches the *pmo*A-DGGE has some drawbacks. The generally used primer pair (A189–A682) also amplifies the *amo*A (α-subunit of the ammonium monooxygenase) thereby adding nitrifying bacteria to the DGGE patterns. In addition, a number of methanotrophs can have multiple copies of the *pmo*A gene on their genome, which can be very different from each other [19]. The latter combined with the fact that the primers are degenerate may result in DGGE patterns where multiple bands originate from

a single bacterial species, further complicating interpretation of the DGGE patterns.

The 16S rRNA-based DGGE assays of Henckel et al. were designed using probes that target all methylotrophic bacteria, including the MOB [20] and are therefore not specific for MOB. Moreover, non-methylotrophic organisms can be amplified [8,6]. The only other 16S rRNA-based DGGE assay has been described by Wise et al (1999) [21]. These authors designed new specific primers, which in combination with a nested universal amplification, allow for DGGE analysis of MOB communities in environmental samples. The specificity of these primers was evaluated by Horz and co-workers [12] and the primers were applied recently in coastal sediments and arctic soils [22,23]. However, the DGGE fragment obtained with the Wise protocol is very small (<160 bp) thus limiting subsequent phylogenetic analyses. In addition, theoretically, the region amplified by this primer pair does not allow for resolving type II MOB species by DGGE because of the lack of sequence difference in that particular region of the 16S rRNA. Furthermore, expansion of the existing culture database on type II MOB [24] has shown that the type II -specific primer used by Wise et al. [21] will not amplify strains of *Methylosinus sporium*.

The aim of the present study was to overcome the shortcomings of the existing 16S rRNA-based DGGE methods for community analysis of MOB. Furthermore, direct vs. nested PCR approaches were explored to possibly omit the second PCR step in nested PCR protocol. The methods, which make use of described primers applied in new combinations, proved applicable to environmental samples and in comparison to the previously described strategies, the strategies we describe have a higher resolving capacity for both type I and type II MOB. Finally, we applied the protocols to determine the diversity of MOB in a freshwater marsh and combined this with abundance and activity measurements to assess structure function relationship with varying depth in the wetland soil profile. The identified MOB are discussed in their ecological context.

2. Materials and methods

2.1. Sampling

Soil samples were taken within a *Glyceria maxima* stand in an oxbow lake along the river Waal, the Netherlands. The area called "Oude Waal" (N 51°52′ E 05°53′) is a former river branch and has been described previously in more detail [25,26]. Within 1 m² plot 4 replicate soil cores were taken randomly (length 20 cm, diameter 3.8 cm) in late summer. Immediately upon arrival in the laboratory the cores were sectioned in layers (0–5, 5–10, 10–20 cm). Roots were removed from the

soil and after homogenisation the samples were stored at $-20\ °C$.

2.2. Potential methane oxidizing activity

The potential CH_4 oxidation activity of the soils was determined as described by Bodelier and Frenzel (1999) [27]. In short, 10 gram of soil was transferred to 150 ml flasks and diluted 1:1 (w/v) with sterile Milli-Q water. The flasks were closed with rubber stoppers and 1.3 ml pure CH_4 (10,000 ppmv) was added. The flasks were incubated on a shaker (150 rpm) at 20 °C. The decrease of CH_4 in the headspace was monitored by GC-FID analysis of regularly taken subsamples. The potential methane oxidation rates were calculated using the slope of the linear parts of the methane depletion curves in regression analysis.

2.3. Most probable numbers

The number of MOB was determined by means of the MPN (most probable number) method described earlier [27]. In order to extract the cells from the sediment particles, 10 gram of soil was diluted 1:5 with phosphate-buffered saline (PBS). The extracts were shaken for 4 h at 150 rpm and subsequently diluted 1:10 with PBS. Subsamples of 100 μl of the suspension were serially diluted 1:1 in sterile microtiter plates (Nunc^{TM}, Denmark) containing nitrate mineral salts (NMS) growth medium for methanotrophs [5]. The plates were incubated for 4 weeks at 25 °C in gastight jars containing 20% methane in air. Inoculated plates without methane served as controls. Wells that were turbid were considered positive. Most probable numbers were obtained from statistical tables [28].

2.4. Strain isolation and culture conditions

To obtain MOB isolates from the soils a traditional MPN analysis was performed as described above. From the least diluted, most diluted and positive wells in between, subsamples were taken, which were transferred to 24-well microtiter plates containing 2 ml NMS medium. The plates were incubated again at 25 °C. This subculturing was repeated three times after which 100 μl subsamples were streaked on NMS-agar plates. The plates contained 1.5% agar (Bacto agar, Difco). Colonies were picked and transferred to liquid NMS medium in 150 ml serum bottles. These bottles were supplied with CH_4 (20%) and incubated at 25 °C. Purity of these cultures was checked microscopically, by incubation on solid as well as in liquid 10% TSB (tryptic soy broth) and by amplifying extracted DNA with bacterial primers (see No. 3 Table 2) and subsequent DGGE analyses.

The obtained isolates as well as the type strains *Methylomonas methanica* S1 (NCIMB 11130), *Methylomonas*

rubra (NCIMB 11913), *Methylobacter luteus* (NCIMB 11914), *Methylomicrobium album* (NCIMB 11123), *Methylococcus capsulatus* Bath (NCIMB 11132), *Methylosinus sporium* (NCIMB 11126), *Methylosinus trichosporium* (NCIMB 11131), *Methylocystis parvus* (NCIMB 11129) were cultivated routinely at 25 °C on a rotary shaker in 25 ml NMS medium in 150 ml serum bottles closed with a rubber stopper. Methane (20%) was supplied aseptically using a syringe and filter.

2.5. DNA extraction and PCR amplification

DNA from cultures and soil samples was extracted using a bead-beating protocol as described by Henckel et al. [16]. DNA from soil samples was re-purified using Wizard DNA clean up columns (Promega, Madison, WI, USA).

The sequences of the primers used for PCR in this study are given in Table 1. PCR amplification was performed in an MBS 0.5 S thermocycler (ThermoHybaid, Ashford, UK) in a 25 μl reaction mixture containing approximately 25 ng of DNA, 10 mM Tris/HCl pH 8.3, 50 mM KCl, 0.04% w/v bovine serum albumin, 200 μM of each deoxynucleotide, 1.5 mM $MgCl_2$, 25 U/ml of *Taq* DNA polymerase and 0.5 μM of each primer. The PCR cycling programs for the different primer combinations are listed in detail in Table 3.

In case of nested PCR designs, depending on the strength of the PCR product in the first round, either 5 μl of undiluted PCR product was used as a template for the second round or 5 μl of 10 or 100 times diluted first round PCR product.

2.6. DGGE profiling

DGGE was performed essentially as described by Muyzer et al. [29]. Briefly, PCR products were separated on a 1.5 mm thick, vertical gel containing 6% (8% in case of DGGE strategy 3, see Table 3) (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 60% at the bottom. Only DGGE with strategy 3 was performed with a gradient of 40–60%.

Here, 100% denaturant is defined as 7 M urea plus 40% v/v formamide. The gels were loaded with 8–10 μl of PCR product in case of cultures and 15–50 μl in case of environmental samples, depending on the band intensity of the PCR product after electrophoresis on 1.5% agarose gels. Before loading, the PCR products were mixed with loading buffer (0.25 μl loading buffer per μl of PCR product). The loading buffer contained 50% Glycerol, 50 mM Tris/HCl pH 7.5, 5 mM EDTA and 0,05% bromophenol blue. Electrophoresis was performed in a buffer containing 40 mM Tris, 40 mM acetic acid, 1 mM EDTA (pH 7.6) (0.5 × Tris–acetate–EDTA

Table 1
Sequences of primers used in PCR amplification

Primer name	Theoretical specificity	Sequence 5′–3′	Reference	*E. coli* position
27f	Universal	AGAGTTTGATCMTGGCTCAG	[37]	8–27
357f[gc]	Eubacterial	CCT ACG GGA GGC AGC AG	[29]	340–357
518r	Eubacterial	ATT ACC GCG GCT GCT GG	[29]	518–534
533f	Universal	GTGCCAGCAGCCGCGGTAA	[38]	513–533
907r[gc]	Universal	AATTCCTTTGAGTTT	[38]	907–922
MethT1dF	Type I MOB	CCTTCGGGMGCYGACGAGT	[21]	84–102
MethT1bR[gc]	Type I MOB	GATTCYMTGSATGTCAAGG	[21]	988–1006
MethT2R[gc]	Type II MOB	CATCTCTGRCSAYCATACCGG	[21]	997–1017
Am976[gc]	Type II MOB	GTCAAAAGCTGGTAAGGTTC	[32]	957–976

[gc]: The following GC-clamp was attached at the 5′-prime end of the primer sequence to facilitate DGGE analysis: cgcccgccgcgccccgcgcccggcccgccgcccccgcccc.

buffer) for 16 h at 100 V. Gels were stained for 1 h in water containing 0.5 µg ml^{-1} ethidium bromide. Images were recorded with a CCD camera mounted on the AutoChemi™ Darkroom (UVP Inc. Upland, CA, USA). A detailed protocol for performing DGGE analyses can be found on http://www.nioo.nl/CL/ME/index.htm.

2.7. Sequencing and sequence analysis

To identify DGGE bands, a small piece of gel from the middle of the target band was excised from the gel using a sterile scalpel and incubated in 50 µl sterile milli-Q purified water for 24 h at 4 °C. After this period the DNA has diffused out of the agarose and the solution can be used as template in a re-amplification PCR. Re-amplification was performed using the original primers but modified PCR programs (see Program Nos. 4 and 5 in Table 2) and run on DGGE to confirm its identity. Only pure bands were used for sequencing by amplifying with primers without a GC-clamp. PCR products for sequencing were purified and sequenced using Applied Biosystems 3730 and 3100 genetic analy-

sers by Baseclear Labservices (Baseclear, Leiden, the Netherlands).

Sequences were aligned to related sequences available in the public databases using the ARB software [30]. Phylogenetic trees were calculated and drawn using the Neighbor-Joining algorithm using Jukes–Cantor correction as implemented in the TREECON software [31]. For tree construction only the aligned positions from the DGGE bands were compared with the sequences from the database. 16S rRNA sequences of DGGE bands and of isolated MOB have been deposited in EMBL database under accession numbers AJ831495–AJ831522.

2.8. Statistical analyses

Effect of soil depth on methane-oxidising activities and most probable numbers was tested using one-way ANOVA followed by the Tukey post-hoc comparison of means. The methane-oxidizing activity data were checked for normality and homogeneity of variances to meet the criteria for performing ANOVA analyses. The test was performed using the STATISTICA (version) software for windows (Tulsa, OK, USA).

Table 2
PCR temperature programs used in this study

	1st step	2nd step			Final step
	Initial denaturation	Denaturation	Annealing	Extension	Extension
No. cycles	1	35			1
Program No. 1	5 min 94 °C	1 min 94 °C	1 min 56 °C	1 min 72 °C	5 min 72 °C
No. cycles	1	30			1
Program No. 2	5 min 94 °C	1 min 94 °C	1 min 60 °C	1 min 72 °C	5 min 72 °C
		Every second cycle the annealing temperature is lowered for 1 °C from 60 °C–50 °C followed by 10 cycles at 50 °C			
No. cycles	1	30			1
Program No. 3	5 min 94 °C	1 min 94 °C	1 min 58 °C	1 min 72 °C	5 min 72 °C
No. cycles	1	25			1
Program No. 4	5 min 94 °C	30 sec 94 °C	30 s 55 °C	30 s 72 °C	5 min 72 °C
No. cycles	1	25			1
Program No. 5	5 min 94 °C	30 s 94 °C	30 s 58 °C	30 s 72 °C	5 min 72 °C

3. Results and discussion

This study was performed in order to improve existing 16S rRNA-based DGGE assays for the analysis of methanotrophic microbial communities and to test and apply these on wetland MOB communities. One of the shortcomings of the existing assays was the lack of resolving power with type II MOB in the nested approach described by Wise [21]. In Fig. 1(a) DGGE analysis (see strategy 3 in Table 3) of type II MOB type cultures and isolates is displayed using the protocol of Wise. The bands of the type cultures *Methylosinus sporium* (NCIMB11126), *Methylosinus trichosporium* (NCIMB 11131) and *Methylocystis parvus* (NCIMB 11129) migrate exactly to the same position because these organisms are absolutely identical in the amplified region. Also the obtained isolates displayed a very similar migration behavior. Isolate H4, L28, H17 and L32 melted at a slightly lower denaturant concentration
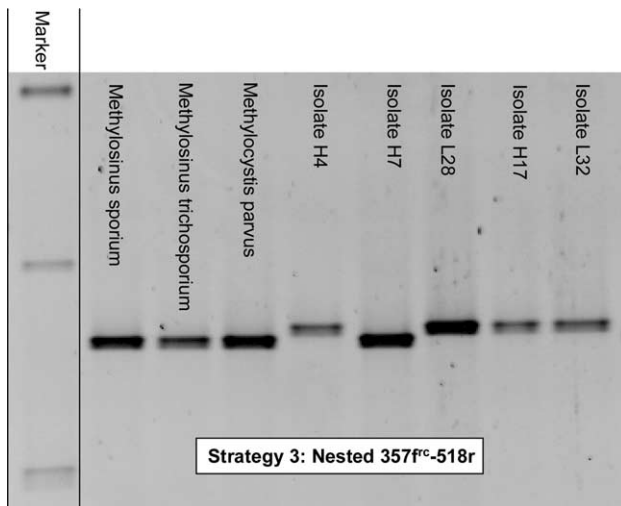
Fig. 1. Denaturing gradient gel electrophoresis of PCR products obtained with the bacterial primer combination 357f^{gc}-518r (strategy 3, Table 3) of type II MOB cultures and isolates.

but could also not be distinguished from each other. Comparison of the nearly complete 16S sequence of these isolates showed clear differences supporting differentiation of these *Methylocystis* strains (data not shown). Actually, similar observations can be seen in the study by Wise et al. [21]; clones and isolates with clear differences in a larger fragment of 16S migrated to the same position in the DGGE.

3.1. Novel DGGE-strategies

We have explored different strategies and combinations of primers as depicted in Table 3. In nested designs the first round of PCR was performed with the primer combinations for type II and I MOB, respectively, as described by Wise and co-workers [21] (see strategies 1–6 Table 3). The first round PCR product was used as a template for a second round of PCR with either universal primers or a combination of a universal with a MOB-specific primer (see strategies 1–6 in Table 3). The first improvement was a strategy, which made use of the universal primer combination 533f-907r^{gc} in the second round PCR (strategy Nos. 4 and 6 in Table 2), replacing the eubacterial primer combination in strategies 3 and 5 (Table 2) as used by Wise et al. This approach yielded larger fragments of approximately 450 bp including the 40 bp GC-clamp in more variable region of the 16S rRNA. The second improvement of existing 16S rRNA-based DGGE assays consisted of combinations of a universal primer with a MOB-specific primer in the second PCR step (strategies 1 and 2, Table 3), to avoid possible amplification of non-MOB by the universal primers. The third strategy of improvement was used to omit the first amplification step and apply the combinations 533f-MethT1bR^{gc} (No. 7, Table 2) and 533f-MethT2R^{gc} (No. 8, Table 2) directly on culture or environmental DNA. For type II MOB strategies 9 and 10 (Table 3) were tested also in nested and direct design. These combinations were made with a primer

Table 3
DGGE strategies, primer combinations used, their mode of application and the reference to the PCR-program used

Strategy No.	DGGE strategies, primer combinations used and their mode of application.	PCR program (see Table 2)	Reference
1	Nested: 1st round 27f-MethT2R (Type II MOB) – 2nd round 533f-MethT2R^{gc} (Type II MOB).	1 (1st) – 2 (2nd)	This study
2	Nested: 1st round MethT1dF-MethT1bR (Type I MOB) – 2nd round 533f-MethT1bR^{gc} (Type I MOB)	1 (1st) – 2 (2nd)	This study
3	Nested: 1st round 27f-MethT2R (Type II MOB) – 2nd round 357f^{gc}-518r (Eubacterial)	1 (1st) – 2 (2nd)	[21]
4	Nested: 1st round 27f-MethT2R (Type II MOB) – 2nd round 533f-907R^{gc} (Eubacterial)	1 (1st) – 2 (2nd)	This study
5	Nested: 1st round MethT1dF-MethT1bR (Type I MOB) – 2nd round 357f^{gc}-518r (Eubacterial)	1 (1st) – 2 (2nd)	[21]
6	Nested: 1st round MethT1dF-MethT1bR (Type I MOB) – 2nd round 533f-907R^{gc} (Eubacterial)	1 (1st) – 2 (2nd)	This study
7	Direct application of 533f-MethT1bR^{gc} (Type I MOB) on environmental or culture DNA	2	This study
8	Direct application of 533f-MethT2R^{gc} (Type II MOB) on environmental or culture DNA	2	This study
9	Nested: 1st round 27f-Am976 (Type II MOB) – 2nd round 533f-Am976^{gc} (Type II MOB)	1 (1st) – 3 (2nd)	This study
10	Direct application of 533f-Am976^{gc} (Type II MOB) on environmental or culture DNA	3	This study

Nested PCR means that the first round of PCR was performed using a MOB-specific primer pair. The PCR product was than subsequently used as template for a second round of PCR performed using either universal primer combinations or combinations of universal and specific primers. The numbers of the PCR programs as indicated in this Table refer to Table 2.

designed for FISH analyses [32], which has no mismatches with all known type II cultures [24].

3.2. Novel type I DGGE strategies applied in wetland soils

Type I MOB type strains were clearly resolved by the three assays used (Fig. 2, panels (a)–(c)). The three different type I strategies yielded identical DGGE profiles (Fig. 2(a)–(c)). The direct amplification from environmental DNA using the 533f-MethT1bR^{gc} combination (Fig. 2C; strategy No. 7, Table 3) basically yielded the same results as the nested approach. Additional bands were formed that appeared to be heteroduplexes after excision,

re-amplification and rerunning on DGGE under identical conditions. The DGGE profile of soil samples retrieved from a freshwater marsh were identical in replicate cores and consisted of three major bands (Fig. 2(a)–(c)). The upper two bands (BDPM 1–2, 4–5, 11–12) belonged to a separate sequence cluster without cultured representatives (Fig. 4) most closely related to *Methylobacter* sp. Bands excised from the same position in gels of different DGGE strategies had similar phylogenetic positions (Fig. 4). Hence, the three strategies used yielded the same profile and apparently only MOB targets were amplified from the soils, confirming the specificity of the MethT1dF-MethT1bR primer set. Comparison of the
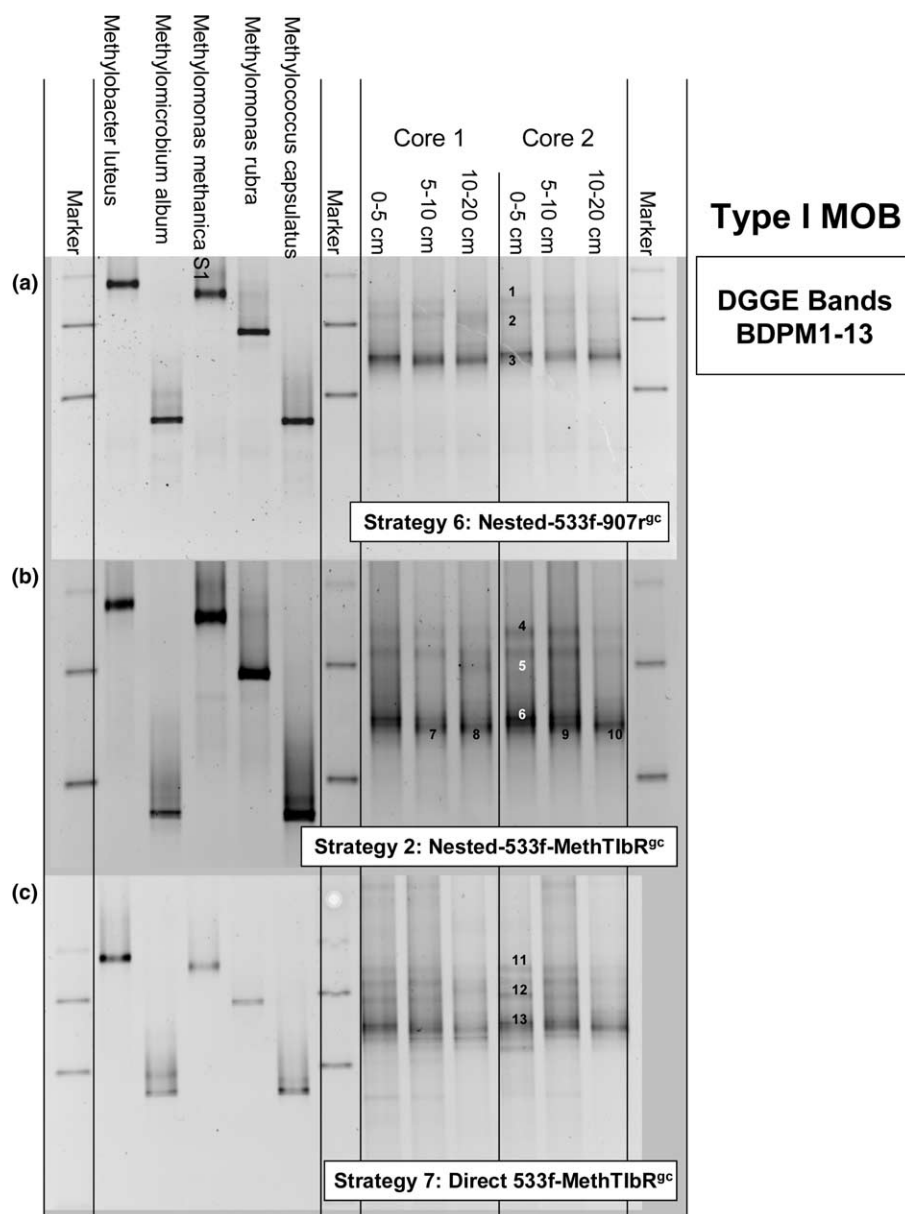
Fig. 2. Denaturing gradient gel electrophoresis of PCR products obtained with specific primers for type I MOB using different DGGE strategies and primer combinations. The upper panel (a) shows the result of strategy No. 6 (Table 3), whereas the panels (b) and (c) display strategies 2 and 7, respectively. The environmental samples were obtained from two replicate soil cores taken within a *Glyceria maxima* stand in a freshwater wetland. These cores were sectioned into three depth layers (0–5, 5–10 and 10–20 cm) before DNA was extracted.
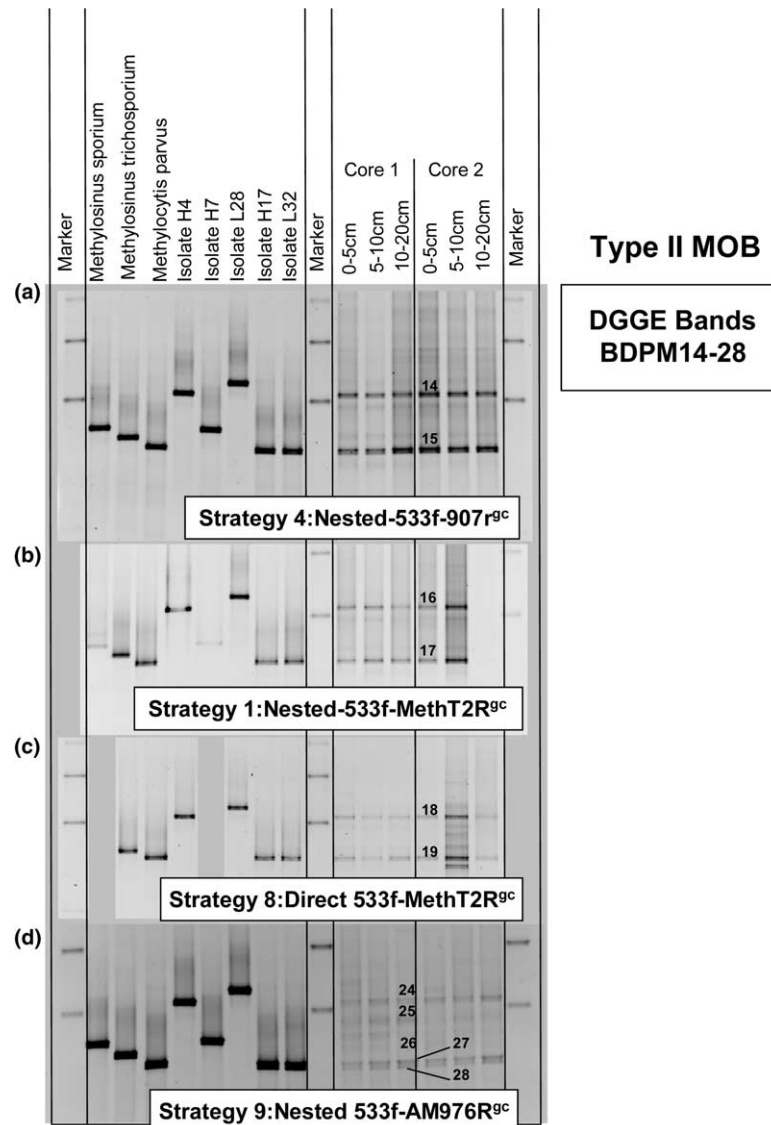
Fig. 3. DGGE gels of PCR products obtained with specific primers for type II MOB using different strategies and primer combinations. Panel A shows the result of strategy No. 4 (Table 3) whereas panel B, C and D display strategies 1, 8 and 9, respectively, as in Table 3. The environmental samples were obtained from two replicate soil cores taken within a *Glyceria maxima* stand in a freshwater wetland. These cores were sectioned into three depth layers (0–5, 5–10 and 10–20 cm) before DNA was extracted. In panel B the 10–20 cm layer of replicate 2 did not contain a sample because a proper PCR product was not obtained.

community with respect to the depth in the soil profile clearly shows that the 0–5 cm layer differs from the two other layers. The lower bands in the 0–5 cm layer (Fig. 2 BDPM 3, 6 and 13) are clearly different from bands BDPM 7 + 8 and 9 + 10 (Fig. 2). Fig. 4 shows indeed that both groups of bands belong to different sequence clusters. Bands BDPM 3, 6 and 13 cluster with non-cultivated clone sequences from rice soil [12] while bands BDPM 7, 8, 9 and 10 cluster with two cultured *Methylobacter* species both being isolated from arctic soils [33,23].

The detected pattern in this wetland soil is relatively low in diversity and therefore comparable to what has been observed in rice field soil [16,13,6]. Eller and co-workers found the same dominance of *Methylobacter*-related sequences in bulk soil, in rhizosphere soil and on the rhizoplane of rice. The authors suggested that the *Azotobacter*-type cysts of *Methylobacter* are more resistant to desiccation than the immature cysts of the other type I genera, thereby ensuring a greater chance of survival of the dry storage of the used rice field soil. However, in the wetland soil investigated desiccation is not of any relevance. Moreover, Horz et al. [12], detected higher diversity of type I MOB on rice roots as compared to bulk soil. The same *Methylobacter*-related sequence cluster was also found in landfill soil [21] and in a basalt aquifer [34], representing high and low methane environments, respectively. Also the type I MOB community in lake sediments has been shown to be dominated by these

Methylobacter-like sequences [35]. It is unclear why the wetland soil we have investigated is dominated by this *Methylobacter*-related bacterium. Isolation of the respective organism may provide further insight into this matter.

Nevertheless, the developed assays improve the existing 16S rRNA DGGE assays with respect to phylogenetic information as well as in the flexibility of the environmental application. The nested design will allow for detection of MOB in environments with low abundance of this bacterial group. The direct approach can be used in high methane environments where the abundance of MOB is expected to be higher. The nested approach apparently did not lead to any biases in the community composition.

3.3. Novel type II DGGE strategies applied in wetland soils

Comparing the type strains and isolates from Fig. 1 with those from Fig. 3(a)–(c) it can be immediately deduced that the resolution of the strategies employed in the present study is much higher than it was for the nested design as described by Wise [21] (see also Fig. 1). Several

Methylocystis strains (*Methylocystis parvus*, Isolate H4, L32, L28) and *Methylosinus* strains (*Methylosinus trichosporium*, *Methylosinus sporium*, Isolate H7) could be clearly separated from each other. The different strategies (nested universal, nested universal-specific, direct universal-specific) yielded exactly the same pattern in the wetland soil cores (Fig. 3(a)–(c)). The type II MOB community consisted of two bands. The pattern was also identical in the different soil layers Fig. 3(a)–(c). Both bands were phylogenetically affiliated with *Methylocytis* strains (Fig. 5). The upper band (Fig. 5, BDPM14, 16 and 18) was identical to an isolate obtained from the wetland soils investigated (Isolate H4). The nearest neighbor was another isolate (L28) obtained in this study and a *Methylocystis* strain (50/54) obtained from the surface sediment at the German Baltic coast [24]. The lower bands (Fig. 5, BDPM 15, 17 and 19) clustered with a *Methylocystis* cluster containing the strains *Methylocystis* Pi5/4, KS3, KS31 and the *Methylosinus trichosporium* strains KS21 and KS24b in case of BDPM 17 and 19 while BDPM15 groups with *Methylocystis* sp. F10V2A. The latter, however, is based on a very small difference in sequence, which might be explained by sequence or PCR errors. It cannot be excluded that in
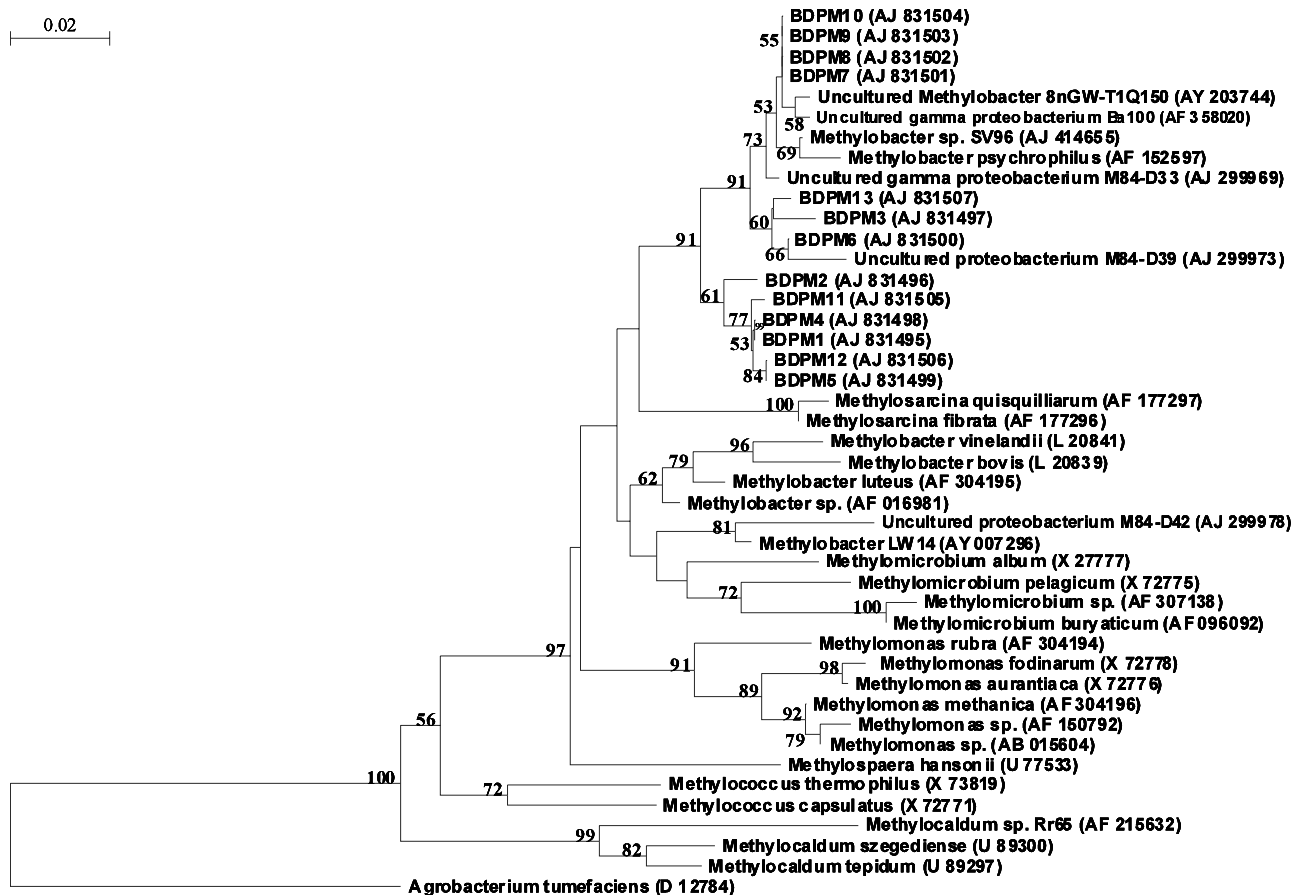

Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences (*E. coli* positions 555–926) showing the relationship of DGGE bands of type I MOB (*Gammaproteobacteria*) as in Fig. 2 with the most closely related members of the gammaproteobacteria. Bootstrap values (percentages) greater than 50% are represented at the nodes (1000 replicates).
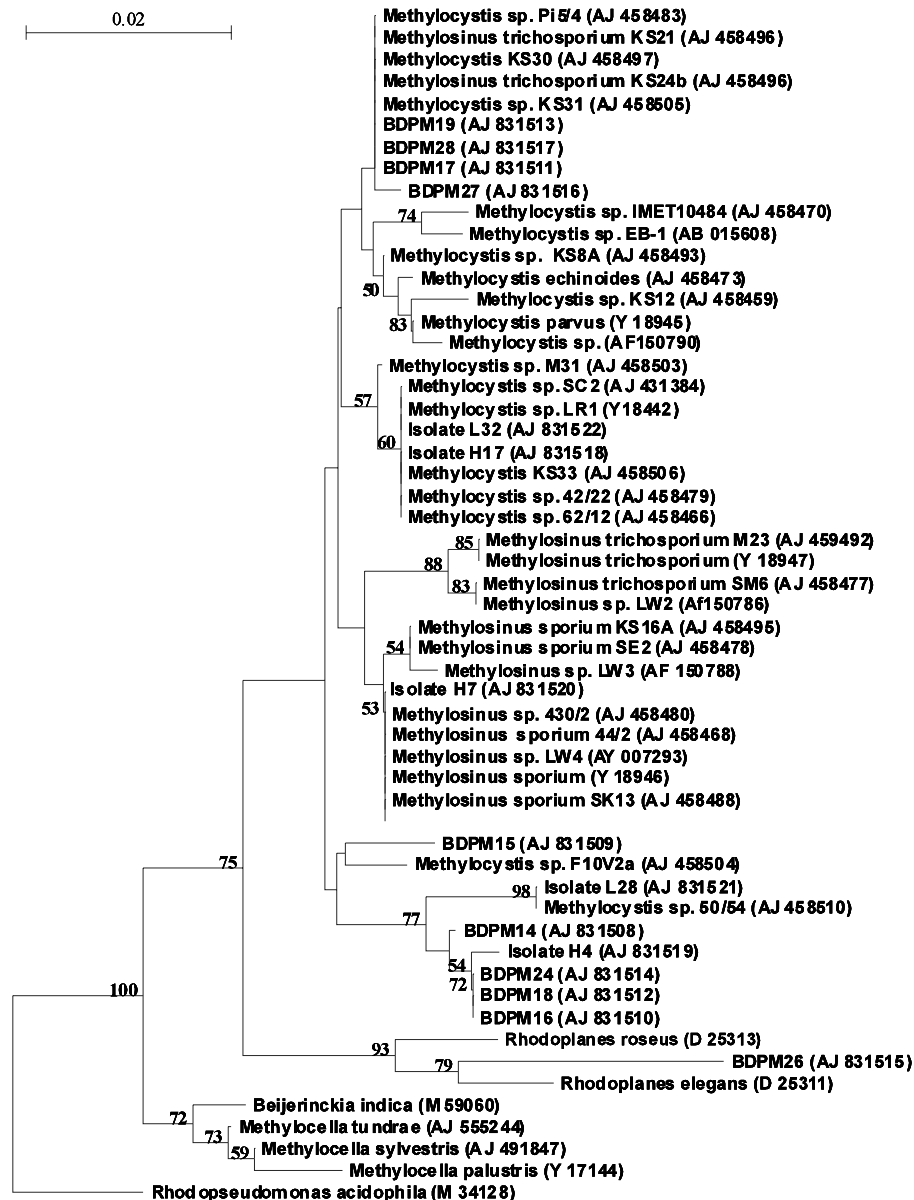
Fig. 5. Phylogenetic tree based on 16S rRNA gene sequences (*E. coli* positions 555–926) showing the relationship of DGGE bands of type II MOB (*Alphaproteobacteria*) from Fig. 3 with the most closely related members of the alphaproteobacteria. Bootstrap values (percentages) greater than 50% are represented at the nodes (1000 replicates).

the assay in panel A of Fig. 2 the lower band consists of more than one very similar sequence, which have an identical melting behavior in DGGE.

Sequences of bands BDPM 15, 17 and 19 do not cluster with the isolates H17 and L32, which in the DGGE profile migrate to the same position. It appeared that the extracted and purified bands had two positions in the sequence (*E. coli* positions 838 and 848) where there were clearly two signals (G or C). Secondary structure analysis predicts that bases at these two positions are paired and therefore should be complementary. Therefore we assume that the band contains a mixture of 838G-848C and 838C-848G, which apparently have equal mobility in DGGE. Theoretical melting curves support

this because the two variant DNA fragments have equal predicted melting behavior and would therefore be inseparable in DGGE. The isolates L32 and H17 had only one base signal on the respective positions and cluster phylogenetically with the 838C-848G variant. Hence, it seems likely that one of the two DNA fragments in the lower band matches the isolated *Methylocystis* sp. L32 and H17.

What was also described by Heyer et al. [24] occurred in the present study. The MethT2R primer system has mismatches with *Methylosinus sporium* sp. From Fig. 3(b) and (c) it is evident that the type strain *Methylosinus sporium* (NCIMB11126) and the Isolate H7, which is affiliated to *Methylosinus sporium* on the basis of

16S rRNA, are not amplified by the MethT2R-based primer combinations. Only in panel B a very weak signal was observed due to the high amount of cycles in the two-step PCR strategy. Therefore, we used the primer AM976R [32], described to have zero mismatches with all known type II cultures [24]. Fig. 3(d) shows the result of the nested approach with the 533f-AM976R combination. Both the *Methylosinus sporium* type strain and the isolate H7 were now amplified as efficiently as the other strains. The banding profiles did not differ significantly from the profiles as in Fig. 3(a)–(c). The upper band 24 clustered with the upper bands from the other assays (see Fig. 5). The lower band appeared to consist of two bands (BDPM27 and 28) that both clustered with the lower bands from the other assays. Apparently, the DGGE resolution of this primer combination is higher than those used in other assays. Also two other bands appeared. Band BDPM25 could not be re-amplified while band BDPM 26 was an alphaproteobacterium not related to methanotrophs (Fig. 5). The latter was to be expected since the primer AM976 matches to all type II methanotrophs but also to non-methanotroph sequences, like in this case to a *Rhodoplanes*-related organism. The direct amplification strategy (strategy No. 10, Table 3) led to a profile with predominantly non-methanotroph species (data not shown). Apparently the nested approach "enriched" for methanotrophic species in the DGGE profile. However, the assay clearly confirms that the dominant genus in these wetland soils is still *Methylocystis* and that *Methylosinus* is most likely a minor member of the community, detected only after enrichment and isolation (Isolate H7).

3.4. Methanotroph ecology in wetland soil cores

Assessment of the activity and abundance of the MOB community in the wetland soil cores clearly indicated that activity decreased with depth in soil (Fig. 6(b)). However, viable counts did not significantly decrease with depth (Fig. 6(a)). The DGGE profiles show that MOB communities consist of both type I and type II MOB related to the genera *Methylobacter* and *Methylocystis*, respectively. While the type II profile is stable with depth, the type I community shifts from band BDPM 3, 6, 13 (0–5 cm) to BDPM 7–10 below 5 cm. Hence, there is a shift from one *Methylobacter*-related species or strain to another. This may be related to the change in activity as observed in Fig. 6(b). The species or strains represented by bands BDPM 7–10 may be the species that are actually active in the soil. Nevertheless, this can only be substantiated using techniques coupling activity to identity. Using incorporation of $^{13}CH_4$ into PLFA indicated the activity of type I MOB exclusively (P.L.E. Bodelier, unpublished data). However, the resolution of this technique is not sufficiently high to couple the activity to a particular species or strain.
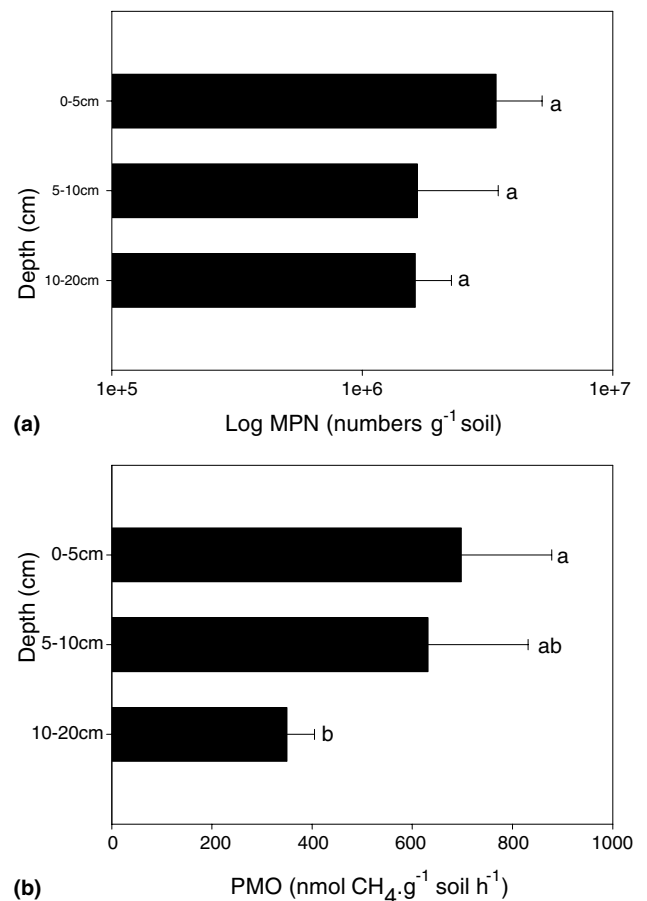

Fig. 6. Viable counts of methanotrophic bacteria (panel a) and potential methane-oxidizing activities (panel b) in different layers of soil cores sampled within a freshwater wetland. Different letters indicate statistically significant differences between means ($p < 0.05$, $n = 4$).

A possible future methodology to do so could be the isotope array approach [36].

The observed DGGE patterns are difficult to define as typical for these types of environments because the data are scarce. Representatives of both genera can be isolated from various environments [24,5]. However, the ability to isolate organisms from an environment does not imply that these are the dominant and active ones. We also isolated strains H7, L28 and others (unpublished results) that were not present in the DGGE profiles. The only comparable studies with respect to methodology and environment originate from rice soils. Eller and co-workers [6] detected the same *Methylobacter*- related cluster in the bulk soil, in the rizosphere, on the rhizoplane and in root homogenates of rice plants. These investigators demonstrated a dominance of type II MOB in the bulk and rhizosphere soil using a FISH that detected both genera, *Methylocystis* and *Methylosinus*. The dominance of *Methylobacter* sp. and *Methylocystaceae* has also been demonstrated in other rice soil experiments [16,8]. Very recently the

Methylobacter sp. cluster we found was also observed in arctic soils [23] and in a basalt aquifer [34]. Taken together both genera are detected in a range of habitats [5,24] thereby making it difficult to assign wetland soils as a possible niche for these groups. It may be speculated that the resistant cysts produced by these genera (i.e., [5]) enable persistence in a wide range of habitats. Other characteristics that can link these groups to specific environments are simply not available. We are currently isolating representatives of the *Methylobacter* cluster to obtain eco-physiological information, which may help to elucidate the biogeography of these organisms.

4. Conclusions

The present study describes new DGGE strategies improving 16S rRNA-based DGGE assays currently available for the study of MOB communities. The strategies increase phylogenetic information as well as the resolving capacity of the DGGE assays themselves. Existing nested PCR assays can be circumvented by the new primer combinations presented. The direct amplification strategies are recommended in environments where the abundance of MOB is anticipated to be high. Non-active methane-consuming environments can be assessed using nested designs. For studies on type II MOB communities, where *Methylosinus sporium* sp. are not of major importance, the 533f-MethT2R[gc] combination (strategies 1 and 8, Table 3) can be used avoiding amplification of non-MOB species. When the whole type II MOB diversity is the target than the 533f-Am976[gc] (strategy 9, Table 3) is recommended, although possible non-specific amplification has to be considered.

Acknowledgements

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