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Use of Stable-Isotope Probing, Full-Cycle rRNA Analysis, and Fluorescence In Situ Hybridization-Microautoradiography To Study a Methanol-Fed Denitrifying Microbial Community

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A denitrifying microbial consortium was enriched in an anoxically operated, methanol-fed sequencing batch reactor (SBR) fed with a mineral salts medium containing methanol as the sole carbon source and nitrate as the electron acceptor. The SBR was inoculated with sludge from a biological nutrient removal activated sludge plant exhibiting good denitrification. The SBR denitrification rate improved from less than 0.02 mg of NO_3^--N mg of mixed-liquor volatile suspended solids (MLVSS)$^{-1}$ h^{-1} to a steady-state value of 0.06 mg of NO_3^--N mg of MLVSS^{-1} h^{-1} over a 7-month operational period. At this time, the enriched microbial community was subjected to stable-isotope probing (SIP) with [^{13}C]methanol to biomark the DNA of the denitrifiers. The extracted [^{13}C]DNA and [^{12}C]DNA from the SIP experiment were separately subjected to full-cycle rRNA analysis. The dominant 16S rRNA gene phylotype (group A clones) in the [^{13}C]DNA clone library was closely related to those of the obligate methylotrophs *Methylobacillus* and *Methylophilus* in the order *Methylophilales* of the *Betaproteobacteria* (96 to 97% sequence identities), while the most abundant clone groups in the [^{12}C]DNA clone library mostly belonged to the family *Saprospiraceae* in the *Bacteroidetes* phylum. Oligonucleotide probes for use in fluorescence in situ hybridization (FISH) were designed to specifically target the group A clones and *Methylophilales* (probes DEN67 and MET1216, respectively) and the *Saprospiraceae* clones (probe SAP553). Application of these probes to the SBR biomass over the enrichment period demonstrated a strong correlation between the level of SBR denitrification and relative abundance of DEN67-targeted bacteria in the SBR community. By contrast, there was no correlation between the denitrification rate and the relative abundances of the well-known denitrifying genera *Hyphomicrobium* and *Paracoccus* or the *Saprospiraceae* clones visualized by FISH in the SBR biomass. FISH combined with microautoradiography independently confirmed that the DEN67-targeted cells were the dominant bacterial group capable of anoxic [^{14}C]methanol uptake in the enriched biomass. The well-known denitrification lag period in the methanol-fed SBR was shown to coincide with a lag phase in growth of the DEN67-targeted denitrifying population. We conclude that *Methylophilales* bacteria are the dominant denitrifiers in our SBR system and likely are important denitrifiers in full-scale methanol-fed denitrifying sludges.

Today the hydrosphere has become the main sink for excess nitrogen due to human activities (37). Nitrogen in its various forms can deplete dissolved oxygen (DO) levels in receiving waters, stimulate aquatic growth, exhibit toxicity towards aquatic life, present a public health hazard, and affect the suitability of wastewater for reuse (37). As a result, stringent standards on total nitrogen levels have been imposed on effluents released from wastewater treatment plants. Biological denitrification (28) is commonly used as the main process to remove nitrogen from wastewater following nitrification, an aerobic process where ammonia is oxidized to nitrate (16).

Denitrification is an anoxic biological process where nitrate and nitrite are reduced to N_2 gas in the presence of an organic carbon source (electron donor) that is typically measured as chemical oxygen demand (COD) in wastewater treatment plants (28). The stringent standards imposed on wastewater treatment plant effluents pose a particular challenge to biological denitrification, especially for treatment plants with a low COD/N ratio in the influent, due to a shortage of suitable electron donors for the nitrate reduction (14, 17, 36). The addition of external carbon sources to the anoxic zone of treatment plants to facilitate denitrification has been one effective solution to this problem (14, 15, 17, 36).

The main external carbon sources used for denitrification include methanol, ethanol, and acetate, of which methanol is generally the most cost-effective (5, 15, 29, 31). However, a complete evaluation of denitrification systems and a comparison of the effects of different carbon sources and operational strategies on their overall performance are largely missing, and this impedes the optimal use of external carbon sources (38).

A major drawback of using methanol intermittently at full-

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scale wastewater treatment plants is the initial long lag period (at least several days to weeks) before an increase in denitrification rates (14, 29). This observation has been made for pre- and postdenitrification processes (14, 15, 32). During the lag period, the wastewater treatment plant suffers from poor denitrification capacity and overall moderate process performance. Although this is considered a major problem in the wastewater industry, little progress has been made so far to identify the likely microbiological reason(s) behind the observed lag period by pure-culture-independent methods.

Studies of pure-culture-dependent methods have demonstrated that, following an initial lag period, highly specialized denitrifying microbial populations develop in wastewater treatment systems in the presence of exogenously added methanol (22). However, since most microorganisms in nature typically cannot be cultured axenically (3), much doubt is placed on these findings, particularly since they have not been verified in situ. Therefore, culture-independent rRNA-based molecular methods, including stable-isotope probing (SIP) (33), were used to identify the dominant methanol-utilizing microorganisms in a laboratory scale activated sludge process fed with methanol and operated with only nitrate as the terminal electron acceptor. The methanol-utilizing denitrifiers were enriched from a full-scale activated sludge biomass.

MATERIALS AND METHODS

Denitrifying SBR. A Setric Genie laboratory fermentor was operated as a sequencing batch reactor (SBR) with a working volume of 1.8 liters. The SBR was seeded with mixed liquor from a biological nutrient removal plant (Wacol, Queensland, Australia) that had exhibited consistently high levels of nitrogen removal for a year prior to sampling without the requirement for an external carbon source to enhance denitrification. The SBR was operated under anoxic conditions for more than 7 months at 22 ± 2°C on a 6-h cycle consisting of 12 min of feeding, 4.7 h of anoxic reaction, and 1.1 h of settling and decantation. The hydraulic retention time was 10.8 h, and, to maintain a sludge age of 7 days, 64 ml of mixed liquor was wasted at the end of each anoxic reaction period of the cycle. A volume of 1 liter of supernatant was decanted during the last 10 min of the settling/decantation stage.

Mixing was achieved by an impeller stirring at 200 rpm. The pH in the reactor was maintained at 7.5 ± 0.3 with a CO_2-N_2 gas mixture. To achieve the desired pH and anoxic conditions, the flow rate of each gas was regulated manually. Periodically the DO level in the mixed liquor was measured, but no DO was detected.

Medium. The basal medium comprised 90 mg of $MgSO_4 \cdot 7H_2O$, 160 mg of $MgCl_2 \cdot 6H_2O$, 42 mg of $CaCl_2 \cdot 2H_2O$, 837 mg of $NaNO_3$, 122 mg of Bacto Peptone (Difco Laboratories, Detroit, Mich.), 20 mg of Bacto Yeast Extract (Difco Laboratories), 50 mg of NH_4Cl, 11 mg of KH_2PO_4, and 26 mg of $Na_2HPO_4 \cdot 12H_2O$ liter^{-1} and 0.3 ml of a nutrient solution prepared as previously reported (6). The medium was made up with reverse-osmosis-deionized water and sterilized by autoclaving. Methanol was used as the carbon source and was separately fed to the SBR to give an initial in-reactor concentration of 263 mg of methanol liter^{-1}. The methanol/NO_3^--N ratio in the medium was maintained at 1.9:1 based on the minimal denitrification stoichiometry (37).

Reactor analysis. Cycle studies were carried out every 2 weeks during the operation of the reactor. Samples were collected every 2 min during the first 30 min and then every 15 min during the 4.7-h anoxic-reaction period of a cycle. The nitrogen removal performance of the SBR was assessed by comparing the supernatant nitrate and nitrite concentrations at the end of a cycle with those concentrations in the influent.

Chemical analysis. Nitrate, nitrite, ammonia, and phosphate were analyzed on filtered (Whatman nitrocellulose membrane, 0.2-μm-pore-size) samples by a flow injection analysis instrument (Lachat, Zellweger Analytical, Milwaukee, Wis.). The mixed-liquor suspended solids and mixed-liquor volatile suspended solids (MLVSS) were determined according to previously published methods (4).

Microbiological analysis. (i) SIP. After 6 months of consistent denitrification operation, the enriched denitrifying microbial community in the SBR was investigated by SIP (33) and by full-cycle rRNA analysis (3). A 50-ml mini-SBR was operated to mimic exactly the 1.8-liter parent SBR. However, in the mini-SBR, [^{13}C]methanol (99% ^{13}C) was substituted for the conventional [^{12}C]methanol and the operation was extended over 24 h or four cycles, during which the mixed liquor in the mini-SBR was sampled four times in one cycle to determine denitrification performance.

(ii) DNA extraction and separation of biomarked DNA. The DNA from the biomass in the mini-SBR (fed with [^{13}C]methanol) and from the 1.8-liter parent SBR (fed with conventional [^{12}C]methanol) was extracted with the Bio 101 FastDNA spin kit (QBiogene). For every milliliter of DNA solution, 1 g of CsCl dissolved in Tris-EDTA buffer (pH 7.6) was added, and 0.8 ml of ethidium bromide (10 mg ml^{-1} in H_2O) was added per 10 ml of DNA-CsCl mixture, such that the final density of the entire mixture was 1.55 g ml^{-1} (34). The mixture was transferred to 5.1-ml Beckman quick-seal centrifuge tubes, and the DNA fractions were resolved by equilibrium centrifugation (200,000 × *g*, 24 h, 20°C). A single DNA band ([^{12}C]DNA band) was observed from the biomass of the parent SBR, which had been exposed to conventional [^{12}C]methanol. The DNA from the mini-SBR biomass produced two bands. One was at the same position as the single [^{12}C]DNA band from the parent SBR, and an additional band approximately 1 cm lower was hypothesized to be as a result of ^{13}C-biomarked DNA ([^{13}C]DNA band). Both bands observed from the mini-SBR were collected with syringes and 20-gauge needles (34). Separate [^{12}C]DNA and [^{13}C]DNA fractions from the mini-SBR biomass were desalted of CsCl and purified from ethidium bromide with Microcon YM-10 centrifugal filter units (Millipore Corporation). The accumulated DNA on the filter device was rinsed two or three times with sterile reverse-osmosis-deionized water and collected to a clean tube.

(iii) PCR amplification, cloning, DNA sequencing, and data analysis. The nearly complete 16S rRNA genes (nucleotides 28 to 1491, *Escherichia coli* numbering) of the [^{12}C]DNA and [^{13}C]DNA fractions from the mini-SBR biomass were amplified and cloned ([^{12}C]DNA and [^{13}C]DNA clone libraries) by methods previously described (8). In brief, primers 27f and 1492r (19) were used to amplify by PCR the nearly complete 16S rRNA genes, and the amplicons were immediately ligated with a TA cloning kit (Invitrogen, San Diego, Calif.). The ligated products were transformed with ultracompetent *E. coli* XL2-Blue MRF′ cells (Stratagene, Sydney, Australia) and screened for positive inserts (clones that contain 16S rRNA gene inserts) with Luria-Bertani agar plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG) (8). Inserts from individual clones were amplified and grouped into operational taxonomic units (OTUs) according to restriction fragment length polymorphism (RFLP) analysis (8). A substantial number of clones from each OTU were partially sequenced with the primer 530f (19) to determine the phylogenetic coverage of each group, and subsequently a representative number of clones were fully sequenced (8). Compilation of DNA sequences and their analysis by BLAST (1) and phylogenetic methods (evolutionary distance analysis and tests of robustness) to construct phylogenetic trees were conducted as previously detailed (9, 12).

(iv) Probe design and synthesis. ARB software (http://www.arb-home.de/) and a database comprising publicly available sequences and our in-house clone sequences were used to design probes (Table 1), as detailed by Hugenholtz et al. (18), to be specific for the microbial communities represented in the [^{12}C]DNA and [^{13}C]DNA clone libraries. The probe design procedure specifically targeted groups of clone sequences from each clone library, which allowed discrimination of other reference sequences. The probe sequences were then confirmed for specificity by using BLAST and were submitted to the probe database ProbeBase (www.probebase.net [25]). In this database, difference alignments, on-line specificity analysis, and other relevant information for published rRNA-targeted probes can be obtained. Finally, the designed oligonucleotides were synthesized and labeled at the 5′ end with the indocarbocyanine dye CY3 (Thermohybaid Interactiva, Ulm, Germany).

(v) Sample fixation and probe optimization. The activated sludge mixed-liquor sample used to seed the reactor, mixed liquor from the parent SBR, and pure cultures (see below) with (positive control) and without (negative control) target sites for the designed probes were fixed and hybridized as previously reported (2, 6, 27). *Burkholderia cepacia* strain H111 (kindly provided by Leo Eberl, Technische Universität München, Munich, Germany) has a single base mismatch with probe DEN67. *Flexibacter elegans* (ATCC 23112) and *Saprospira grandis* (ATCC 23119) are targeted by the two different versions of the SAP553 probe (Table 1). Fluorescence in situ hybridization (FISH) of fixed samples was carried out as previously described (27). For the newly designed probes, the formamide concentrations for optimum stringency were determined by performing a series of FISH experiments at 5% formamide increments starting at 0% formamide. Due to a lack of pure cultures whose 16S rRNA would bind the DEN67 probe (Table 1), the optimum formamide concentration was determined with reference to the enriched parent SBR sludge and with the 1-base-mismatch *B. cepacia* negative-

TABLE 1. FISH oligonucleotides used in this study

Probe	Sequence (5′–3′)	rRNA target site[a]	Specificity	% Formamide	Reference or source
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338–355	Most but not all *Bacteria*	20	2
EUB338-II	GCAGCCACCCGTAGGTGT	16S, 338–355	Bacterial groups not covered by EUB338 and EUB338-III	20	11
EUB338-III	GCTGCCACCCGTAGGTGT	16S, 338–355	Bacterial groups not covered by EUB338 and EUB338-II	20	11
ALF1b	CGTTCG(C/T)TCTGAGCCAG	16S, 19–35	Many *Alphaproteobacteria* and many other bacteria[b]	20	27
BET42a	GCCTTCCCACTTCGTTT	23S, 1027–1043	*Betaproteobacteria*	35	27
GAM42a	GCCTTCCCACATCGTTT	23S, 1027–1043	*Gammaproteobacteria*; used as a competitor for BET42a	35	27
HYP1241	GCTGCSCATTGTCACCGCC	16S, 1241–1259	*Hyphomicrobium* spp.	40	20
PAR1457	CTACCGTGGTCCGCTGCC	16S, 1457–1474	*Paracoccus* spp.	35	28
DEN67	CAAGCACCCGCGCTGCCG	16S, 67–86	Methanol-utilizing denitrifying cluster group A[c]	35	This study
MET1216	TTACGTGTGAAGCCCTGGC	16S, 1216–1234	*Methylophilales* order[c]	ND[d]	This study
SAP553	TAAACCCAGTGAWTCCGGAT	16S, 553–572	Mostly *Saprospiraceae* family	35	This study

[a] *E. coli* numbering (7).
[b] See reference 24.
[c] See Fig. 3.
[d] ND, not determined.

control culture. A similar approach of using mixed microbial biomass in probe optimization has been previously described (9, 13, 35).

(vi) FISH and MAR. A sludge sample obtained from the end of a reactor cycle was diluted with reactor effluent to achieve MLVSS concentrations of 1 to 2 g liter^{-1}. To demonstrate active carbon substrate uptake, a control experiment was performed with pasteurized (80°C for 15 min) biomass. For the experiment, a minimum of three sets of 9-ml glass vials were used. Set 1 was inoculated with 4 ml of pasteurized sludge, while sets 2 and 3 were inoculated with nonpasteurized sludge. A volume of 500 μl of sterile reactor feed which included the electron acceptor (nitrate) but not the electron donor (carbon source) was added to vials of sets 1 and 2 to mimic the exact conditions that prevailed in the reactor. To set 3, with nonpasteurized sludge, 500 μl of sterile reactor feed not containing both the electron acceptor (nitrate) and electron donor (carbon source) was added to demonstrate whether the target cells could carry out any anaerobic uptake of the carbon source. The vials were then sealed with butyl rubber stoppers and flushed with oxygen-free nitrogen for 5 to 10 min. To remove any further traces of oxygen from within these vials, they were placed on a rotary shaker at 200 rpm for 30 min. Finally 500 μl of carbon (nonradioactive methanol and 20 μCi of [^{14}C]methanol [Amersham, Little Chalfont, England]) was introduced into each vial to provide electron donor concentrations equivalent to that of the reactor. Strict anaerobic techniques were used on these anoxic samples at all times, and the samples were incubated at room temperature (20°C) on a rotary shaker (200 rpm). The duration of incubation of multiple nonpasteurized samples (in individual 9-ml vials) ranged from 1 to 4 h. A time series was necessary to determine the minimum duration of incubation since overincubation could result in false interpretation due to a possible uptake of radioactive by-products by nontarget organisms. The control vials were incubated for the entire 4-h period. The subsequent steps of the FISH-microautoradiography (MAR) protocol were then carried out as detailed in Lee et al. (21).

(vii) Microbial quantification by biovolume measurement. The shifts in microbial communities in the parent SBR sludge were monitored and quantified with published probes along with those designed for this study and from previous studies of denitrifying activated sludges (Table 1). The methods used for microbial quantification have already been described by Zeng et al. (39).

RESULTS

Denitrifying performance. The denitrification performance of the parent SBR was assessed by measuring effluent nitrate and nitrite levels. During the initial 3 months of operation, the denitrification performance was determined to be poor (less than 0.02 mg of NO_3^--N mg of MLVSS^{-1} h^{-1}). Denitrification rates then increased to as high as 0.06 mg of NO_3^--N mg of MLVSS^{-1} h^{-1} at steady state and remained around that level for the last 6 weeks of operation. During the 7-month operational time, the NO_2^--N concentrations within SBR cycles were typically less than 1 mg liter^{-1}, the PO_4^{-3}-P concentration was in the range of 4.5 to 7.6 mg liter^{-1}, and the NH_3-N concentration increased from 20.3 to 26.0 mg liter^{-1}. The initial NO_3^--N concentration in the parent SBR at the start of the cycle was as high as 269.4 mg liter^{-1} but decreased to less than 0.2 mg liter^{-1} within 2 h of the anoxic reaction period (Fig. 1).

The mini-SBR fed with 250 mg of NO_3^--N liter^{-1} and [^{13}C]methanol demonstrated a denitrification rate of 0.06 mg of NO_3^--N mg of MLVSS^{-1} h^{-1}, demonstrating that its denitrification performance was similar to that of the steady-state parent SBR.

Clone libraries and probe development. The sludge was subjected to SIP and full-cycle rRNA analysis once the reactor reached steady-state operation.

(i) [^{13}C]DNA clone library. A total of 55 clones of the [^{13}C]DNA clone library from the mini-SBR DNA was screened by RFLP analysis, and these were assigned to 19 different OTUs, although differences in banding patterns were often subtle (data not shown). Three OTUs contained in total 33 clones (26, 4, and 3 clones), while the remaining 16 OTUs contained 1 or 2 clones each. Representatives of most OTUs (32 clones) were partially sequenced to reveal that 25 clones from 10 OTUs had sequences closely related to 16S rRNA gene sequences from organisms in the class *Betaproteobacteria*. The single largest OTU was called group A and comprised 26 clones or 47% of the library. Phylogenetic analysis of these partial sequences revealed that all group A clone sequences clustered closely together. By evolutionary distance analysis, the nearly complete sequences of seven group A clones clustered with the obligate methylotrophs *Methylobacillus* and *Methylophilus* in the order *Methylophilales* of the *Betaproteobacteria* (Fig. 2). Due to the high abundance of this group in the clone library and their phylogenetic affiliation with obligate methylotrophs (which have the capacity to grow on methanol or methylamine but not on methane), they became a focus for oligonucleotide probe design. Only nearly complete 16S rRNA gene sequences (positions 28 through 1491; *E. coli* numbering) of group A clones were used for probe design. Two probes were designed, namely, DEN67 and MET1216 (Fig. 2 and Table 1). DEN67 was highly specific for group A clone sequences, whereas MET1216 targeted all members of the *Methylophilales* (Fig. 2). The DEN67 probe highlighted a single type of morphologically distinct cell (short rods) in the parent SBR sludge up to the determined optimal formamide concen-

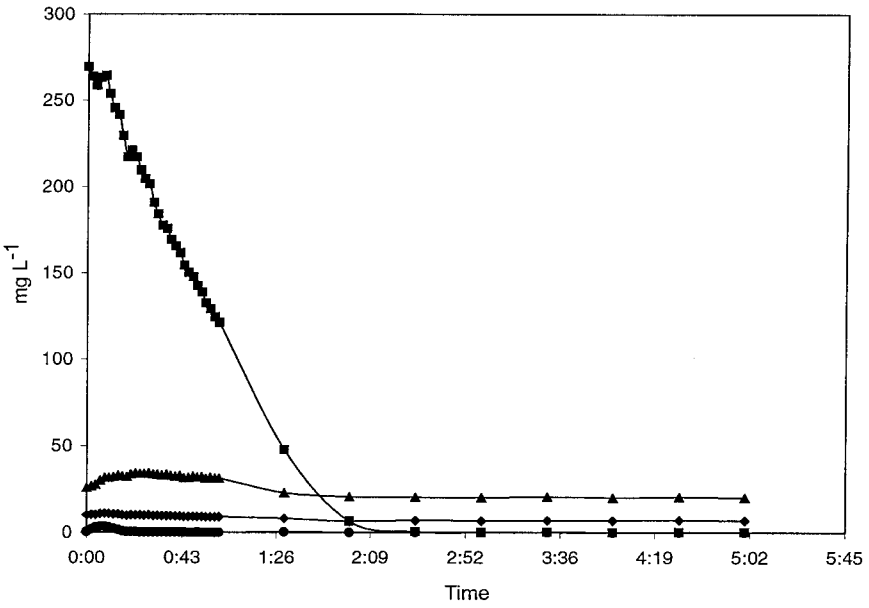

FIG. 1. Profiles of NO_3^--N (■), NO_2^--N (●), PO_4^{-3}-P (♦), and NH_3-N (▲) in an anoxic reactor cycle during the enrichment of the denitrifying sludge.

tration (35%). At 35% formamide in FISH, *B. cepacia*, with 1 nucleotide mismatch in the probe target site, was not detected. The MET1216 probe did not hybridize to the DEN67-targeted cells or any other cell types in the SBR sludge at any formamide concentration and was not further evaluated for FISH. The MET1216 probe is included in Table 1, since it may be a useful probe for specifically targeting the *Methylophilales* by other methods, such as membrane hybridization. Simultaneous FISH with DEN67 and BET42a (broadly specific for *Betaproteobacteria*) confirmed that DEN67-targeted cells are members of the *Betaproteobacteria* (see Fig. 5A).

(ii) [^{12}C]DNA clone library. The [^{12}C]DNA clone library contained 92 clones broadly distributed among 24 OTUs (2 to 10 clones per OTU). The different unique RFLP patterns

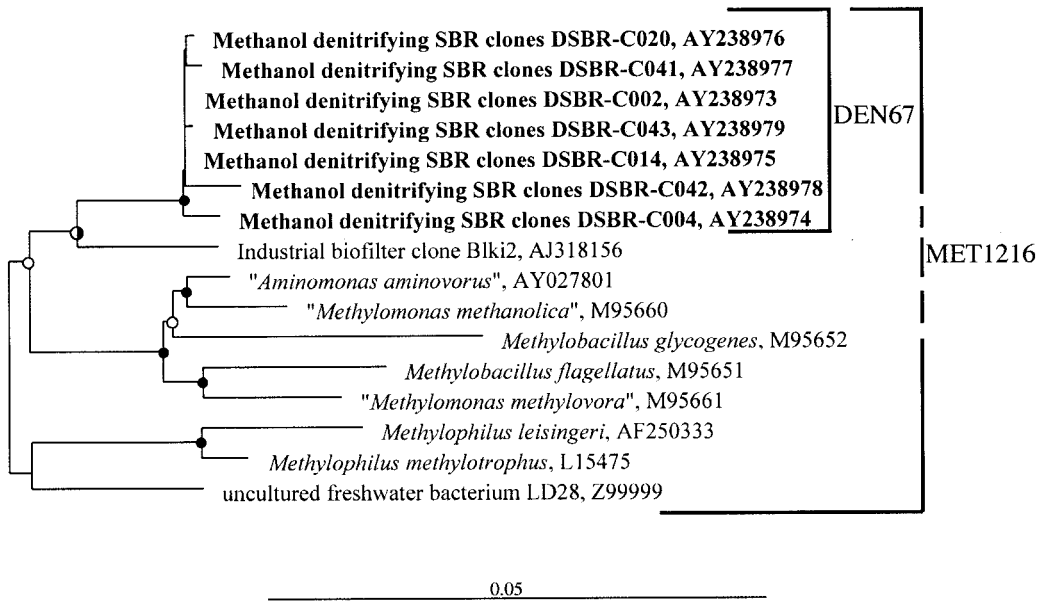

FIG. 2. Phylogenetic tree of the group A clones derived from the [^{13}C]DNA clone library and from publicly accessible databases. Evolutionary distance and parsimony analyses were carried out in PAUP*, version 4.0b10, by employing 2,000 bootstrap resamplings. Filled circles at nodes, >75% bootstrap support from both analyses; open circles, 50 to 75% support from both analyses; half-filled circles, analyses where one algorithm gave >75% bootstrap support and the other gave 50 to 75%. Solid lines, specificities of the DEN67 and MET1216 probes designed in this work; dashed lines, probe that does not have 100% identity with some sequences. *Neisseria denitrificans* (M35020) was used as the outgroup in the analysis but is not shown in the tree. The scale indicates 0.05 nucleotide change per nucleotide position.

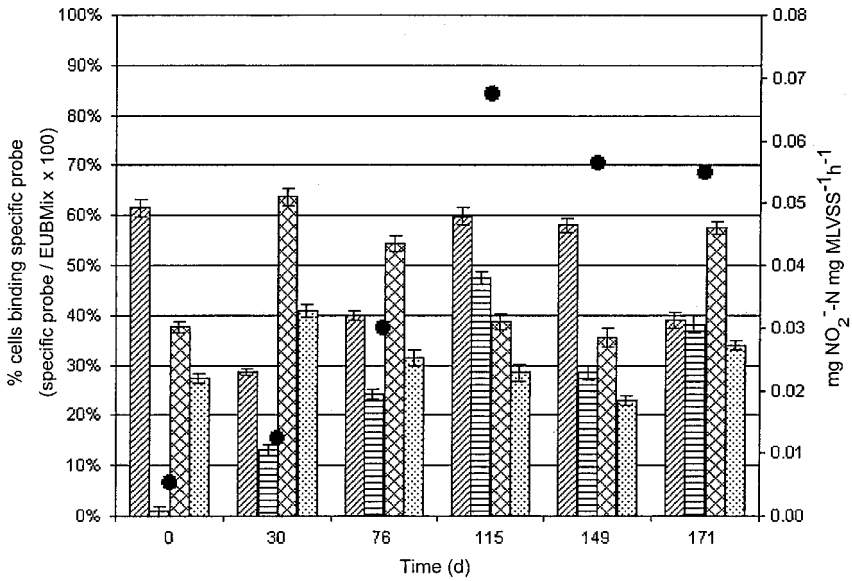

FIG. 3. Bacterial community analysis of the reactor sludge as determined by FISH biovolume measurement. The values obtained with *Betaproteobacteria* ▨, DEN67 ▤, *Alphaproteobacteria* ▩, and *Hyphomicrobium* ▥ probes are expressed as percentages of the area of cells detected with EUBMix (a mixture of EUB338, EUB338-II, and EUB338-III probes). The denitrification rate (●) for each time point analyzed by FISH is indicated.

showed that a wider diversity of organisms was represented in the [^{12}C]DNA clone library than in the [^{13}C]DNA clone library. Partial sequencing and phylogenetic analysis of 43 clones representing all OTUs confirmed the presence of many bacterial species in the [^{12}C]DNA clone library, as suggested by RFLP. In contrast to the composition of the [^{13}C]DNA clone library, only 1 of the 92 [^{12}C]DNA library clones belonged to the group A cluster. Approximately 67% (62 clones) of the [^{12}C]DNA library clones were members of the family *Saprospiraceae* in the *Bacteroidetes* phylum, and 5% (5 clones) were *Alphaproteobacteria*. The SAP553 probe was designed to target mostly *Saprospiraceae*, including a large number of clones closely related to *Haliscomenobacter* spp. identified in the [^{12}C]DNA clone library.

Use of designed and other group-specific probes. Laboratory scale SBR sludge samples were fixed every 2 weeks and evaluated with the DEN67 and SAP553 probes together with other published FISH probes specific for microbial groups relevant to denitrification (Table 1). Figure 3 shows the shifts that occurred in the SBR microbial community with changes in the measured denitrification rate. The DEN67-targeted population (part of the *Betaproteobacteria*) initially comprised just 1 to 2% of all bacteria but rose to 38% at SBR steady-state operation. During this time, the BET42a-targeted *Betaproteobacteria* decreased from 62 to 29 to 39% of all bacteria. The DEN67-targeted-population changes were quite different from these, highlighting the limited utility of broad-specificity probes for studying specific populations of bacteria. The SAP553-targeted population remained at a low level of around 3 to 4% (results not shown in Fig. 3) despite being well represented among the OTUs in the [^{12}C]DNA clone library. The population of the HYP1241-targeted *Hyphomicrobium* sp. (in the class *Alphaproteobacteria* and previously associated with denitrification with methanol) remained at approximately 30% of all bacteria, showing only a marginal increase of 2% from its initial population (Fig. 3). The PAR1457-targeted *Paracoccus* sp. (in the class *Alphaproteobacteria*, also previously associated with denitrification with methanol) was only occasionally observed in the sludge (data not shown). Figures 3 and 4 demonstrate a significant positive correlation {Pearson's correlation [$r(4)$] = 0.953; $P < 0.01$} between the abundance of the DEN67-targeted organisms and the increase in denitrification rates measured in the SBR. Figure 4 shows that there is no significant correlation between *Hyphomicrobium* sp. abundance and denitrification rates [$r(4) = -0.389$; $P = 0.5$]. The DEN67-targeted organisms are observed as clusters of cells in Fig. 5A, while Fig. 5B demonstrates the in situ abundance of *Hyphomicrobium*.

DEN67-targeted microorganisms and MAR with methanol. FISH-MAR was conducted with biomass from the methanol-fed SBR using the DEN67 probe and [^{14}C]methanol. Slides (10 to 15) for FISH-MAR were prepared and observed. The results clearly indicate that DEN67-targeted cells were able to take up methanol under anoxic conditions, as shown in the overlap of the yellow cluster in Fig. 5C (DEN67-targeted cells) with the dark area in Fig. 5D (silver grains in the autoradiographic film). Figure 5C and D are representative images of the FISH-MAR observed on 10 to 15 separate slides. Some DEN67-targeted cells did not take up radioactive methanol, as indicated by clear autoradiographic film above these cells on the MAR slides (Fig. 5C and D). As the autoradiographic film remained clear in all experiments with pasteurized control biomass and nonpasteurized anaerobic control biomass (data not shown), the MAR signals obtained with living biomass could not be caused by adsorption, precipitation, or anaerobic uptake of labeled methanol but must have been results of active methanol uptake by DEN67-targeted cells.

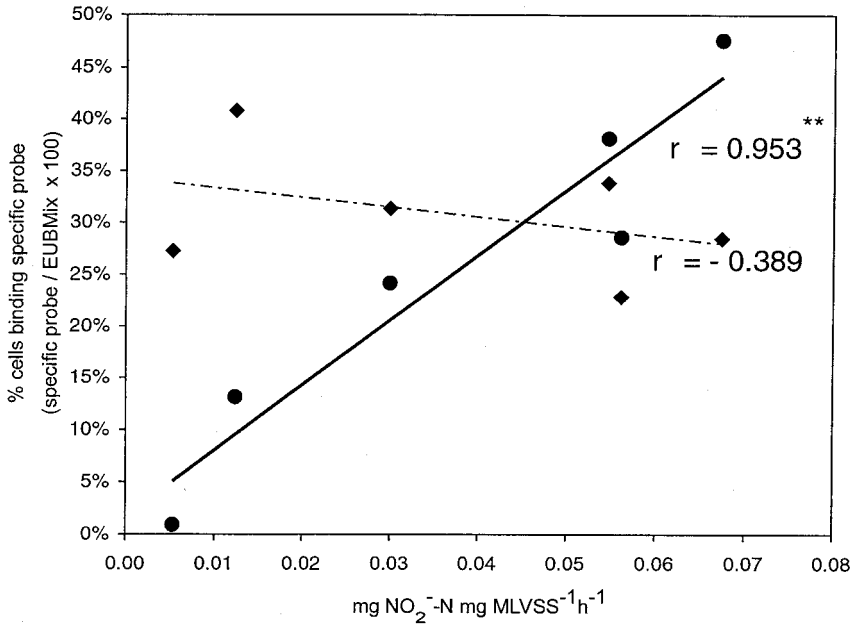

FIG. 4. Relationship between denitrification rate and DEN67-targeted cells (●) and HYP1241-targeted (*Hyphomicrobium*) cells (◆) as a percentage of the EUBMix (a mixture of EUB338, EUB338-II, and EUB338-III probes)-positive cells. **, significant correlation at the $P = 0.01$ level.

DISCUSSION

This study reports, to the best of our knowledge, the first combined use of SIP (33), full-cycle rRNA analysis (3, 30), which includes FISH probe design, and FISH-MAR (21) to establish a link between phylogeny and physiology. The aim was to discover the identity of methanol-utilizing denitrifiers in a laboratory scale SBR activated sludge system. SIP with [^{13}C]methanol was used to biomark the DNA of denitrifiers from the steady-state, anoxically operated SBR. The marked DNA was used as a template for 16S rRNA gene amplification, cloning, RFLP analysis, and phylogenetic analysis of cloned DNA sequences. The largest group (47%) of the analyzed sequences formed a monophyletic clade (group A) in the *Betaproteobacteria* closely related to methylotrophic bacteria in the family *Methylophilales*. Oligonucleotide probe DEN67 was designed to target the group A clones (Fig. 2). Since it is well accepted that clone libraries are biased in several steps, FISH probing with DEN67 was used to explore the in situ abundance of the group A organism. At bioreactor steady state, 38% of all bacteria bound the DEN67 probe. A clear correlation between SBR denitrification rates and the abundance of the DEN67-targeted bacteria (Fig. 3 and 4) was demonstrated. FISH-MAR using the DEN67 probe and [^{14}C]methanol confirmed that the DEN67-targeted microorganisms were taking up methanol in the SBR. Taken together, this information strongly suggests that the group A *Methylophilales* are the dominant methanol-utilizing denitrifiers in the laboratory scale system under study. The combination of methods enables the linkage between the identity of microorganisms (via clone library and FISH probing) and their function (through process performance, SIP, and MAR), which is of major benefit when studying the specific activity of mixed microbial populations. It could be that other microorganisms in the mixed microbial community are also able to carry out denitrification with methanol, but these were not explored in this study.

Three previous studies exploring the identities of methanol-utilizing denitrifiers relied partially on pure-culture-based approaches (20, 23, 28), with a significant drawback being that many microorganisms cannot yet be isolated in pure culture (3). Neef et al. (28) also designed FISH probes for *Paracoccus* and *Hyphomicrobium* and applied them to study a methanol-fed, postdenitrification biofilm system. A correlation between the relative abundance of these genera and denitrification performance was used to suggest their active participation in denitrification (28). Lemmer et al. (23) found that the most highly abundant isolate from a methanol-fed, postdenitrification sand filter biofilm was *Hydrogenophaga* (from *Betaproteobacteria* and known to be unable to grow on C1 compounds such as methanol), followed by *Paracoccus* and *Hyphomicrobium* (*Alphaproteobacteria*), both recognized denitrifiers. Layton et al. (20) carried out a full-cycle rRNA analysis of pooled sludge samples from a full-scale industrial wastewater treatment plant, where the ability to carry out denitrification with C1 carbon sources such as methanol was an important feature. According to FISH with probe HYP1241, *Hyphomicrobium* was found to be abundant within and outside the activated sludge flocs. Although these reports demonstrated the presence of substantial microbial diversity in the analyzed systems, they failed to directly demonstrate that the identified microorganisms were indeed respiring anaerobically with nitrate within the systems, since none of the applied methods was suited to study the phenotype of the microorganisms in their ecological niche.

According to FISH with HYP1241, *Hyphomicrobium* comprised 22 to 40% of the total bacterial population in the laboratory scale SBR (Fig. 3), but its slight shifts in abundance showed no correlation with the rates of denitrification ob-

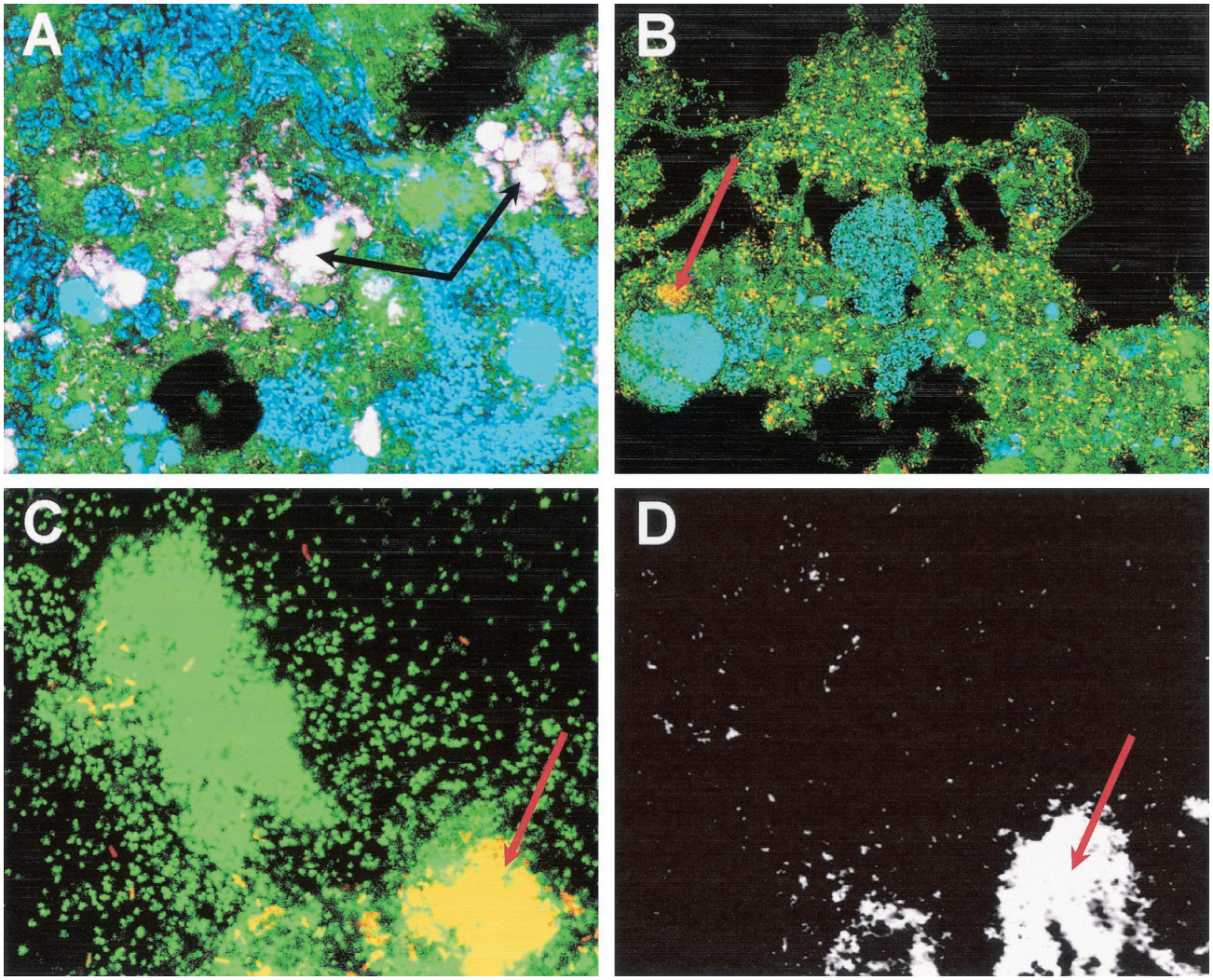

FIG. 5. In situ analyses of denitrifying bacteria in reactor sludge. (A) Confocal laser scanning micrograph of reactor sludge triple hybridized with EUBMix (25 ng of fluorescein-labeled probe, a mixture of EUB338, EUB338-II, and EUB338-III), BET42a (25 ng, Cy5 labeled), and DEN67 (25 ng, Cy3 labeled) probes. The white cell aggregates (arrows), comprising densely packed 1- to 2-μm-diameter cells, are the DEN67-targeted organisms since they are tripled labeled with EUBMix (green), BET42a (blue), and DEN67 (red) probes. The cyan-colored cells are other *Betaproteobacteria* since they are only dual labeled with EUBMix (green) and BET42a (blue) probes. (B) Confocal laser scanning micrograph of reactor sludge triple hybridized with EUBMix (25 ng, fluorescein labeled), BET42a (25 ng, Cy5 labeled), and HYP1241 (25 ng, Cy3 labeled) probes. The yellow cell aggregates (arrow), composed of 1- to 2-μm-diameter cells, comprise *Hyphomicrobium* cells since they are dual labeled with EUBMix (green) and HYP1241 (red) probes. The cyan-colored cells are *Betaproteobacteria* since they are dual labeled with EUBMix (green) and BET42a (blue) probes. (C) Confocal laser scanning micrograph of [^{14}C]methanol-fed reactor sludge dual hybridized with EUBMix (25 ng of fluorescein) and DEN67 (25 ng, Cy3 labeled) probes. The yellow cell aggregates (arrow) are the DEN67-targeted organisms since they are dual labeled with EUBMix (green) and DEN67 (red) probes. The image was compiled by obtaining a stack of images by confocal laser scanning microscopy and combining them by orthographic projection into a single image. The image stack was acquired to ensure that all organisms that contributed to the MAR signal (D) would be visible in the final image. (D) A micrograph of the radiographic film at the same position as that of panel C. Magnification, ×500.

served (Fig. 4). The *Hyphomicrobium* present might have adapted to the methanol feedstock, but, if so, this potential adaptation was extremely slow since, even after 30 days of operation, the denitrification rate was still very low (Fig. 3). Although FISH-MAR was not carried out with HYP1241, nearly all bacteria that took up [^{14}C]methanol were targeted by DEN67. The absence of any *Hyphomicrobium* sequences in the [^{13}C]DNA clone library also does not support the concept that *Hyphomicrobium* adapted to methanol and was the responsible denitrifier. It is unlikely that *Paracoccus* played any role in denitrification in the methanol-fed SBR, since PAR1457-targeted cells were only occasionally observed in the sludge. This finding is further supported by the absence of clones related to *Paracoccus* in the [^{13}C]DNA clone library. These two previously well-reported methanol-utilizing denitrifiers were thus shown not to be responsible for denitrification in our methanol-fed SBR. SAP553 was designed from the [^{12}C]DNA clone library sequences, but the abundance of these *Cytophagales* was not correlated with denitrification, nor did they appear as a significant group in the [^{13}C]DNA clone library.

The group A clones by evolutionary distance analysis grouped with the obligate methylotrophs *Methylobacillus* and *Methylophilus* (Fig. 2), which are able to grow on methanol or methylamine but not on methane. Other methylotrophs, such as *Methylomonas*, *Methylococcus*, *Methylobacter*, *Methylosinus*, *and Methylocystis*, are able to grow on both methane and methanol and hence are also methanotrophs (10, 26). The denitrification activity of methylotrophs has not previously been explored, but at least the group A clone cluster from bacteria closely related to *Methylobacillus* and *Methylophilus* has been demonstrated in this study to be denitrifiers. This extends the known phenotype of methylotrophs.

Previous researchers (14, 29) hypothesized that the initial lag period in denitrification activity observed in methanol-amended activated sludge systems might be explained by the development of specific methanol-utilizing populations. This study confirms that DEN67-targeted organisms comprise one bacterial group that was present only at very low levels in the original full-scale denitrifying sludge. They were then enriched to become the dominant group in the biomass, which demonstrated a much higher denitrification rate using methanol than the original reactor inoculum. This establishes that a microbial population shift is the most likely cause for the slow development of denitrification capacity when a particular carbon source, for example, methanol, is used. This finding is highly relevant to the practical operation of denitrification in wastewater treatment processes, as the selection of carbon source added needs to be done with due recognition of the associated operational effects caused by bacterial population changes. The use of external carbon addition is becoming more widespread to meet increasingly demanding effluent concentration limits for total nitrogen in many parts of the world. Since methanol is usually the cheapest carbon source, it is commonly used in such situations, although with various degrees of success. Recognizing the influence of the bacterial community composition on the denitrification performance is therefore highly valuable for the study and optimization of denitrification processes using external carbon sources. Further studies determining the impact of other carbon sources on the microbial population are also important in this context and are under way in our research group.

CONCLUSIONS

Our study employed a combination of culture-independent methods to reveal the identity of a new microbial community able to utilize methanol as a carbon substrate for denitrification. The combination of SIP with full-cycle rRNA analysis enabled us to bias the clone library such that it was enriched in clones coming from [^{13}C]methanol-utilizing denitrifiers. A FISH probe designed from the full-cycle rRNA analysis (DEN67) was used in MAR to reconfirm anoxic methanol uptake of DEN67-targeted microorganisms. SIP enabled the design of molecular probes for methanol-utilizing denitrifiers, while FISH-MAR provided visual evidence for the in situ function of the microorganisms already identified with the probe. The application of SIP together with FISH-MAR is suggested as an effective approach to address the in vivo physiology of microorganisms in mixed-population environments, such as activated sludge systems. This is the first report of members of the family *Methylophilales* being implicated in denitrification, and we predict that these bacteria will be important denitrifiers in full-scale wastewater treatment processes on a global scale. This prediction can be tested by using the specific probes presented in this study.

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