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Differential detection of type II methanotrophic bacteria in acidic peatlands using newly developed 16S rRNA-targeted fluorescent oligonucleotide probes

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Abstract

Based on an extensive 16S rRNA sequence database for type II methanotrophic bacteria, a set of 16S rRNA-targeted oligonucleotide probes was developed for differential detection of specific phylogenetic groups of these bacteria by fluorescence in situ hybridisation (FISH). This set of oligonucleotides included a genus-specific probe for *Methylocystis* (Mcyst-1432) and three species-specific probes for *Methylosinus sporium* (Msins-647), *Methylosinus trichosporium* (Msint-1268) and the recently described acidophilic methanotroph *Methylocapsa acidiphila* (Mcaps-1032). These novel probes were applied to further characterise the type II methanotroph community that was detected in an acidic *Sphagnum* peat from West Siberia in a previous study (Dedysh et al. (2001) Appl. Environ. Microbiol. 67, 4850–4857). The largest detectable population of indigenous methanotrophs simultaneously hybridised with a group-specific probe targeting all currently known *Methylosinus*/*Methylocystis* spp. (M-450), with a genus-specific probe for *Methylocystis* spp. (Mcyst-1432), and with an additional probe (Mcyst-1261) that had been designed to target a defined phylogenetic subgroup of *Methylocystis* spp. The same subgroup of *Methylocystis* was also detected in acidic peat sampled from *Sphagnum*-dominated wetland in northern Germany. The population size of this peat-inhabiting *Methylocystis* subgroup was $2.0 \pm 0.1 \times 10^6$ cells g^{-1} (wet weight) of peat from Siberia and $5.5 \pm 0.5 \times 10^6$ cells g^{-1} of peat from northern Germany. This represented 60 and 95%, respectively, of the total number of methanotroph cells detected by FISH in these two wetland sites. Other major methanotroph populations were *M. acidiphila* and *Methylocella palustris*. Type I methanotrophs accounted for not more than 1% of total methanotroph cells. Neither *M. trichosporium* nor *M. sporium* were detected in acidic *Sphagnum* peat.

Keywords: Fluorescent in situ hybridisation (FISH); Oligonucleotide probe; Type II methanotroph; *Methylocystis* sp.; Acidic peatlands; *Methylocapsa acidiphila*; *Methylocella palustris*

1. Introduction

Methane-oxidising bacteria (MOB) colonise *Sphagnum*-dominated acidic wetlands of the northern hemisphere and reduce the potential flux of methane from these environments to the atmosphere. The taxonomic identity of the methanotrophs present in peatlands has remained elusive for a long time. The approaches that have been used to identify MOB in this acidic habitat have included analyses of methanotroph-specific phospholipid fatty acids (PLFAs) [1], and analyses of total community DNA [2–4] directly extracted from peat. The first approach showed that both type I and type II MOB were present in peat. However, the concentration of type II-specific PLFAs greatly exceeded the corresponding value for type I-specific PLFAs. Polymerase chain reaction-based retrieval of methanotroph-specific marker genes revealed clusters of environmental 16S rDNA, *pmoA*, and *mxaF* sequences that grouped closely to, but were distinct from, sequences of cultured type II MOB. Recently, an effective approach based on the fluorescence in situ hybridisation (FISH) technique [5,6] was applied to determine the abundance of distinct phylogenetic groups of methanotrophs in a

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Sphagnum peat from West Siberia [7]. The set of methanotroph-specific rRNA-targeted oligonucleotide probes used in that study included probes with reported group specificity for type I MOB (probes M-84 and M-705) or for the *Methylosinus*/*Methylocystis* group of type II MOB (probes MA-221 and M-450), as well as two probes (Mcell-1026 and Mcell-181) for the acidophilic methanotroph *Methylocella palustris* [8]. It was shown that acidic peat is colonised mainly by type II MOB, namely by *M. palustris* and an uncharacterised *Methylocystis* population. These comprised approximately 40 and 60%, respectively, of the total number of MOB cells detected by FISH.

The occurrence of an uncharacterised population of peat-inhabiting *Methylocystis* spp. was concluded based on conflicting cell counts obtained using two different type II MOB-specific probes, MA-221 [9] and M-450 [10]. The cell counts obtained with probe MA-221 were only 10^4 type II MOB cells g^{-1} (wet weight) of peat, while the probe M-450 revealed more than 10^6 type II MOB cells g^{-1} of the same samples [7]. This discrepancy was explained by the finding that probe M-450 targets all currently known strains of *Methylosinus* and *Methylocystis*, whereas the probe MA-221, originally described as group-specific, fails to detect many *Methylocystis* strains, and a minority of *Methylosinus* strains [11]. Thus, it was assumed that a subgroup of *Methylocystis* accounted for most (about 99%) of the cells detected by probe M-450 in peat.

Until recently, the sequences of 16S rRNA genes of type II MOB were quite poorly represented in public-domain databases. This made it impossible to formulate probes enabling the differentiation between *Methylocystis* spp. and *Methylosinus* spp. Even the most recently published 16S rRNA-targeted oligonucleotide probes detect these methanotrophs only as a group [12]. This situation has changed due to a comprehensive database of 16S rRNA gene sequences recently obtained for a large collection of *Methylocystis* and *Methylosinus* strains isolated from diverse environments [11]. Thus, the aim of this study was to design a set of oligonucleotide probes for differential detection of bacteria within the *Methylosinus*/*Methylocystis* group, and to apply these probes to further characterise type II MOB populations in the acidic peat examined in our previous study [7]. In addition, we extended the set of methanotrophs detectable by FISH by developing oligonucleotide probes specific for another recently described acidophilic methanotroph, *Methylocapsa acidiphila* [13]. Similar to *M. palustris*, this bacterium was isolated from a *Sphagnum* peat bog, but it was targeted neither by the *Methylocella*-specific probe Mcell-1026 nor by any of the group-specific methanotroph probes applied in our previous study. The updated set of 16S rRNA-targeted oligonucleotide probes was used to compare the abundance of distinct subgroups of MOB in acidic peat sampled from *Sphagnum*-dominated wetlands of two different geographical sites, West Siberia and northern Germany.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Methylosinus trichosporium OB3b (ATCC 35070^T), *M. acidiphila* strain B2 (DSM 13967^T and NCIMB 13765^T), and *M. palustris* strain K (ATCC 700799^T) were the type strains used in this study. In addition, 11 *Methylosinus* and *Methylocystis* strains isolated from various environments [11] were selected as reference organisms for the adjustment of the newly designed probes. These strains included: *M. trichosporium* strain SM6 (IMV B-3060), *Methylosinus sporium* strains SC8, SM27a, and 44/2, and *Methylocystis* sp. strains 42/22, 51, 62/38a, H9a, 62/12, SD5, and SC2. *M. acidiphila* B2 and *M. palustris* K were grown on twice diluted nitrogen-free and nitrogen-containing M1 mineral medium [8,13], respectively; *M. trichosporium* OB3b was grown on NMS medium [14], while all other methanotrophs were cultivated on a basal salt-NH_4Cl medium ('medium 10') [11]. All methanotroph cultures were cultivated under a gas headspace containing 20% methane (vol/vol). The cultures were shaken at 120 rpm and incubated at 24°C. *Bradyrhizobium japonicum* (DSM 30131^T) and *Azorhizobium caulinodans* (DSM 5975^T) were used as non-target control strains and were grown on media recommended by the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) catalogue. To ensure constant exponential growth and high cell ribosome content, all cultures were serially transferred at least three times prior to harvesting.

2.2. Peat samples

Two *Sphagnum* peat samples from different geographical sites were examined in this study. The first one was a peat sample used in our previous study [7]. It was collected from 10–15 cm below the surface of an acidic peat (pH of 3.6–4.5) underlying a *Sphagnum-Carex* plant community (Bakchar bog, Plotnikovo field station in West Siberia, 56°N, 82°E). The second sample was collected from 15 cm below the surface of *Sphagnum* peat (pH 4.2) on the bank area of the dystrophic peat bog lake Kleine Fuchskuhle (northeastern Germany, 53°10′N, 13°02′E). The sampling depth in both sites was determined by the water table level (WTL), i.e. the sampling was done from the layer located just below WTL as this peat layer was shown to be most abundantly inhabited by methanotrophs [1,2].

2.3. Fixation procedure

2.3.1. Bacterial strains

Cells growing in the logarithmic phase were harvested by centrifugation and resuspended in 0.5 ml of phosphate-buffered saline (PBS) (g l^{-1}: NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.44; NaH_2PO_4, 0.2; pH 7.0). Cell suspensions

were mixed with 1.5 ml of 4% (wt/vol) freshly prepared paraformaldehyde solution (Sigma, Deisenhofen, Germany) and fixed for 1.5 h at room temperature. The cells were then collected by centrifugation (6600×*g* for 1 min) and washed twice with PBS to ensure removal of paraformaldehyde. The resulting pellet was resuspended in 0.5 ml of 50% ethanol–PBS (vol/vol) and this cell suspension was stored at −20°C until use.

2.3.2. Peat samples

The cells were serially extracted from peat using a previously described procedure [7]. Two successive fractions (peat water fraction and peat debris fraction obtained after the first round of homogenisation and extraction) were prepared with each *Sphagnum* sample, fixed as described above and used for cell quantification. The total number of cells in a peat sample was determined as a sum of the corresponding cell numbers in peat water and peat debris fractions.

2.4. Oligonucleotide probes

2.4.1. Methylosinus/Methylocystis group

The 16S rDNA sequence database of type II MOB used for probe design was the one described by Heyer et al. (2002). It consisted of strains isolated and described for that study, along with most other public-domain sequences from type II MOB isolates. It included 19 sequences from *M. trichosporium* strains, 22 from *M. sporium* strains, and 54 from strains of the genus *Methylocystis*. Several of the *Methylosinus* isolates for which 16S rDNA sequences are available were not identified morphologically to the species level, but in the present study were considered to belong to either *M. sporium* or *M. trichosporium* based on the observation that these species cluster into two distinct phylogenetic groups [11].

The probe design tool of the ARB program package (developed by O. Strunk and W. Ludwig; available online at http://www.arb-home.de) was used to identify conserved nucleotide sequence regions that were unique for each of these three taxa and allowed their discrimination from all non-target reference sequences in the public-domain 16S rRNA sequence database. The final selection of suitable target sites was done using the in situ accessibility map of *Escherichia coli* 16S rRNA [15]. According to standard nomenclature [16], two newly designed species-specific probes for detection of *M. trichosporium* and *M. sporium*, and one genus-specific probe for detection of all representatives of the genus *Methylocystis* were designated S-S-Msint-1268-a-A-18 (Msint-1268), S-S-Msins-0647-a-A-18 (Msins-647), and S-G-Mcyst-1432-a-A-18 (Mcyst-1432), respectively. For use in FISH these probes were labelled with indocarbocyanine dye (Cy3). To provide further evidence for correct target identification, each of these three probes was used in combination with the *Methylosinus/Methylocystis* group-specific oligonucleotide probe M-450 [10], labelled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS).

2.4.2. M. acidiphila

An oligonucleotide probe was designed for specific detection of the recently described acidophilic methanotroph *M. acidiphila* B2. This probe targeted positions 1032–1049 (based on the *E. coli* numbering system). It was designated S-St-Mcaps-1032-a-A-18 (Mcaps-1032) and labelled with Cy3. A second, FLUOS-labelled oligonucleotide probe, AcidM-181, was used for simultaneous hybridisation of cells along with Mcaps-1032. The probe AcidM-181 was originally described as Mcell-181 and used for specific detection of *M. palustris* [7]. However, probe AcidM-181 targets both *M. palustris* and *M. acidiphila* B2 and, thus, it was renamed S-*-AcidM-0181-a-A-18 (AcidM-181).

2.4.3. Peat-inhabiting subgroup of Methylocystis

The oligonucleotide probe targeting positions 1261–1278 was developed to detect some of the *Methylocystis* strains that escaped detection by the type II MOB group-specific probe MA-221, but were targeted by the type II group-specific probe M-450. The newly designed probe was designated S-*-Mcyst-1261-a-A-18 (Mcyst-1261) and, depending on the experimental setup, was applied in FISH with Cy3 or Cy5 label. Similar to other probes developed for the *Methylosinus/Methylocystis* group (see above), Mcyst-1261 was used in combination with the FLUOS-labelled group-specific probe M-450 to guarantee the confidence of cell identification.

2.4.4. Other probes used in this study

Three probes with reported group specificity for either type I MOB (M-84, M-705) or the *Methylosinus/Methylocystis* group of type II MOB (M-450) [10], were labelled with Cy3 and used in this study. We also used the Cy3-labelled oligonucleotide probe Mcell-1026, which was developed for specific detection of the acidophilic methanotroph *M. palustris* [7]. Depending on the experimental setup, the bacterial probe EUB338 [17] was applied in FISH with a Cy3 or FLUOS label. Oligonucleotide probes were purchased from MWG Biotech (Ebersberg, Germany). Probe sequences, target sites, formamide concentrations in the hybridisation buffer, hybridisation temperature, and NaCl concentrations in the washing buffer used for FISH are given in Table 1.

2.5. Whole-cell hybridisation

Hybridisations were performed as described elsewhere [7,18]. Briefly, 2 μl of the fixed cell suspension was spread on a six-well Teflon-coated slide, air-dried, and dehydrated by successive passages through an ethanol series (50, 80, and 100% (vol/vol) for 3 min each). A 9-μl aliquot of hybridisation buffer (0.9 M NaCl, 20 mM Tris/HCl (pH 7.2), 0.01% sodium dodecyl sulphate (SDS), and formam-

Table 1
Oligonucleotide probes used in this study

Probe	Specificity	Probe sequence (5′–3′)	Target site[a] (16S rRNA positions)	% FA[b]	*T* (°C)[c]	NaCl (mM)[d]	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338–355	0	46	900	[17]
M-450	*Methylosinus*/*Methylocystis* spp.	ATCCAGGTACCGTCATTATC	450–470	30	46	112	[10]
M-84, M-705	type I MOB	CCACTCGTCAGCGCCCGA	84–103	20	46	225	[10]
		CTGGTGTTCCTTCAGATC	705–724				
Mcell-1026	*M. palustris*	GTTCTCGCCACCCGAAGT	1026–1043	0	45–50	900	[7]
AcidM-181	*M. palustris*+*M. acidiphila*	TCTTTCTCCTTGCGGACG	181–198	0	45–50	900	[7]
Mcaps-1032	*M. acidiphila*	CACCTGTGTCCCTGGCTC	1032–1049	0	45–50	900	this study
Msint-1268	*M. trichosporium*	TGGAGATTTGCTCCGGGT	1268–1285	20	46	225	this study
Msins-647	*M. sporium*	TCTCCCGGACTCTAGACC	647–664	30	46	112	this study
Mcyst-1432	all *Methylocystis* spp.	CGGTTGGCGAAACGCCTT	1432–1449	0	50	900	this study
Mcyst-1261	peat-inhabiting subgroup of *Methylocystis* spp.	TTGCTCGAGGTCGCCCTT	1261–1278	0	50–55	900	this study

[a]Position numbers refer to the *E. coli* 16S rRNA sequence.
[b]Percent formamide in the hybridisation buffer.
[c]Temperature of hybridisation.
[d]Concentration of sodium chloride in the washing buffer.

ide concentration as given in Table 1) was placed on each spot of fixed cells. The slide was transferred to the equilibrated hybridisation chamber and prehybridised for 30 min. Following prehybridisation, 1 μl of fluorescent probe solution (50 ng probe $\mu l^{-1}$of double-distilled water) was added to each spot and the slide was returned to the hybridisation chamber for 2 h. Slides were then washed at the appropriate hybridisation temperature for 10 min in washing buffer (20 mM Tris/HCl, 0.01% SDS, NaCl concentration as given in Table 1) and rinsed with twice distilled water. The slides were air-dried, stained with the universal DNA stain 4′,6-diamidino-2-phenylindole (DAPI, 2 μM) for 10 min in the dark, rinsed again with distilled water, and finally air-dried. Each well of the slide was mounted with a drop of Citifluor AF1 antifadent (Citifluor Ltd., Canterbury, UK), covered with a coverslip and viewed immediately.

2.6. Optimisation of hybridisation conditions

Our previous study [7] had shown that the optimisation of hybridisation conditions by the addition of formamide to the hybridisation buffer [19] was not appropriate for some methanotrophs. For example, the presence of formamide clearly inhibited the whole-cell hybridisation of *M. palustris* with all fluorescent probes used, including EUB338. In order to check whether the same effect occurred during the whole-cell hybridisation of *M. acidiphila* B2 and other type II MOB, two different approaches were used to optimise the hybridisation conditions: (i) gradually increasing the hybridisation stringency by the addition of formamide to the hybridisation buffer in 5% (vol/vol) steps as described by Manz et al. (1992); (ii) increasing the hybridisation temperature from 30 to 70°C in 5°C steps without formamide in the hybridisation buffer. In each case hybridisation was performed with target organisms as well as non-target organisms that displayed the smallest number of mismatches within the target region (Table 2).

2.7. Counting of methanotroph cells by FISH

Cell counts of *M. trichosporium*, *M. sporium*, all *Methylocystis* spp. and the subgroup of peat-inhabiting *Methylocystis* spp. were performed on slides with fixed peat extracts simultaneously hybridised with FLUOS-labelled M-450 (universal to the *Methylosinus*/*Methylocystis* group)

Table 2
Reference strains used for stringency adjustment of the newly designed oligonucleotide probes

Probe	Target organism(s)	Reference strain(s)	
		Positive control	Negative control (no. of mismatches)
Msint-1268	*M. trichosporium*	*M. trichosporium* strains OB3b, SM6	*Methylocystis* spp. strains 62/12, SD5, H9a (2); *M. palustris* K (2)
Msins-647	*M. sporium*	*M. sporium* strains SC8, SM27a, 44/2	*M. trichosporium* strains OB3b, SM6 (1); *Methylocystis* spp. strains 42/22, 51, H9a, 62/12, SD5, SC2 (1)
Mcyst-1432	all *Methylocystis* spp.	*Methylocystis* spp. strains 42/22, 51, H9a, 62/12, SD5, SC2, 62/38a	*M. trichosporium* strains OB3b, SM6 (1); *M. sporium* strains SC8, SM27a, 44/2 (1)
Mcyst-1261	peat-inhabiting subgroup of *Methylocystis* spp.	*Methylocystis* spp. strains 51, SC2, 42/22, 62/38a	*Methylocystis* spp. strains 62/12, SD5, H9a (1); *M. palustris* K (2)
Mcaps-1032	*M. acidiphila* B2	*M. acidiphila* B2	*B. japonicum* (2)
AcidM-181	*M. palustris*+*M. acidiphila* B2	*M. palustris*, *M. acidiphila* B2	*A. caulinodans* (1)

and the corresponding group-specific Cy3-labelled probes (i.e. Msint-1268, Msins-647, Mcyst-1432, or Mcyst-1261). The numbers of *M. acidiphila* and *M. palustris* cells were determined on preparations that were simultaneously hybridised with the FLUOS-labelled group-specific probe AcidM-181 and the corresponding Cy3-labelled probes Mcaps-1032 and Mcell-1026, respectively. In addition, the number of cells stained with the bacterial probe EUB338, type I MOB probes (M-84 and M-705) and the *Methylosinus*/*Methylocystis* group-specific probe M-450 were determined. Cell counting was performed on 100 randomly chosen fields of view (FOV) for each test sample, which was sufficient for a statistically valid quantification of methanotrophs in a peat sample [7]. The number of target cells per gram of wet peat was determined from the area of the sample spot, the FOV area, the volume of the fixed sample used for hybridisation, and the volume of the peat water extracted from the sample.

2.8. Total cell counts

Total cell counts from peat samples were obtained using DAPI staining (see above). Dilutions that resulted in approximately 20–200 cells per FOV were used. The total cell number in 100 randomly chosen FOV was determined as described above.

2.9. Microscopy

Slides with different mixtures of target and non-target

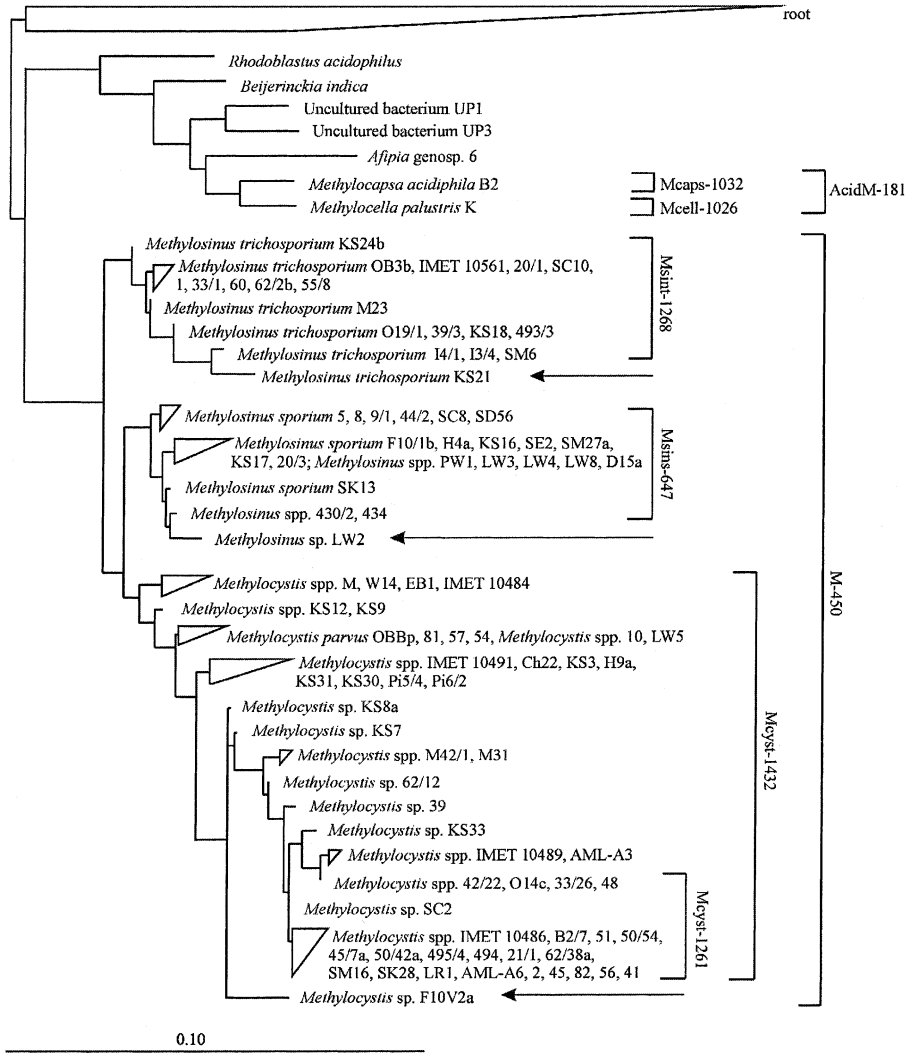

Fig. 1. Maximum-likelihood phylogenetic tree showing the relative positions of 16S rRNA gene sequences of type II MOB (*Methylosinus*/*Methylocystis* group), acidophilic methanotrophs *M. acidiphila* and *M. palustris*, and the non-MOB *Beijerinckia indica*, *Afipia* genospecies 6, *R. acidophilus*, and two sequences (UP1, UP3) retrieved from soil by cultivation-independent methods. The selection of species and tree construction is exactly as described by Heyer et al. [11]. The brackets indicate the scope of each of the newly designed oligonucleotide probes (Msint-1268, Msins-647, Mcyst-1432, Mcyst-1261, and Mcaps-1032) and of the previously developed M-450 probe with group specificity for all *Methylosinus*/*Methylocystis* spp. Arrows indicate the three strains not detected by the species-specific or genus-specific probes Msint-1268, Msins-647, and Mcyst-1432. The root was composed of 24 sequences from (non-methanotrophic) α-*Proteobacteria*. The distance bar represents 0.1 substitution per nucleotide position.

methanotroph cells as well as peat samples hybridised with the newly designed probes were examined by confocal laser-scanning microscopy with a Leica DMR XE microscope, 63× oil immersion lenses, and TCS NT 1.6.582 software. The cell counts in peat samples were carried out with a Zeiss Axioplan 2 microscope (Carl Zeiss, Oberkochen, Germany) equipped with the following filter sets: HQ light filter AHF/AF 41001 (AHF Analysentechnik, Tübingen, Germany) for FLUOS-labelled probes (excitation 460–500 nm, emission 510–560 nm), AHF/F 41007 for Cy3-labelled probes (excitation 510–560 nm, emission 572.5–642.5 nm), and Zeiss Filter 02 for DAPI staining (excitation 365 nm, long pass emission 420 nm).

3. Results and discussion

3.1. Design of oligonucleotide probes

The public-domain 16S rRNA sequence data set for type II MOB has been greatly expanded by the recent description of a large culture collection [11]. Based on this data set, and on the public-domain 16S rRNA database of the ARB program package, three oligonucleotide probes were designed for the differential detection of the three major monophyletic subgroups within the *Methylosinus*/*Methylocystis* group: *Methylocystis* spp., *M. trichosporium*, and *M. sporium*. Thus, the newly designed probes included a genus-specific probe for *Methylocystis* (Mcyst-1432) and two species-specific probes for *M. trichosporium* (Msint-1268) and *M. sporium* (Msins-647) (Fig. 1, Table 1).

The sequence of the Mcyst-1432 probe matched 53 of 54 currently available sequences from isolates of the genus *Methylocystis*. The single exception was strain F10V2a. The probe showed one mismatch (G:U at position 1444) with the 16S rRNA sequences of several *M. trichosporium* and *M. sporium* strains, and with *Rhodoblastus acidophilus*. All other non-target reference sequences displayed at least two mismatches to this probe.

A detailed analysis of the 16S rRNA database of type II MOB and reference organisms showed that it is not possible to design a probe that specifically targets all representatives of the genus *Methylosinus*. Instead, two separate probes were developed, one targeting 18 of 19 known strains of *M. trichosporium* (strain KS21 was the exception) and the other targeting 21 of 22 known strains of *M. sporium* (*Methylosinus* sp. LW2 was the exception). The combination of these two probes coupled to the same fluorophore allowed detection of all *Methylosinus* spp. (excepting the two strains noted above). The probe with intended target specificity for *M. trichosporium*, Msint-1268, displayed at least two mismatches to 16S rRNA sequences of all other non-target reference sequences from cultured organisms. The probe with intended target specificity for *M. sporium*, Msins-647, exhibited one mismatch to the 16S rRNA sequences of all *Methylocystis* strains (T:C at position 652) and strains of *M. trichosporium* (C:A at position 648).

An additional oligonucleotide probe, Mcyst-1261, was designed to detect a subcluster of *Methylocystis* strains (Fig. 1, Table 1). This subcluster contains 16S rRNA sequences that were absent from the public-domain databases until recently. As a consequence, many of the currently existing type II MOB group-specific probes (except M-450) contain mismatches to sequences of these strains of *Methylocystis* [11]. Based on the conflicting data obtained with the probes MA-221 and M-450 [7], we assumed that this subgroup might contain acidophilic/acid-tolerant *Methylocystis* spp., and furthermore might account for most (about 99%) of the cells detected by probe M-450 in native peat.

We have also designed a probe with intended target specificity for the recently described acidophilic methanotroph *M. acidiphila* B2 (Mcaps-1032, Table 1). Mcaps-1032 matched the sequence of the only known isolate of *M. acidiphila* (strain B2), while all other non-target reference sequences from cultured organisms displayed at least two mismatches to this probe.

The specificity of each newly designed probe was also confirmed in silico using the probe match tool of the Ribosomal Database Project [20]. The organisms whose 16S rRNA sequences exhibited the next smallest number of mismatches in the target region of the newly designed probes were chosen as negative control strains for optimisation of the probe hybridisation conditions (Table 2).

3.2. Optimisation of hybridisation conditions

We first attempted to determine optimal hybridisation conditions for all newly designed probes by increasing the concentration of formamide in the hybridisation buffer according to Manz et al. [19]. The range of formamide concentrations used was from 0 to 40%, in 5% increments. This approach worked well with the two species-specific probes designed for *M. trichosporium* and *M. sporium*, Msint-1268 and Msins-647. Specific detection was achieved at a concentration of 20 and 30% formamide in the hybridisation buffer, respectively (Table 1). The combined use of both probes labelled with the same fluorescent dye at 20% formamide in the hybridisation buffer allowed detection of all representatives of the genus *Methylosinus*.

In contrast to whole-cell hybridisation of *Methylosinus* strains, the approach based on the use of formamide was not applicable for *M. acidiphila* B2 and for some of the *Methylocystis* strains. Similar to the effect observed earlier for *M. palustris* K [7], the addition of formamide to the hybridisation buffer strongly inhibited the whole-cell hybridisation of *M. acidiphila* B2 with all fluorescent probes tested, Mcaps-1032, AcidM-181, and EUB338 (data not shown). In contrast, without formamide in the hybridisa-

tion buffer but at the same stringency conditions achieved by raising the temperature, all cells were properly stained with the same probes. The same effect was observed for some representatives of *Methylocystis* (strains 51, H9a, 62/38a, 62/12) isolated from freshwater environments, i.e. boggy waters, oligotrophic and eutrophic lakes. Interestingly, the use of formamide did not show any negative effect on whole-cell hybridisation of *Methylocystis* strains isolated from brackish water (strains 42/22 and 33/26). Thus, formamide was not further applied for the whole-cell hybridisations of *M. acidiphila* B2 and cultures of *Methylocystis*, and the optimal hybridisation conditions were determined by increasing the hybridisation temperature in 5°C steps. The optimal hybridisation temperature ranges that provided high target specificity were 45–50°C for Mcaps-1032, 50°C for Mcyst-1432, and 50–55°C for Mcyst-1261.

3.3. Analysis of cell mixtures of different type II MOB

Most newly designed probes showed high signal intensities of comparable value for all target strains used in this study (see Table 2). Only the genus-specific probe for *Methylocystis* (Mcyst-1432) gave a signal of slightly less intensity, but this was still sufficient enough to enable reliable target cell detection. The target specificity of the probes Msint-1268, Msins-647, Mcyst-1432, and Mcyst-1261 was confirmed using different two- and three-component cell mixtures of methanotrophs from the *Methylosinus/Methylocystis* group: (i) *M. trichosporium*+*M. sporium*, (ii) *M. trichosporium*+*Methylocystis* sp., (iii) *M. sporium*+*Methylocystis* sp., (iv) *M. trichosporium*+*M. sporium*+*Methylocystis* sp. Each of these cell mixtures was prepared in three to four combinations using different strains of the same methanotroph species (see Section 2.1). Application of each probe allowed clear discrimination of the target population from cells of other type II MOB in each of these cell mixtures (Fig. 2).

3.4. Identification of indigenous type II MOB populations in Sphagnum peat

Each of the probes developed for specific detection of the type II MOB subgroups, Msint-1268, Msins-647, and Mcyst-1432, was applied to a *Sphagnum* peat sample from West Siberia, which had been analysed in a previous study [7]. The aim was to more precisely identify a methanotroph population that had been detected by the group-specific probe M-450, but not detected with probe M-221. While M-450 targets all known type II MOB of the genera *Methylosinus* and *Methylocystis*, M-221 targets only 11 of 19 *M. trichosporium* strains in our database, 20 of 22 *M. sporium* strains, and eight of 54 *Methylocystis* strains.

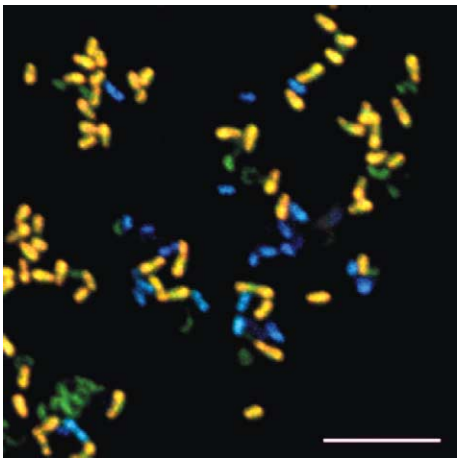

Fig. 2. Three-component cell mixture of *M. trichosporium* OB3b, *M. sporium* SM27a, and *Methylocystis* sp. 62/12 hybridised with the species-specific probe Msint-1268-Cy3 (red) and Mcyst-1261-Cy5 (blue) in parallel to the group-specific probe for all *Methylosinus/Methylocystis* spp., M-450-FLUOS (green). Target cells of methanotrophs hybridised with two group-specific probes and showed up as orange-yellow (*M. trichosporium* OB3b) or green-blue (*Methylocystis* sp. 62/12), while the reference cells (*M. sporium* SM27a) hybridised only with the group-specific probe M-450 and showed a green colour. The scale bar is 10 μm.

No cells were detected in acidic peat with the two species-specific probes for *M. trichosporium* (Msint-1268) and *M. sporium* (Msins-647). In contrast, hybridisation with the *Methylocystis*-specific probe Mcyst-1432 detected a population that was as numerous ($1.8 \pm 0.1 \times 10^6$ cells g^{-1} of wet peat) as the methanotrophic population detected in our previous study with the M-450 probe (Table 3). Moreover, the same number of cells was detected with the probe Mcyst-1261, which was newly designed to specifically target a defined subgroup of *Methylocystis* strains (Fig. 1). We presume that this subgroup contains acidophilic/acid-tolerant strains. To check whether this presumed acidophilic/acid-tolerant population of *Methylocys-*

Table 3
The number of DAPI-stained cells detected by FISH with EUB338 and with different methanotroph-specific oligonucleotide probes in acidic peat of two *Sphagnum*-dominated peatlands of different geographic location

Sampling site location	Number of DAPI-stained cells, $N \times 10^8$ g^{-1} of wet peat	Number of cells detected with different oligonucleotide probes (g^{-1} of wet peat)[a]						
		EUB338, $N \times 10^8$	M-450, $N \times 10^6$	Mcyst-1432, $N \times 10^6$	Mcyst-1261, $N \times 10^6$	Mcaps-1032, $N \times 10^5$	Mcell-1026, $N \times 10^5$	M-84+M-705, $N \times 10^4$
West Siberia	4.5 ± 0.4	2.9 ± 0.3	1.8 ± 0.1	1.8 ± 0.1	2.0 ± 0.1	1.4 ± 0.5	11.9 ± 1.1	0.2 ± 0.2
North Germany	1.7 ± 0.2	0.5 ± 0.1	5.7 ± 0.6	5.5 ± 0.6	5.5 ± 0.5	1.4 ± 0.9	0.6 ± 0.4	6.1 ± 3.6

[a]No cells were detected with Msint-1268 and Msins-647 probes.

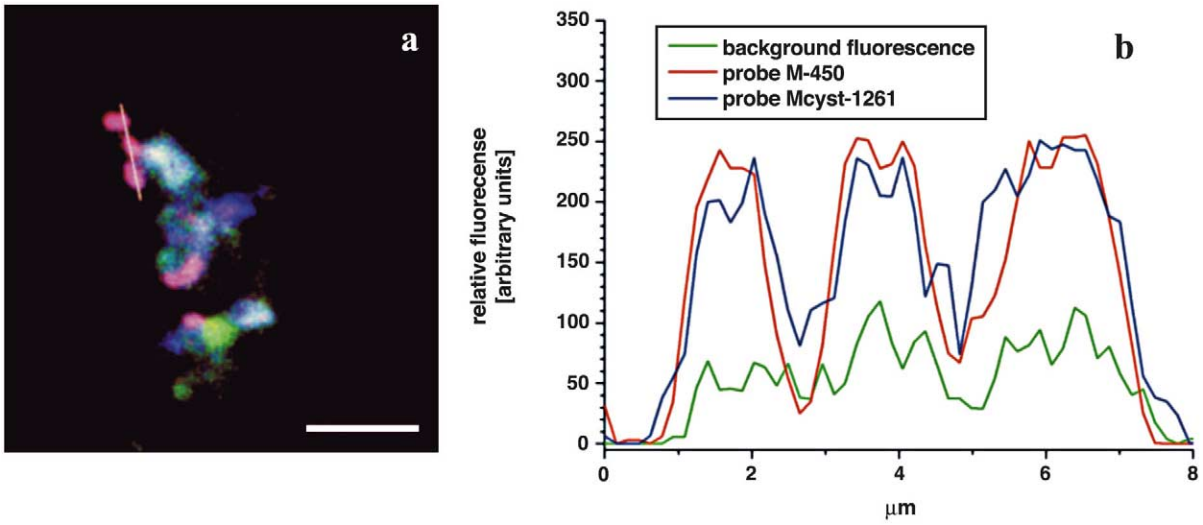

Fig. 3. a: In situ hybridisation of a *Sphagnum* peat sample with two oligonucleotide probes, M-450 and Mcyst-1261, marked with Cy3 (red) and Cy5 (blue), respectively. Methanotroph cells hybridised with both probes and showed up as pink-coloured cells. The scale bar is 10 µm. b: Intensity profiles of the fluorophores determined in a transect through three target cells (indicated by line in a), showing high and equal signal intensities for the probes M-450 and Mcyst-1261.

tis spp. might also be abundant in other *Sphagnum* peatlands, we have examined an acidic peat in northern Germany. In this sample FISH with the M-450 probe detected an even more abundant population of type II methanotrophs ($5.7 \pm 0.6 \times 10^6$ cells g^{-1} of wet peat). The cell counts with probes Mcyst-1432 and Mcyst-1261 were nearly identical to those with the M-450 probe (Table 3). Final proof that the majority of MOB cells detected in peat belonged to this defined subgroup of presumably acidophilic/acid-tolerant *Methylocystis* spp. was obtained by parallel hybridisation of both peat samples with Cy3-labelled probe M-450 and Cy5-labelled probe Mcyst-1261. Examination by confocal laser-scanning microscopy showed that all target cells were stained with both fluorophores (Cy3 and Cy5), indicating simultaneous hybridisation of target cells with both oligonucleotide probes (Fig. 3).

3.5. Detection of *M. acidiphila* in *Sphagnum* peat by FISH

The preparations obtained with native peat samples were hybridised with the Cy3-labelled probe Mcaps-1032 in conjunction with FLUOS-labelled EUB338 and stained with DAPI (Fig. 4). The microscopic examination revealed that cells of *M. acidiphila* were present in both peat samples used in this study. The population sizes of this acidophilic methanotroph were $1.4 \pm 0.5 \times 10^5$ and $1.4 \pm 0.9 \times 10^5$ cells g^{-1} of wet peat in samples from West Siberia and northern Germany, respectively.

3.6. Comparison of methanotroph community structure in *Sphagnum* wetlands of different geographic location

In our previous study we were able to determine the major methanotroph populations in acidic peat sampled from a West Siberian wetland. The indigenous methanotrophs in that sample were represented mainly by type II MOB, while type I MOB accounted for less than 0.1% of the total methanotroph cells detected by FISH [7]. In this study, we also examined a *Sphagnum* peat sample from northern Germany. The peat samples from both sites had almost the same pH (4.2). The number of type I

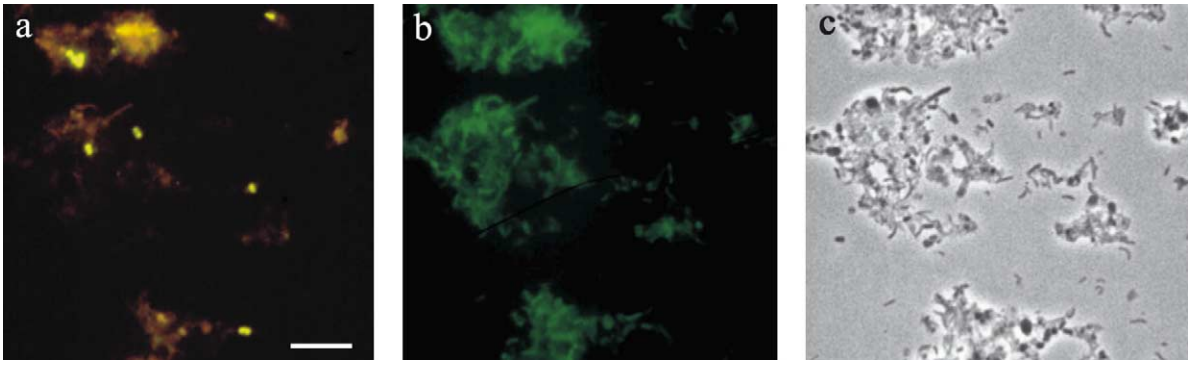

Fig. 4. Specific detection of *M. acidiphila* in a peat sample by FISH. Pictured are epifluorescence micrographs of in situ hybridisation with probes Mcaps-1032 labelled with Cy3 (a) and EUB338 labelled with FLUOS (b), and the phase-contrast image (c). The scale bar (10 µm) applies to all images.

MOB cells detected with probes M-84 and M-705 in the peat sample from northern Germany was more than one order of magnitude higher than that detected in the West Siberian peat sample ($6.1 \pm 3.6 \times 10^4$ cells g^{-1} of wet peat, Table 3). Nevertheless, this accounted for only 1% of total methanotrophs detected by FISH. Thus, indigenous methanotrophic populations in both wetland sites were represented mainly by type II MOB.

The numerically largest MOB group, accounting for 60 and 96% of total methanotroph cells detected by FISH in peat from Siberia and Germany, respectively, was *Methylocystis* spp., belonging specifically to a phylogenetic subgroup of *Methylocystis* detectable by probe Mcyst-1261. A large population size of these bacteria in both acidic wetlands suggests an acidophilic or, at least, acid-tolerant nature of these organisms. However, at present it is not possible to draw any final conclusions because none of the cultured strains used for design and evaluation of target specificity of probe Mcyst-1261 were isolated from acidic *Sphagnum* wetlands. The cultivated representatives of this phylogenetic subgroup of *Methylocystis* spp. were isolated on neutral media (pH 7.0–7.4) from different environmental samples of generally neutral pH, such as lake sediments, freshwaters, and brackish waters [11]. Thus, the phenotypic and physiological characterisation of *Methylocystis* spp. inhabiting acidic peatlands requires the isolation of the organisms in pure culture.

Two other populations of type II MOB detected in both wetland sites were *M. acidiphila* and *M. palustris*. *M. acidiphila* was enumerated in both samples at greater than 10^5 cells g^{-1} of peat. This accounted for 5 and 2% of total methanotroph cells detected by FISH in peat from Siberia and Germany, respectively. In contrast, the population sizes of *M. palustris* were significantly different in *Sphagnum* peat from West Siberia (10^6 cells g^{-1} of peat) and northern Germany (10^4 cells g^{-1} of peat) (Table 3). An explanation for such a profound difference might be that the probe Mcell-1026 targets the only described species of *Methylocella*, namely *M. palustris*, while the *Sphagnum* wetland around lake Kleine Fuchskuhle might be inhabited by some other, yet unknown, species of *Methylocella*. The same assumption might be true for the probe Mcaps-1032, which has been developed based on the only existing isolate of the genus *Methylocapsa*. Thus, the possibility that some unknown species of acidophilic *Methylocella* and *Methylocapsa* still escaped detection by FISH with the currently available set of oligonucleotide probes cannot be excluded. The total numbers of methanotrophs detected in our study were $3.1 \pm 0.2 \times 10^6$ and $5.7 \pm 0.4 \times 10^6$ cells g^{-1} (wet weight) of peat in *Sphagnum* peat samples from West Siberia and northern Germany, respectively. However, these values refer to single-time, single peat soil depth sample measurements. Significant variations in methanotroph community structure and abundance might occur in different subsites and depths as well as on a seasonal scale. In addition, some environmental factors (variation in temperature, aeration conditions, substrate availability) might also have a strong impact on population dynamics of different MOB in acidic peatlands.

In summary, this study has extended the set of rRNA-targeted oligonucleotide probes available for specific detection and enumeration of MOB in environmental samples by FISH. The major emphasis was given to differential detection of type II MOB, as they represent the dominant methanotroph population in acidic *Sphagnum* peatlands of different geographic locations. The application of the newly developed oligonucleotide probes allowed us to more closely identify the numerically largest group of indigenous methanotrophs. These belonged to a phylogenetically defined subcluster of *Methylocystis* spp. Future studies will address questions concerning the phenotypic traits of this novel subgroup of *Methylocystis* spp. and the environmental factors that have a major impact on their population dynamics in situ.

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References

[1] Sundh, I., Borda, P., Nilsson, M. and Svensson, B.H. (1995) Estimation of cell numbers of methanotrophic bacteria in boreal peatlands based on analysis of specific phospholipid fatty acids. FEMS Microbiol. Ecol. 18, 103–112.

[2] McDonald, I.R., Hall, G.H., Pickup, R.W. and Murrell, J.C. (1996) Methane oxidation potential and preliminary analysis of methanotrophs in blanket bog peat using molecular ecology techniques. FEMS Microbiol. Ecol. 21, 197–211.

[3] McDonald, I.R. and Murrell, J.C. (1997) The methanol dehydrogenase structural gene *mxaF* and its use as a functional gene probe for methanotrophs and methylotrophs. Appl. Environ. Microbiol. 63, 3218–3224.

[4] McDonald, I.R. and Murrell, J.C. (1997) The particulate methane monooxygenase gene *pmoA* and its use as a functional gene probe for methanotrophs. FEMS Microbiol. Lett. 156, 205–210.

[5] Amann, R.I., Krumholz, L. and Stahl, D.A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

[6] Amann, R.I., Ludwig, W. and Schleifer, K.-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

[7] Dedysh, S.N., Derakshani, M. and Liesack, W. (2001) Detection and enumeration of methanotrophs in acidic *Sphagnum* peat by 16S rRNA fluorescence in situ hybridization, including the use of newly developed oligonucleotide probes for *Methylocella palustris*. Appl. Environ. Microbiol. 67, 4850–4857.

[8] Dedysh, S.N., Liesack, W., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Bares, A.M., Panikov, N.S. and Tiedje, J.M. (2000) *Methylocella palustris* gen. nov., sp. nov., a new methane-

oxidizing acidophilic bacterium from peat bogs representing a novel sub-type of serine pathway methanotrophs. Int. J. Syst. Evol. Microbiol. 50, 955–969.

[9] Bourne, D.G., Holmes, A.J., Iversen, N. and Murrell, J.C. (2000) Fluorescent oligonucleotide rDNA probes for specific detection of methane oxidising bacteria. FEMS Microbiol. Ecol. 31, 29–38.

[10] Eller, G., Stubner, S. and Frenzel, P. (2001) Group-specific 16S rRNA targeted probes for the detection of type I and type II methanotrophs by fluorescence in situ hybridisation. FEMS Microbiol. Lett. 198, 91–97.

[11] Heyer, J., Galchenko, V.F. and Dunfield, P.F. (2002) Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. Microbiology 148, 2831–2846.

[12] Gulledge, J., Ahmad, A., Steudler, P.A., Pomerantz, W.J. and Cavanaugh, C.M. (2001) Family- and genus-level 16S rRNA-targeted oligonucleotide probes for ecological studies of methanotrophic bacteria. Appl. Environ. Microbiol. 67, 4726–4733.

[13] Dedysh, S.N., Khmelenina, V.N., Suzina, N.E., Trotsenko, Y.A., Semrau, J.D., Liesack, W. and Tiedje, J.M. (2002) *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. Int. J. Syst. Evol. Microbiol. 52, 251–261.

[14] Whittenbury, R., Phillips, K.C. and Wilkinson, T.F. (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. J. Gen. Microbiol. 61, 205–218.

[15] Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W. and Amann, R. (1998) Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. 64, 4973–4982.

[16] Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A. and Raskin, L. (1996) The oligonucleotide probe database. Appl. Environ. Microbiol. 62, 3557–3559.

[17] Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

[18] Stahl, D.A. and Amann, R. (1991) Development and application of nucleic acid probes. In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 205–248. Wiley, New York.

[19] Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992) Phylogenetic oligonucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.

[20] Maidak, B.L., Cole, J.R., Parker, C.T., Garrity, J.M., Larsen, N., Li, B., Lilburn, T.G., McCaughey, M.J., Olsen, G.J., Overbeck, R., Pramanik, S., Schmidt, T.M., Tiedje, J.M. and Woese, C.R. (1999) A new version of the RDP (Ribosomal Database Project). Nucleic Acid Res. 27, 171–173.