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Population Analysis in a Denitrifying Sand Filter: Conventional and In Situ Identification of *Paracoccus* spp. in Methanol-Fed Biofilms

ALEXANDER NEEF,[1] ANITA ZAGLAUER,[2] HARALD MEIER,[1] RUDOLF AMANN,[1]* HILDE LEMMER,[2] AND KARL-HEINZ SCHLEIFER[1]

Lehrstuhl für Mikrobiologie, Technische Universität München, D-80290 Munich,[1] and Institut für Wasserforschung, Bayerisches Landesamt für Wasserwirtschaft, D-80539 Munich,[2] Germany

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The microbial community of a denitrifying sand filter in a municipal wastewater treatment plant was examined by conventional and molecular techniques to identify the bacteria actively involved in the removal of nitrate. In this system, denitrification is carried out as the last step of water treatment by biofilms growing on quartz grains with methanol as a supplemented carbon source. The biofilms are quite irregular, having a median thickness of 13 to 20 μm. Fatty acid analysis of 56 denitrifying isolates indicated the occurrence of *Paracoccus* spp. in the sand filter. 16S rRNA-targeted probes were designed for this genus and the species cluster *Paracoccus denitrificans-Paracoccus versutus* and tested for specificity by whole-cell hybridization. Stringency requirements for the probes were adjusted by use of a formamide concentration gradient to achieve complete discrimination of even highly similar target sequences. Whole-cell hybridization confirmed that members of the genus *Paracoccus* were abundant among the isolates. Twenty-seven of the 56 isolates hybridized with the genus-specific probes. In situ hybridization identified dense aggregates of paracocci in detached biofilms. Probes complementary to the type strains of *P. denitrificans* and *P. versutus* did not hybridize to cells in the biofilms, suggesting the presence of a new *Paracoccus* species in the sand filter. Analysis using confocal laser scanning microscopy detected spherical aggregates of morphologically identical cells exhibiting a uniform fluorescence. Cell quantification was performed after thorough disruption of the biofilms and filtration onto polycarbonate filters. An average of 3.5% of total cell counts corresponded to a *Paracoccus* sp., whereas in a parallel sand filter with no supplemented methanol, and no measurable denitrification, only very few paracocci (0.07% of cells stained with 4′,6-diamidino-2-phenylindole) could be detected. *Hyphomicrobium* spp. constituted approximately 2% of all cells in the denitrifying unit and could not be detected in the regular sand filter. This clear link between in situ abundance and denitrification suggests an active participation of paracocci and hyphomicrobia in the process. Possible selective advantages favoring the paracocci in this habitat are discussed.

The ionic nitrogen oxides, nitrate and nitrite, are converted into the gaseous products nitrogen and nitrous oxide during denitrification. In wastewater treatment, denitrification is the final step of nitrogen elimination succeeding nitrification, the aerobic conversion of ammonia to nitrate. In the activated sludge process, denitrification can be accomplished either during the degradation of carbonaceous substrates (simultaneous denitrification) or preceding this step (predenitrification). In both cases, the carbon sources for the process are derived from the wastewater. Alternatively, postdenitrification can be performed in an activated sludge unit following the aeration step or in a separate fixed-bed system. In both cases, an external carbon source must be supplied.

The wastewater treatment plant in Munich-Dietersheim is equipped with a sand filter system for the removal of suspended solids from the secondary effluent before being released to the river Isar. A postdenitrification process was introduced in this sand filter system in 2 of 24 filter cells to fulfill high nitrogen elimination standards without costly reconstruction. Pilot studies for optimizing the denitrification process parameters have been performed by the city of Munich since 1992 (11). In the sand filter, the secondary effluent is supplemented with methanol as an additional substrate to provide sufficient reduced carbon compounds for nitrate reduction. Methanol is often used as an inexpensive carbon source for postdenitrification. McCarty et al. (25) calculated a demand of 2.5 g of methanol per g of NO_3^--N eliminated for efficient nitrate removal, and similar ratios have been determined by other groups (29, 44).

Despite the widespread application of methanol-supported postdenitrification systems (14), knowledge about the bacteria actually involved in the process is relatively limited, although attempts have been made to identify and enumerate denitrifiers in activated sludge. Members of the genera *Pseudomonas*, *Alcaligenes*, and *Hyphomicrobium* have been isolated as a predominant part of the denitrifying flora in one study (39), whereas Vedenina and Govorukhina (50) isolated *Paracoccus* and *Hyphomicrobium* spp. Additionally, Knowles (16) reported the involvement of *Bacillus* and *Methylobacterium* spp. Sperl and Hoare (41), as well as Attwood and Harder (4), have shown that enrichments for methylotrophic denitrifiers from various habitats including soil, mud, and polluted water selected for *Hyphomicrobium* spp. Hyphomicrobia have been identified by cell morphology in denitrifying activated sludge (29, 44). On the basis of earlier results of cultivation, it has been suggested (16) that *Hyphomicrobium* spp. may be the sole

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Technische Universität München, Arcisstrasse 16, D-80290 Munich, Germany. Phone: 49 89 2892 2373. Fax: 49 89 2892 2360. Electronic mail address: amann@mbitum2.biol.chemie.tu-muenchen.de.

TABLE 1. Range of operational, physical, and chemical parameters of the sand filter from the wastewater treatment plant Munich-Dietersheim

Parameter[a]	Minimal–maximal value range[b]
Capacity (PE/filter cell)	40,000
Q_d (m^3 day^{-1})	9,000
Surface load (mh^{-1})	6–9
Grain size of quartz sand (mm)	2.0–3.2
Bumping frequency (h)*	Every 3–5[c]
Backwashing frequency (h)*	Approx. every 24[d]
NO_3^- influent (mg of NO_3^--N $liter^{-1}$)	10–45
NO_3^- effluent (mg of NO_3^--N $liter^{-1}$)*	6–12[e]
NO_3^- elimination (%)*	44–67[e]
Methanol dosage into influent (mg/mg of NO_3^--N within influent)*	2.4–3.6[e]
Actually degraded methanol (mg/mg of eliminated NO_3^--N)*	3.6–5.4[e]
COD in influent except methanol (mg $liter^{-1}$)	17–24
Total P (mg $liter^{-1}$)	0.5–0.9
NH_4^+ (mg of NH_4^+-N $liter^{-1}$)	0.1–0.7
Temp (°C in winter–°C in summer)	12–21[f]
Dissolved oxygen (mg $liter^{-1}$)	Up to 9.5 during backwashing[f]
pH	6.7–7.2[e]

[a] Parameters marked with an asterisk refer only to filter cell 18. Q_d, dry-weather flow; COD, chemical oxygen demand; PE, population equivalents.
[b] Ranges of monthly mean values from 1994 and 1995.
[c] Up to 24 h depending on the test regime.
[d] Up to once every 72 h depending on the test regime.
[e] Depending on the target value and test regime.
[f] Measurements in 60-cm depth within the filter bed.

group active as a denitrifier in natural habitats where methanol is available. Currently, it is known that members of other genera, such as *Paracoccus*, *Rhodobacter*, and *Blastobacter*, are also able to denitrify with methanol as a carbon source (13, 40, 49). All other known methanol utilizers are obligate aerobes (13, 17).

Modern identification of bacteria in environmental samples often takes advantage of rRNA molecules as phylogenetic markers (3, 21). Fluorescently labeled 16S and 23S rRNA targeted probes have been used for single-cell identification of bacteria in samples such as activated sludge (51), biofilms (32), and aerosols (27). Population studies of denitrifiers in the environment are normally based on the selection of this trait by cultivation-dependent approaches (7, 12, 39) and are thus limited to the culturable part of the population. Application of in situ hybridization has shown that cultivation-based methods often fail to represent the true microbial community structure (51). Therefore, the aim of this study was to combine conventional isolation of denitrifiers with the application of specific rRNA-targeted probes for the analysis of the microbial community structure of a methanol-supplemented, denitrifying sand filter with a stable rate of nitrate removal.

MATERIALS AND METHODS

The sand filter units of the wastewater treatment plant Munich-Dietersheim. The downflow sand filter system consists of 24 filter cells. Each cell covers an area of 81.1 m^2. The active filter bed is 1.50 m thick. The grain size of the carrier material is 2.0 to 3.2 mm. This layer is situated on a supporting quartz sand layer with a grain size of 5 to 8 mm. The water level is approximately 2.5 m above the active filter bed. In filter cell 18, a dose of undiluted methanol was added approximately 30 cm underneath the water level for support of denitrification. Upflow bumping with effluent water was performed to expunge developed gases from this filter cell to keep denitrification rates stable. Additionally, during the backwashing process, air was blown throughout the filter cell to prevent clogging by excess biofilm and thus maintain a constant flow rate. Operation parameters and physical and chemical data of the sand filters are presented in Table 1.

Organisms and growth conditions. Reference strains used in this study and their sources are given in Table 2. *Paracoccus denitrificans* CECT 694 and *Gluconobacter oxydans* DSM 3503^T were kindly provided by C. Belloch, CECT, Universidad de Valencia, Spain, and M. Sievers, Institut für Lebensmittelwissenschaft, Zürich, Switzerland, respectively. The media and conditions recommended in the respective catalogs of strains were used for cultivation. Cultures were harvested in the logarithmic growth phase (optical density at 600 nm, 0.2 to 0.8) for whole-cell hybridizations. Cells were fixed with 4% paraformaldehyde by the method of Amann et al. (1).

Filter bed samples. Samples were taken after operation of the filter cell was stopped. After complete drainage, samples were collected from the sand bed with a core driller. Sand grains were carefully sampled with a spatula from layers at different depths of the nondenitrifying filter cell 16, i.e., at 30 (sample A), 60 (sample B), and 100 (sample C) cm, and at different depths of the denitrifying filter cell 18 supplemented with methanol, at 40 (A) and 100 (B) cm. Samples were immediately fixed by the addition of 1 part of phosphate-buffered saline (PBS) and 3 parts of 4% paraformaldehyde solution, as described by Amann et al. (1), for in situ hybridization and the examination of the biofilm thickness. Alternatively, samples were fixed with ethanol as described by Roller et al. (36). Sand grains were transferred into sterile plastic tubes and transported into the laboratory on ice for subsequent cultivation.

Isolation. Prior to plating, the biofilms were removed from the sand grains and disrupted by vortexing 1 g of the sample vigorously for 1 min with 10 ml of 0.85% sodium chloride solution supplemented with 0.28% $NaPP_i$. Appropriate dilutions of the supernatant were plated on R2A agar (33) and Trypticase soy agar (Becton-Dickinson, Heidelberg, Germany). The plates were incubated aerobically for 5 days at 28°C.

Denitrification capability. Cultures pregrown on Trypticase soy agar were inoculated into 5 ml of nitrate bouillon (Merck, Darmstadt, Germany) supplied with a Durham vial and cultured anaerobically at 28°C for 5 to 15 days. Negative control assays for the production of gases other than nitrogen gases were made.

Analysis of fatty acid compositions from whole-cell hydrolysates. Cell cultivation and fatty acid extraction were performed as described by Moss et al. (26). It was necessary to vary growth conditions slightly or to prolong growth to obtain sufficient cell mass for analysis of some isolates. Data interpretation was performed with the MIS (TSBA database version 3.8; MIDI, Newark, Del.) as described by Sasser (37).

Oligonucleotides. The oligonucleotide probes used in this study are listed in Table 3. Labeling of 5′-amino-linked oligonucleotides (MWG Biotech, Ebersberg, Germany) with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide-ester (FLUOS; Boehringer GmbH, Mannheim, Germany), 5(6)-carboxytetramethylrhodamine-*N*-hydroxysuccinimide-ester (CT; Molecular Probes, Eugene, Oreg.), or the 5,5′-disulfo-1,1′-di(γ-carbopentynyl)-3,3,3′,3′-tetramethylindolocarbocyanine-

TABLE 2. Reference strains

Species	Strain and source[a]	Other strain designations[a]
Paracoccus denitrificans	DSM 65^T	ATCC 17741, LMG 4218, NCIMB 11627
	CECT 694	DSM 413, ATCC 19367, LMG 1627, NCIMB 8944
	DSM 1408	
Azorhizobium caulinodans	LMG 6465^T	
Azospirillum amazonense	DSM 2787^T	
Blastobacter denitrificans	LMG 8443^T	
Brevundimonas diminuta	DSM 1635	
Gluconobacter oxydans	DSM 3503^T	
Hyphomicrobium vulgare	ATCC 27500	
Hyphomonas jannaschiana	ATCC 33882	
Mycoplana bullata	ATCC 4278^T	
Rhodobacter sphaeroides	DSM 158^T	
Rhodobium marinum	DSM 2698^T	
Rhodomicrobium vannielii	LMG 4299^T	
Rhodospirillum salexigens	LMG 2132^T	
Sphingomonas capsulata	LMG 2830^T	
Sphingomonas parapaucimobilis	LMG 10923^T	
Sphingomonas yanoikuyae	LMG 11252^T	

[a] Abbreviations: T, type strain; ATCC, American Type Culture Collection, Rockville, Md.; CECT, Collection Española de Cultivos Tipo, Universidad de Valencia, Valencia, Spain; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, United Kingdom.

TABLE 3. Probe data

Probe	Sequence 5′–3′	Binding position[a]	Specificity	Applied stringency (formamide concn [%])	Reference
PAR651	ACCTCTCTCGAACTCCAG	16S, 651–668	Genus *Paracoccus*	40	This study
PAR1244	GGATTAACCCACTGTCACC	16S, 1244–1283	Genus *Paracoccus*	20	This study
PAR1457	CTACCGTGGTCCGCTGCC	16S, 1457–1474	Genus *Paracoccus*	35	This study
Pdv198	CTAATCCTTTGGCGATAAATC	16S, 198–232	*P. denitrificans* and *P. versutus*	20	This study
Pdv1031	CCTGTCTCCAGGTCACCG	16S, 1031–1048	*P. denitrificans* and *P. versutus*	35	This study
Hvu1034	GCACCTGTCCCACTGCCT	16S, 1034–1051	*H. vulgare*	20	This study
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338–355	*Bacteria*	0	2
ARCH915	GTGCTCCCCCGCCAATTCCT	16S, 915–934	*Archaea*	35	42
ALF1b	CGTTCG(C/T)TCTGAGCCAG	16S, 19–35	Alpha subclass of *Proteobacteria* and some organisms of the delta subclass of *Proteobacteria* and some gram-positive bacteria with low G+C content of DNA	20	23
BET42a	GCCTTCCCACTTCGTTT	23S, 1027–1043	Beta subclass of *Proteobacteria*	35	23
GAM42a	GCCTTCCCACATCGTTT	23S, 1027–1043	Gamma subclass of *Proteobacteria*	35	23
CF319a+b	TGGTCCGT(G/A)TCTCAGTAC	16S, 319–336	*Cytophaga-Flexibacter* cluster	35	24
HGC69a	TATAGTTACCACCGCCGT	23S, 1901–1918	Most gram-positive bacteria with high G+C content of DNA	25	36
Ps	GCTGGCCTAGCCTTC	23S, 1432–1446	Most rRNA group I pseudomonads	35	38
Met9a	CCCTGAGTTATTCCGAAC	16S, 142–159	Genera *Methylobacterium*, *Methylocystis*, *Methylosinus*, and *Beijerinckia*	20	46

[a] *E. coli* rRNA numbering (6).

N-hydroxysuccinimide-ester (Cy3.18; Biological Detection Systems, Pittsburgh, Pa.) and subsequent purification of the oligonucleotide-dye conjugates were performed as described by Amann et al. (2).

Whole-cell hybridization. Whole-cell hybridization with fluorescently labeled oligonucleotides was performed as described by Manz et al. (23). The hybridization buffers contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate (SDS), and different amounts of formamide. Hybridization was performed for 1.5 h at 46°C in an isotonically equilibrated humid chamber; this was followed by a 20-min washing step at 48°C. To achieve the same stringency during washing as that during hybridization, the washing buffer contained between 0.9 M and 7 mM NaCl (according to the formula of Lathe [18], applied with the destabilization increment for DNA-rRNA hybrids of 0.5°C per percent of formamide concentration [52]), 20 mM Tris-HCl (pH 7.4), and 0.01% SDS. For combinations of probes with different optimal hybridization stringencies, two hybridizations were done successively. The first hybridization was done with the probe which required the higher formamide concentration and was followed by a second hybridization at a lower stringency.

Evaluation of probe specificities. Optimal stringencies for the designed probes were adjusted by hybridization against cells of reference strains with stepwise, increasing amounts of formamide (5% each step). Sufficient stringency for total discrimination of nontarget sequences was achieved when the fluorescence intensity of the cells was in the background range. Quantification of cell-conferred fluorescence signals with FLUOS-labeled probes was performed with a charge-coupled device camera (CF 15/2; Kappa, Gleichen, Germany) attached to an epifluorescence microscope and the fg-cell image analysis program (Captec, Malhide, Ireland), as described by Trebesius et al. (45). Between 50 and 250 cells were analyzed for each value. Probe-conferred signal intensities of the cells were divided by the cell area to determine the intensity values independent of cell size. Mean values of signal intensities between different reference strains were normalized relative to the signal of the probe EUB338-FLUOS to correct for the different contents of ribosomes.

Microscopy and documentation. After hybridization, the slides were mounted in Citifluor (Citifluor Ltd., London, United Kingdom). An Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with filter sets 01, 09, 15 (Zeiss, Oberkochen, Germany), HQ-FITC, and HQ-Cy3 (Chroma Tech. Corp., Brattleboro, Vt.) was used for examination. Photomicrographs were taken with Kodak TMAX 400 black-and-white film (0.05 s for phase contrast; 15 to 20 s for epifluorescence).

Digital microscopy was performed with a model 410 laser-scanning microscope (Carl Zeiss, Jena, Germany) equipped with internal lasers (excitation wavelengths, 488 and 543 nm) and an external UV laser (364 nm). Image processing was carried out with the accompanying software package and the program Corel Draw (version 5.0; Corel Corp., Dublin, Ireland). Reconstructed images were documented on a Kodak color reversal film (EE6 100 Professional) by using an Agfa forte slide exposure device (Agfa Deutschland, Cologne, Germany).

In situ observation of biofilms and measurements of biofilm thickness. Small compartments were formed on glass slides with plasticine. Biofilm-covered sand grains were carefully placed into these compartments, without contacting the plasticine mass, and covered with 4′,6-diamidino-2-phenylindole (DAPI) solution (1 μg/ml) for 15 min. After washing with double-distilled water, sand grains were embedded in Citifluor and the compartment was sealed with a coverslip. Estimates of biofilm thickness were obtained by focusing on the uppermost and lowermost cell layers within an area of 12 μm by 12 μm. The distances between these two focus planes were taken as estimates for the thickness of the biofilm. Biofilms were also scanned in the vertical direction (z-scan) with the confocal laser scanning microscope (CLSM). Thickness values were taken as the distances between the highest and lowest recorded DAPI signals from cells.

Total and probe-conferred cell counts. It is difficult to obtain accurate total cell counts in cell clusters. Therefore, the biofilms were detached from the grains and converted as efficiently as possible to a suspension of individual cells, or at least to smaller cell clusters, by passing 100 μl of detached biofilms through a 0.65-mm-wide needle 50 times, vortexing it thoroughly for 5 min, again passing it through the needle 100 times, vortexing it for another 5 min, and again passing it through the needle 50 times. Subsequently, 3 μl of the sample was diluted in 2 ml of PBS and filtered onto a polycarbonate filter (0.2-μm pore size; Millipore Corp., Bedford, Mass.). After air drying, the filters could be stored at room temperature for several weeks. Hybridization with Cy3-labeled probes on pieces of the filters, washing, and DAPI staining were performed as described by Neef et al. (27). Total cell counts were determined by evaluating at least 20 microscopic fields with at least 100 cells per field. Probe-positive cells were determined relative to DAPI counts.

RESULTS

Biofilm structure. Phase-contrast microscopy and DAPI staining of detached denitrifying biofilms from the methanol-fed filter cell revealed thick conglomerates in which cells and extracellular material were tightly packed. Within this dense matrix, single cells could hardly be resolved by phase-contrast microscopy. However, after DAPI staining, single cells were visible by their fluorescent signal. Total cell counts, as determined by staining, were $5.8 \times 10^9 \pm 4 \times 10^8$ and $2.4 \times 10^9 \pm 7 \times 10^8$ cells per ml of sand filter material at depths of 40 and 100 cm, respectively.

The biofilm distribution on the sand grains was very irregular. Areas in which no cells were detected by DAPI staining alternated with areas densely covered with multiple cell layers. Likely as a result of abrasion caused by the frequent bumping, exposed edges had no, or very thin, biofilms, whereas protected pits supported thicker biofilms. Two attempts were made to determine average thickness. Both avoided dehydration, which would result in a collapse of the extracellular matrices and, thereby, in a severe underestimation of the overall thickness.

When a paraformaldehyde-fixed, DAPI-stained sample was examined by regular epifluorescence microscopy, the thickness was 13 ± 7 μm (median value of 100 individual measurements on five sand grains). A second fixed sample was stained with DAPI and examined by CLSM. The z-scans yielded 20 individual values of 10 to 50 μm with a median of 20 ± 12 μm.

Cultivation and characterization of denitrifying bacteria. Detached biofilm samples were plated on a nutrient-rich (Trypticase soy) as well as on a nutrient-poor (R2A) agar. Of 75 pure cultures examined, 56 produced gas when grown in nitrate bouillon. Denitrification capability was confirmed by the lack of gas production in the nitrate-free controls. Analysis of these 56 isolates by fatty acid profiling with the MIS identification system (MIDI, Newark, Del.) (see reference 37) yielded matching values with reference organisms of 0.042 to 0.768. Only 15 of the 56 denitrifying isolates were above the confidence level of 0.6 (37), six matched *Hydrogenophaga* spp., five matched *Paracoccus* spp., and four matched *Acidovorax* spp. For the isolates with matching values below the confidence value of 0.6, *Paracoccus denitrificans* was the closest match most often (19 isolates). Less frequently, *Hydrogenophaga* spp. (eight isolates), *Methylobacterium* spp. (seven isolates), *Agrobacterium* spp. (four isolates), and *Acidovorax* spp. (one isolate) were the organisms with the closest match.

Design and optimization of rRNA-targeted probes. Because most of the matching values by the MIS system were too low for a clear identification, fluorescently labeled rRNA-targeted oligonucleotides were applied as a genotypic identification method. On the basis of the results of the fatty acid analysis and on knowledge about potential methylotrophic denitrifiers, new probes targeted for members of the genus *Paracoccus*, as well as for *Hyphomicrobium vulgare*, were designed.

The search for specific 16S rRNA-targeted oligonucleotide probes was performed against an alignment of over 4,000 complete and partial sequences (TUM sequence database [19, 22]) by using the ARB software (43). This alignment contained nearly complete sequences of the seven validly described *Paracoccus* species (15). Three genus-level probes could be designed and were named PAR651, PAR1244, and PAR1457. Additionally, two phylogenetically narrower probes, specific for *P. denitrificans* and its closest relative *Paracoccus versutus*, could be designed and were named Pdv198 and Pdv1031. A single probe was constructed for *Hyphomicrobium vulgare*, the only species of this genus sequenced thus far. It was named Hvu1034. Probe sequences are given in Table 3.

The ARB probe check program lists organisms by number and localization of mismatches within the target sequence (43). Probes were checked against more than 6,000 complete and partial 16S rRNA sequences available in the TUM database in June 1996 (19). Representative results of the probe check are shown in Fig. 1. The probes designed for the same monophyletic group (genus and species cluster) were not recording entirely identical groups of organisms. The three genus-specific probes recorded only *Paracoccus* species, and two of the probes recorded additional unique clone sequences located within or in the direct vicinity of the genus. Single *Paracoccus* species escape detection by two of the probes (*Paracoccus alcaliphilus* by PAR651 and *Paracoccus kocurii* by PAR1244), whereas PAR1457 detects all seven species. In the database, 30, 4, and 23 sequences showed a single mismatch with PAR651, PAR1244, and PAR1457, respectively, whereas 75, 26, and 187 sequences had two bases changed with these probes, respectively. All of the other sequences had at least three mismatches. Pdv198 and Pdv1031 are located on relatively variable target sites and detect identical phylogenetic clusters. Three and 1 of the sequences in the database had one mismatch with Pdv148 and Pdv1031, respectively; 54 and 4 sequences had two mismatches with Pdv198 and Pdv1031, respectively. All sequences from other *Paracoccus* species had at least three mismatches, except *P. kocurii*, which had one mismatch with Pdv1031. Hvu1034 is currently only complementary to the rRNA sequence available for *H. vulgare*. All other 16S rRNA sequences except for that of an unnamed *Hyphomicrobium*-like species have more than two mismatches. However, it has to be taken into account that 16S rRNA sequences of other hyphomicrobia are currently not available. The exact specificity of probe Hvu1034 therefore remains to be established.

The optimum hybridization conditions for discrimination of target and nontarget organisms were evaluated by increasing the formamide concentration in the hybridization buffer, which increased the stringency of hybridization (42). The effect of increasing the concentrations of formamide on the fluorescent signal conferred by fluorescein-labeled probe PAR651 to the target organism, *P. denitrificans* DSM 65^T, as well as two species with related target sequences, *Azorhizobium caulinodans* LMG 6465^T and *Gluconobacter oxydans* DSM 3503^T, was quantified (Fig. 2). Probe binding to the type strain of *P. denitrificans* decreased with the addition of formamide at concentrations between 35 and 55%, and high fluorescent signal intensities decreased to background levels. Half-maximal probe binding (42) was reached at 46% formamide. Hybridization of probe PAR651 to *A. caulinodans* demonstrated a corresponding drop with the addition of 20 to 40% formamide (half-maximal probe binding at 29% formamide), indicating that even a lateral mismatch (U instead of G at position 3) is effectively discriminating. The resulting single C-U mismatch between probe and rRNA yielded a decline in hybrid stabilization of approximately 17% formamide, which corresponds to an 8.5°C decrease in dissociation temperature. *G. oxydans*, possessing a C-A mismatch at position 3 of PAR651 and a less destabilizing A-G mismatch (G instead of U) at position 18 of PAR651, could be discriminated readily at low formamide concentrations. Qualitative estimation of probe binding determined that probe PAR651 requires 40% formamide for optimal discrimination (Table 4). For all other probes, the hybridization parameters were adjusted in a formamide gradient by using reference strains showing few sequence mismatches. Discriminating formamide concentrations are displayed in Table 4. For example, one central C-C mismatch is commonly detected at position 7 of PAR1457, e.g., in *Rhodobacter sphaeroides*, *Blastobacter denitrificans*, and *Brevundimonas diminuta*. This mismatch is sufficient for discrimination at 20% formamide in all three organisms. Complete discrimination of the nontarget organisms which were tested was achieved by the addition of 20% formamide for PAR1457 and Pdv198, 10% formamide for Pdv1031, and no formamide for PAR1244 and Hvu1034. Pdv198, even at high formamide concentrations, failed to discriminate *Rhodomicrobium vannielii*, since the 16S rRNA of this organism has only two weak lateral mismatches at the probe target site. Even though the available reference cells were discriminated at lower formamide concentrations, probes PAR1457, Pdv1031, PAR1244, and Hvu1034 were used in situ at 35, 35, 20, and 20% formamide, respectively. Signal intensities were not decreased significantly when these concentrations of formamide were used.

A well-documented phenotypic and genotypic heterogeneity exists for those strains classified as *P. denitrificans* (5, 28), which was further complicated by the reclassification of *Thiosphaera pantotropha* as *P. denitrificans* (20). Therefore, the type strain and two additional strains classified as *P. denitrificans* were tested for probe binding. All three genus-specific probes hybridized equally well to each of the three strains. However,

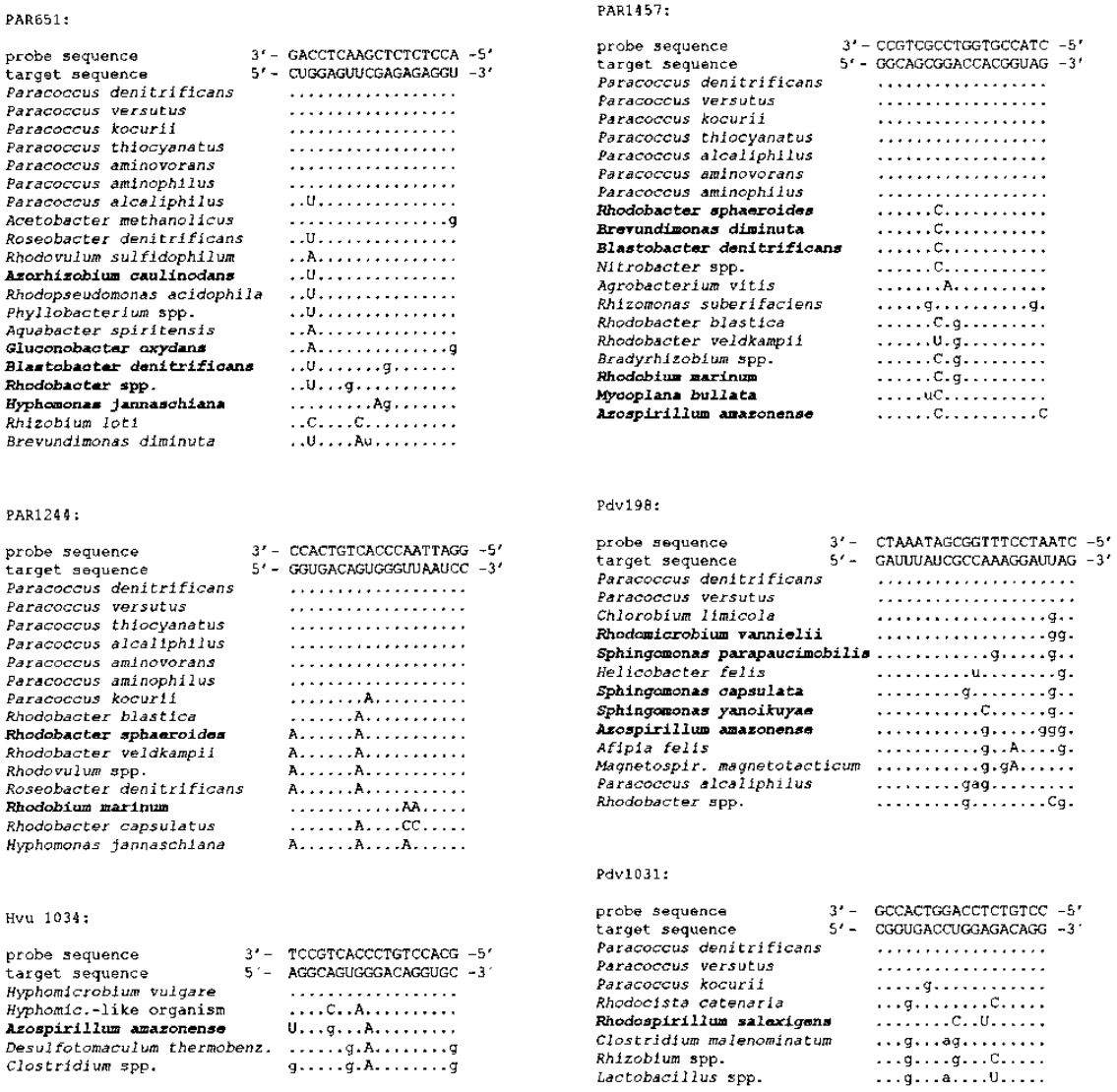

FIG. 1. Difference alignment showing target sequences, matching target organisms, and exemplary nontarget organisms for the designed probes. All matching species as well as all species with one mismatch and exemplary species with two or more mismatches in the target site are shown. Strong and weak mismatches are indicated by capital and lowercase letters, respectively, by the method of Stahl and Amann (42). Organisms used for evaluation of probe specificities are shown in bold letters.

only the type strain *P. denitrificans* DSM 65 hybridized with the probes Pdv198 and Pdv1031. The other two strains, CECT 694 and DSM 1408, demonstrated no signals at the appropriate stringencies. These results indicate sequence heterogeneity within the 16S rRNA of different strains classified as *P. denitrificans.*

Characterization of denitrifying sand filter isolates with rRNA probes. By using probes for the major subclasses of *Proteobacteria* (23), 35 of the 56 isolates from the denitrifying sand filter were assigned to the alpha subclass, 17 were assigned to the beta subclass, and 0 was assigned to the gamma subclass. Four isolates did not hybridize to any of the proteobacterial probes. The isolates which gave signals with ALF1b (63%) were further examined with the probes specific for the genus *Paracoccus* and *H. vulgare*. Twenty-three isolates showed strong signals with all three probes specific for the genus *Paracoccus*. An additional four isolates showed strong signals only with PAR651 and PAR1457 but weak signals with PAR1244. None of these 27 isolates hybridized with the more-specific probes Pdv198 and Pdv1031. Most of the isolates possessed a coccoid morphology occurring as single cells, pairs, or chains. Only one of the alpha-subclass isolates hybridized with Hvu1034. None of the isolates gave a signal with the probe Met9a (46), which is specific for serine pathway methylotrophs and some other bacteria. Hybridization results and fatty acid analysis did not always correspond. Although 24 isolates were classified as *Paracoccus* spp. by both identification approaches, the MIS system missed three paracocci which were detected by whole-cell hybridization.

In situ identification of *Paracoccus* spp. and *Hyphomicrobium* spp. in denitrifying biofilms. After in situ probing with fluorescein- and rhodamine-labeled oligonucleotides, even small cells were clearly visible within the biofilm by their fluorescent signal, although autofluorescence in both the green and the red emission ranges was present in the surrounding matrix. Detached biofilms were examined by using a set of rRNA-targeted probes following the top-to-bottom approach.

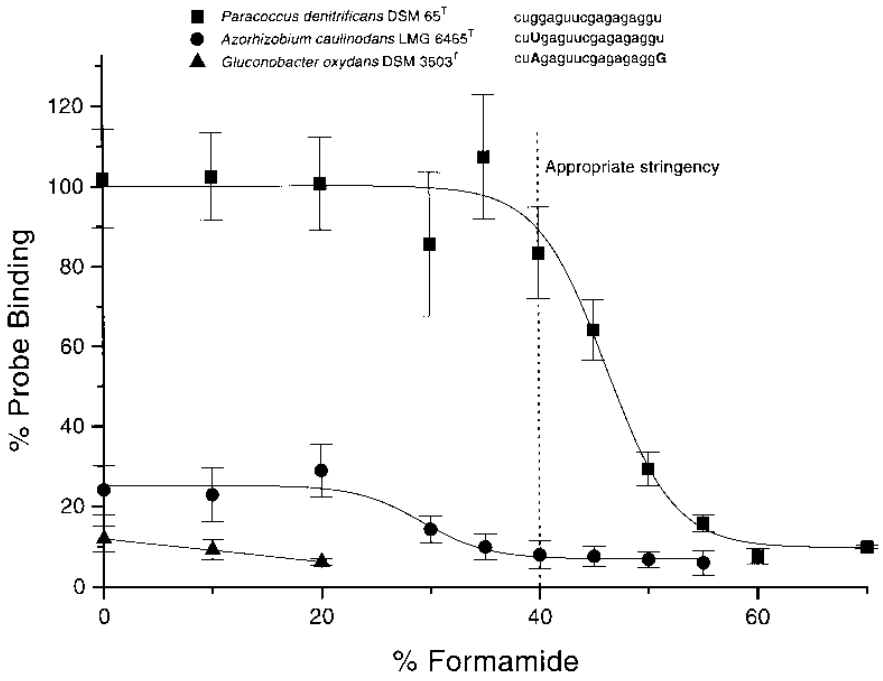

FIG. 2. Signal quantification of probe binding of PAR651-FLUOS to reference organisms. Fixed cells were hybridized with increasing concentrations of formamide (10 and 5% increments). Fluorescence intensities were normalized relative to the signal strength of EUB338 at 0% formamide. Brightness of *P. denitrificans* DSM 65^T exhibiting maximal probe binding is set as 100%. 16S rRNA sequences at the target site are shown at the top of the figure, with base changes in capital letters.

In a semiquantitative evaluation, hybridized samples were analyzed by two independent observers for the fraction of probe-positive cells relative to those visualized by a more universal stain (with DAPI, EUB338, and subclass-specific probes). These estimates are summarized in Table 5. The *Bacteria*-specific probe EUB338 visualized approximately 70 to 80% of DAPI-stained cells. Approximately two of every three cells detected by the bacterial probe hybridized with the probe for members of the beta subclass of *Proteobacteria* (assessed to 60 to 70% of EUB338-positive cells). A probe complementary to members of the alpha subclass of *Proteobacteria*, ALF1b, detected 10 to 15% of EUB338-positive cells. Approximately 5 to

TABLE 4. Specificity testing by whole-cell hybridization and formamide gradient[a]

Probe	Organism	Discriminating stringency (%)[b]
PAR651	*Azorhizobium caulinodans*	40
	Gluconobacter oxydans	20
	Blastobacter denitrificans	15
	Rhodobacter sphaeroides	10
	Hyphomonas jannaschiana	5
PAR1244	*Rhodobacter sphaeroides*	0
	Rhodobium marinum	0
PAR1457	*Brevundimonas diminuta*	20
	Blastobacter denitrificans	20
	Rhodobacter sphaeroides	20
	Rhodobium marinum	10
	Mycoplana bullata	10
	Azospirillum amazonense	10
Pdv198	*Rhodomicrobium vannielii*	ND[c]
	Sphingomonas parapaucimobilis	20
	Sphingomonas capsulata	15
	Azospirillum amazonense	10
	Sphingomonas yanoikuyae	0
Pdv1031	*Rhodospirillum salexigens*	10
Hvu1034	*Azospirillum amazonense*	0

[a] Fixed cells were hybridized with an increasing formamide concentration (increment, 5%).
[b] Discriminating stringency is the formamide concentration necessary for complete discrimination of the reference strain.
[c] ND, no discrimination possible, even at a higher stringency.

TABLE 5. In situ probing of biofilms from sand filter cells 16 and 18 with group-specific probes

Specificity and probe	Relative abundance (%) in[a]:	
	Filter cell 18 with methanol	Filter cell 16 without methanol
Domain		
ARCH915	<0.1	<0.1
EUB338	70–80	40–60
Group		
ALF1b	10–15	5–10
BET42a	60–70	10–15
GAM42a	1–2	0.1–0.5
CF319a+b	5–10	1–2
HGC69a	0.5–1	0.5–1
Subgroup		
PAR651	40–50	0.5–1
Hvu1034	15–25	<0.1
Met9a	0.5–1	<0.1
Ps	<0.1	ND[b]

[a] Assessment of relative abundances of probe-positive cells relative to those visualized by DAPI staining (domain-specific probes) or by probing with EUB338 (group-specific probes) or with Alf1b and GAM42a for sand filter cells 16 and 18, respectively (subgroup-specific probes).
[b] ND, not determined.

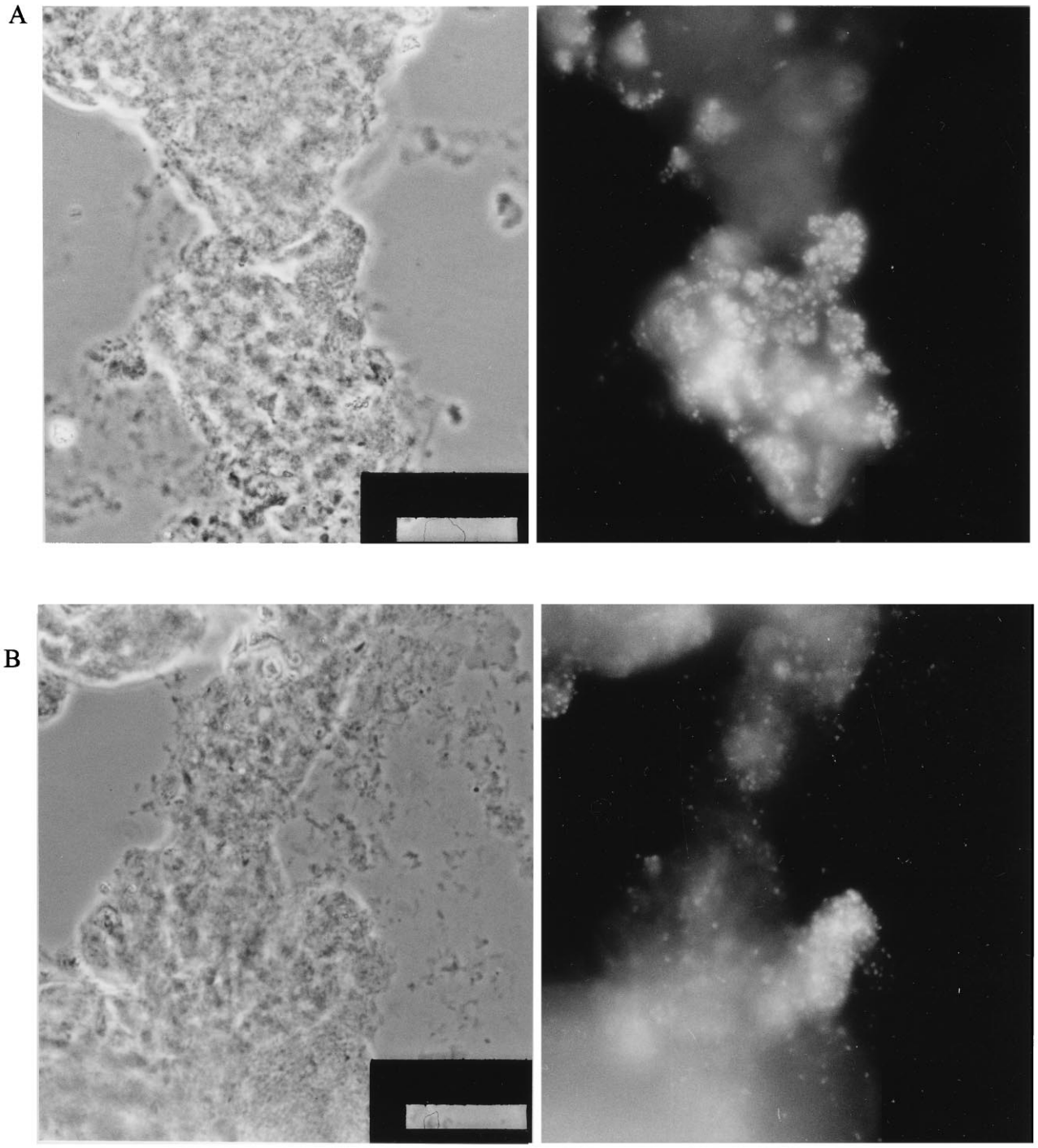

FIG. 3. In situ identification of a *Paracoccus* sp. and a *Hyphomicrobium* sp. in detached biofilms from methanol-fed filter cell 18. Phase-contrast (left) and epifluorescence (right) photomicrographs are shown for identical microscopic fields at ×1,000 magnification. (A) Hybridization with CT-labeled probe PAR651; (B) hybridization with CT-labeled probe Hvu1034. Bars, 20 μm.

10% of EUB338-positive cells bound probes specific for the *Cytophaga-Flexibacter* cluster. Members of the gamma subclass of *Proteobacteria* and gram-positive bacteria with high G+C contents formed only a minor part of the community, constituting about 1% relative abundance. rRNA-group I pseudomonads could not be detected with an appropriate probe. Significant differences in the abundances of the groups were not observed between samples from two different depth layers (40 and 100 cm).

The probes for the genus *Paracoccus* hybridized to dense aggregates of coccoid cells in the denitrifying biofilms (Fig. 3A) and showed relatively bright signals. The large majority of the detected cells were in clusters of as many as several hundred cocci, although scattered cells were detected as well.

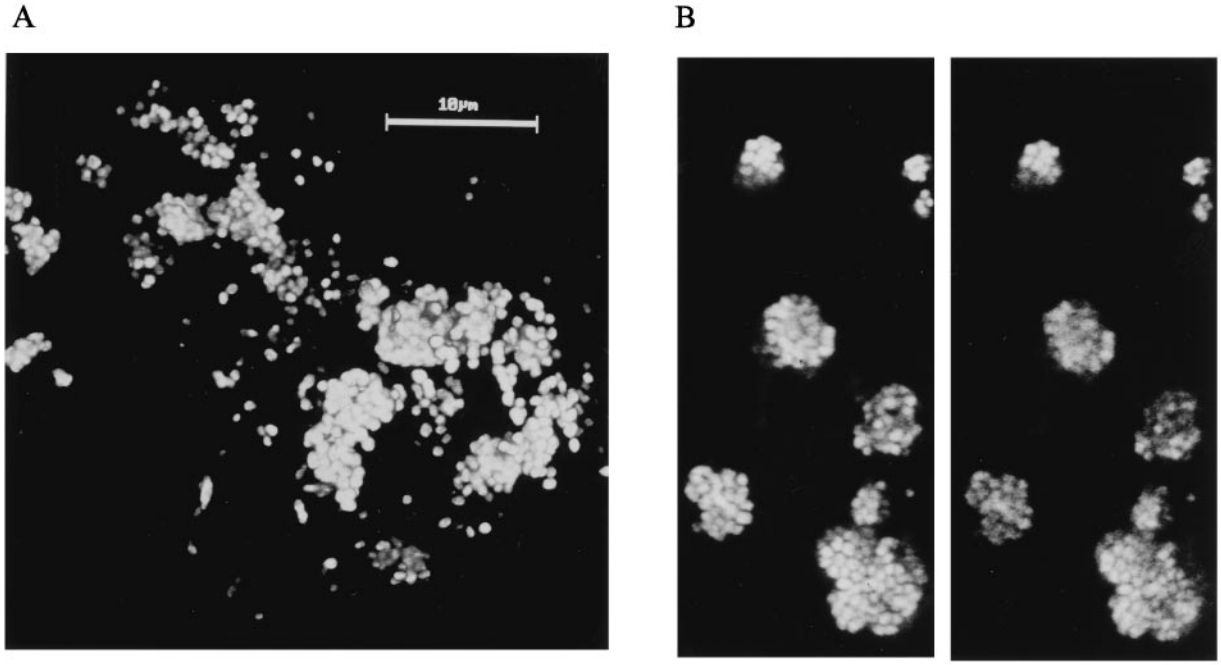

FIG. 4. Analysis of *Paracoccus* sp. clusters in detached biofilms from filter cell 18 by CLSM. (A) Spatial structure of clusters. Hybridization was with the CT-labeled probe PAR651. Two-dimensional reconstruction (all in focus) of 15 optical sections which were recorded at intervals of 0.7 μm. (B) Double identification of *Paracoccus* sp. cells. Hybridization was with the CT-labeled probe PAR651 (left) and FLUOS-labeled probe PAR1457 (right). Single images of the same optical section are displayed. Scales of panels A and B are identical.

In total, approximately half of all ALF1b-stained cells could be assigned to the genus *Paracoccus* with the genus-specific probes. Aggregates of paracocci were not distributed randomly throughout the biofilm but were concentrated in distinct areas, whereas in other regions of the biofilm, few, if any, paracocci were located. Paracocci aggregates were localized at the surface of, as well as inside, the biofilm matrix. Further analysis, using a CLSM, resolved the spatial arrangement of the cell aggregates in greater detail. Digital microscopy revealed that the roughly spherical clusters consisted of uniform cocci with cell diameters of 0.5 to 1 μm (Fig. 4A). Cells were evenly fluorescent, independent of their position within the aggregates, indicating similar cellular ribosome contents. Simultaneous hybridization with different combinations of two of the three genus-specific probes usually resulted in the binding of both probes to all detected cells. This could be examined most reliably by CLSM (Fig. 4B). Since three independent sites of the 16S rRNA are examined, CLSM results in an unequivocal identification of probe-positive cells as members of the genus *Paracoccus* since no other sequences are recorded by all three probes. Few single cells hybridized with only one of the genus-specific probes. Again, with the two more-specific probes, Pdv198 and Pdv1031, no cells were detected.

Probe Hvu1034, specific for *H. vulgare*, hybridized to a significant fraction of cells as well (assessed as approximately 15 to 25% of all ALF1b-stained cells). This population was not tightly clustered but occurred mainly as small, individual ovoid rods (Fig. 3B). These cells produced weaker signals than the paracocci did and were located mostly within the biofilm matrix. The characteristic morphology, the presence of hyphae, could be visualized only by phase-contrast microscopy for cells resolved from the biofilm matrix.

The relative abundance of *Paracoccus* spp. and *Hyphomicrobium* spp. in the biofilm and the ability of members of both genera to denitrify with methanol suggest a significant function in the overall process. This is supported by a bacterial community analysis of filter cell 16, a unit operated without methanol supplementation for the removal of suspended solids only. Because of the lack of readily available carbon sources, measurable denitrification did not occur here and hybridization signals of the cells were generally lower, indicating lower overall activities. Additionally, the total number of DAPI-stained cells per biofilm volume was also lower ($1.4 \times 10^9 \pm 8 \times 10^8$ cells ml of sand filter material^{-1}). The bacterial probe EUB338 detected only about 50% of DAPI-stained cells. Smaller fractions of cells than that in filter cell 18 were detected with the group-specific probes (Table 5). Again, no significant differences were evident between samples from three depth layers, i.e., 30, 60, and 100 cm. Only very few *Paracoccus* cells could be detected with all three probes. These cells, mostly small rods, were not clustered together but appeared to be distributed randomly throughout the biofilm. No hyphomicrobia could be detected. Thus, the comparative analysis of the bacterial communities of a denitrifying and a nondenitrifying filter cell, indeed, indicates a correlation between community composition and function.

The abundance of *Paracoccus* spp. in biofilms from the two filter cells was determined more accurately by quantification of PAR651-positive cells than total cell counts could be determined by DAPI staining. Because accurate counting of cells is difficult within dense flocs, the biofilms were mechanically disrupted to obtain smaller aggregates. The procedure was effective and resulted in smaller flocs ($<10^3$ cells per floc) and single cells. Nevertheless, the numbers of probe-positive cells varied considerably between different counting areas. Consequently, the standard deviations of the numbers of cells for the PAR651-fractions remained high. Many microscopic fields were examined so that high numbers of DAPI-stained cells could be determined. In samples from depths of 40 and 100 cm in the denitrifying sand filter, 3.6% ± 5.2% and 3.3% ± 5.6%

TABLE 6. Proportion of *Paracoccus* spp. to total cells as determined by quantification on polycarbonate filters

Filter cell no.	Sample[a]	No. of evaluated microscopic fields	No. of PAR651-positive cells	Total no. of DAPI-stained cells	Proportion (mean ± SD)[b]
16	B	24	2	3,053	0.07 ± 0.23
18	A	20	427	11,799	3.6 ± 5.6
18	B	35	373	11,174	3.3 ± 5.2

[a] See Materials and Methods for details.
[b] Proportion of PAR651-positive cells to total DAPI-stained cells.

of all cells were paracocci, whereas in the medium layer of the nondenitrifying sand filter, fewer than 0.1% of the cells counted were PAR651 positive (Table 6). Their abundance was not significantly higher in the samples from the other two depth layers of this sand filter.

DISCUSSION

Rationale of the design of multiple probes. In this study, multiple rRNA-targeted probes have been used to identify unequivocally paracocci in a denitrifying community. When probes targeted against variable regions of the rRNA are applied in complex environmental samples, false-positive binding to nontarget organisms, whose sequences had not been retrieved to date and were, therefore, not considered during probe design, cannot be ruled out. A way out of this dilemma is to use two or more probes detecting the same group of organisms. Chances of coincident false-positive detection of the same organisms by two or even three probes against independent, variable target sites are considerably lower.

The genus *Paracoccus* forms a monophyletic branch within the alpha-3 subgroup of the *Proteobacteria* clearly separate from other members of this cluster such as the genera *Rhodobacter*, *Rhodovulum*, and *Roseobacter* (15). Therefore, three probes with almost identical specificities but targeted to three independent sites on the 16S rRNA could be found. The taxa of bacteria which showed one or two mismatches with these probes were quite different in size and composition. The conformity of the hybridization results with all three genus-specific probes in our samples strongly implies that the detected clusters are paracocci.

Attempts to develop a second probe for hyphomicrobia based on the 16S rRNA sequence of *H. vulgare* were not successful. Two oligonucleotides (located at *Escherichia coli* positions 61 to 78 and 812 to 829) were evaluated but showed no signals with *H. vulgare* ATCC 27500, indicating a limited accessibility of the respective rRNA target sites.

Comparison of probe-based classification and fatty acid analysis. By fatty acid analysis, only 15 of the 56 denitrifying isolates had matching values above the confidence value of 0.6. The most important reason for the failure of this system to yield more accurate identification is the insufficient representation of environmental isolates in the database. Furthermore, bacterial identification by the MIS system strongly relies on the comparative analysis of a relatively variable phenotypic character, the composition of cytoplasmic membrane fatty acids. High matching values require standardized growth conditions, e.g., medium composition and temperature. However, many environmental isolates do not readily grow under these conditions. In contrast, the probe identification system used in this study is clearly more reliable since it is based on stable genotypic characters (21).

Optimization of hybridization conditions for rRNA probes. Stringencies of whole-cell hybridizations were optimized by using representative reference organisms which possessed commonly occurring individual mismatches or mismatch patterns within the respective target site (Fig. 1). The discriminating value of a mismatch depends, e.g., upon its position within the target site and the relative destabilizing effect of the base change (42). The comparative quantification of the probe binding of PAR651 to whole fixed cells of three reference strains under different stringencies, as determined by concentration of formamide added, demonstrates these effects nicely. Since the quantification of probe binding is very time-consuming, we have used a qualitative approach to determine optimal hybridization stringencies for the other probes. Again, probes were tested with reference strains possessing exemplary mismatch patterns. When otherwise standardized conditions were used, hybridization stringencies were varied solely by changing the formamide concentration. Formamide concentrations and thereby hybridization stringencies were regarded as optimal when target organisms yielded strong probe-conferred fluorescence but nontarget organisms with high sequence similarity were discriminated. The qualitative determination of the optimal formamide gradient for probe PAR651 yielded the same result that the quantitative probe binding profile did, confirming the experimental reliability of the second approach.

Comparisons of binding by PAR651 and PAR1457 to nontarget sequences show that central mismatches have a higher discriminating value than lateral mismatches. The 20% formamide difference in the discrimination stringencies of *Sphingomonas parapaucimobilis* and *Sphingomonas yanoikuyae* with Pdv198 is an example of the influence of the discriminating strength of nonmatching bases. Additionally, it has been shown, with probe PAR1457, that different organisms with the same mismatch require the same stringency for discrimination, demonstrating that it is sufficient to test one reference organism for each type of mismatch during the optimization of hybridization parameters.

Probes as a tool for the identification of *P. denitrificans* strains. The failure of two strains classified as *P. denitrificans* to hybridize with the two probes complementary to the type strains of both *P. denitrificans* and *P. versutus*, which have a 16S rRNA complete sequence similarity of 99.3% and DNA-DNA similarity of approximately 50% (15), was unexpected. However, there are reports (5, 28, 53) of extensive intraspecies heterogeneity within *P. denitrificans*. Auling et al. (5) reported DNA-DNA similarity values of only 40 to 68% for 12 strains classified as *P. denitrificans* and grouped them in four distinct DNA similarity groups. Approximately 50% of all denitrifying isolates hybridized with all three genus-specific probes for a *Paracoccus* sp., but none of them hybridized with the two more-specific probes for *P. denitrificans* and *P. versutus*. This was surprising since, except for *P. denitrificans* and *P. versutus*, none of the other five described *Paracoccus* species is expected to grow under the conditions present in the sand filter: *Paracoccus aminovorans* and *Paracoccus aminophilus* are not able to utilize methanol nor do they have the potential for denitrification (47). *P. alcaliphilus* is able to utilize methanol but shows only weak growth at pH 7 and does not denitrify (48). *P. kocurii* and *Paracoccus thiocyanatus* are able to denitrify but cannot grow with methanol as the sole carbon source (15, 30). This indicates that the isolates do not belong to any of these species. Consequently, the detected cells should be referred to as a *Paracoccus* sp. Our isolates might belong to a new species within the genus *Paracoccus*. However, further studies are required to resolve their taxonomic position.

In situ identification of methylotrophic denitrifiers with rRNA-targeted probes. Identification of all or most denitrify-

ing bacteria by use of one or a few rRNA-targeted probes is impossible because of the phylogenetically widespread distribution of this trait. All prominent phyla within the class *Bacteria* contain organisms capable of denitrification (54). For this reason, in situ probing was targeted to genera which have demonstrated their capability of methylotrophic denitrification in the laboratory and have been observed in the respective environments.

Methanol supplements constituted the main part of exogenous utilizable carbon substrate in filter cell 18 to support denitrification. Residual chemical oxygen demand (COD) in the secondary effluent varies between 17 and 24 mg liter^{-1}, whereas the addition of between 60 and 90 mg of methanol liter^{-1} is equal to 90 to 135 mg of COD liter^{-1} (29). Probe Met9a (46) targeted to strictly aerobic, obligate, or facultative methylotrophs from the alpha subclass of *Proteobacteria* failed to detect cells. On the other hand, members of the genera *Paracoccus* and *Hyphomicrobium*, which are known to possess the potential for denitrification with methanol, could be identified in the biofilms. Both constituted significant parts of the population. The paracocci identified with three genus-specific probes were arranged mostly in aggregates. The uniformly bright probe-conferred signals throughout the clusters demonstrated by CLSM analysis suggest that all cells have similar metabolic activities.

The identification of hyphomicrobia with probe Hvu1034 is supported by the morphology of the detected cells. They were ovoid and occurred mostly as individual cells. The exact specificity of probe Hvu1034 remains to be established since sequence information of other *Hyphomicrobium* species is lacking and the majority of existing isolates could not be obtained from public culture collections. However, we believe that most hyphomicrobia present in the sand filter were detected by Hvu1034 since this probe hybridized with the hyphomicrobial isolates obtained from the system in this study.

The fact that no cells were detected with probe Ps complementary to a signature present in most rRNA group I pseudomonads rules out a major contribution of this group, which is assumed to be of major importance in denitrification in soil (12). Other denitrifiers, which use other carbon sources in the residual COD or secondary substrates derived from the lysis of microorganisms and carry out a metabolism termed "endogenous denitrification" (8), could not be excluded. However, the contribution of such metabolisms to total denitrification activities could not be high since no denitrification was detected in reference filter cell 16.

An indication of which bacteria are responsible for nitrate removal could be found in the comparison of the relative abundances of different bacterial groups in the methanol-supplemented and the nonsupplemented filter cells. The exclusive occurrence of distinct methylotrophic denitrifying populations of paracocci and hyphomicrobia in the methanol-supplemented filter cell is a strong indication that these two groups are actually involved in methanol utilization and denitrification. To our knowledge, the only descriptions to date of *Paracoccus* spp. in wastewater-related denitrification processes were given by Claus and Kutzner (9), who described a denitrifying coculture of paracocci and hyphomicrobia in a bioreactor operated for nitrate and nitric acid removal and which had been inoculated with a soil sample, and by Vedenina and Govorukhina (50), who isolated paracocci and hyphomicrobia from a denitrifying activated sludge.

Ecological implications for the high abundance of *Paracoccus* spp. A typical feature of the investigated system is that, on average, once daily a cleaning process is performed by flushing it with water in an upflow mode and subsequently blowing air through the filter cell to prevent clogging by the augmenting biomass. By this backflushing process, part of the biofilm material is removed from the substratum and high flow rates through the filter bed can be maintained. During this process, oxygen concentrations which are usually 0 mg liter^{-1} quickly increase to 6 to 9 mg liter^{-1} for about 10 to 15 min, values which are near saturation.

There are various reports on bacteria capable of denitrification under different degrees of oxygen partial pressure (7, 10, 31, 35). *P. denitrificans* is able to denitrify in the presence of oxygen up to levels of 90% air saturation (10, 34, 35). Growth rates are higher as a result of the simultaneous use of both oxygen and nitrate as terminal electron acceptors (34, 35). It is probable that other representatives of the genus are capable of aerobic denitrification. Such paracocci would possess selective advantages in habitats exposed to varying oxygen concentrations such as those present in the examined sand filter. Furthermore, whereas other denitrifiers are expected to lose their denitrification potential because of oxygen inactivation (16) and must first rebuild their denitrification capacity when shifted back to anaerobiosis, the paracocci could denitrify continuously. Their metabolism seems to be perfectly suited for the niche present in the investigated discontinuously aerated, methanol-fed denitrifying sand filters.

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