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Enrichment and Molecular Detection of Denitrifying Methanotrophic Bacteria of the NC10 Phylum▽

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Anaerobic methane oxidation coupled to denitrification was recently assigned to bacteria belonging to the uncultured phylum NC10. In this study, we incubated sediment from a eutrophic ditch harboring a diverse community of NC10 bacteria in a bioreactor with a constant supply of methane and nitrite. After 6 months, fluorescence in situ hybridization showed that NC10 bacteria dominated the resulting population. The enrichment culture oxidized methane and reduced nitrite to dinitrogen gas. We assessed NC10 phylum diversity in the inoculum and the enrichment culture, compiled the sequences currently available for this bacterial phylum, and showed that of the initial diversity, only members of one subgroup had been enriched. The growth of this subgroup was monitored by quantitative PCR and correlated to nitrite-reducing activity and the total biomass of the culture. Together, the results indicate that the enriched subgroup of NC10 bacteria is responsible for anaerobic methane oxidation coupled to nitrite reduction. Due to methodological limitations (a strong bias against NC10 bacteria in 16S rRNA gene clone libraries and inhibition by commonly used stopper material) the environmental distribution and importance of these bacteria could be largely underestimated at present.

Atmospheric concentrations of methane have risen 2.6-fold since preindustrial times (10). After several years of stagnation, there was again a clear increase in the methane concentration in 2007 (29). Currently, it is uncertain whether an increase in the number of sources and production or a decrease in the number of sinks and consumption is responsible for this reversal of the trend.

Freshwater habitats like natural wetlands and rice fields are a major source (38% [9]) of atmospheric methane. In the absence of other documented electron donors, aerobic methane oxidation is assumed to be the most important sink in these habitats, but the role of alternative electron donors is not well understood (19, 30). Anaerobic methane oxidation coupled to denitrification is energetically favorable, but evidence that it occurs is scarce. In marine, methane-containing sediments, nitrate and nitrite are usually not quantitatively important electron acceptors; in freshwater sediments the denitrifying and aerobic zones are in close proximity (3, 22, 35), possibly masking the process from detection. To our knowledge, concomitant methane and nitrate profiles of sediments have never been published.

So far, methane oxidation coupled to denitrification has received the most attention in the field of hydrogeology. In groundwater, contamination with nitrate and nitrite occurs frequently, whereas electron donors are limiting. Methane plumes often form around landfills, and their attenuation has sometimes been attributed to denitrification (2, 37). So far, a single previous study unambiguously demonstrated anaerobic oxidation of methane coupled to denitrification in a contaminated freshwater aquifer (32). The first in vitro observation of anaerobic methane oxidation coupled to denitrification came from a laboratory-scale sludge digestor (11). The use of a laboratory enrichment culture also eventually resulted in identification of the organisms involved; bacteria of the NC10 phylum and archaea of the order *Methanosarcinales* dominated a mixed culture carrying out anaerobic methane oxidation coupled to denitrification (27). This culture was enriched from a freshwater canal sediment after 1 year of continuous supply of methane and nitrite. Subsequently, the archaea were shown to be dispensable, as they disappeared after prolonged incubation of the same culture (7). Mass balance calculations showed that methane oxidation was coupled to the reduction of nitrite with a 3:8 stoichiometry, in accordance with theoretical expectations. The bacteria that dominated the mixed culture and apparently oxidized methane anaerobically are members of the NC10 phylum, one of the many phyla having no members in pure culture (8, 28). The 16S rRNA gene sequences of such organisms, however, have been found in a number of environmental surveys of aquatic environments; e.g., the most closely related sequences have been found in aquifers (1, 23) and lake sediments (13, 17). Sequence similarity and phylogenetic affiliation may indicate similar metabolic capacities of organisms, but by itself this is not sufficient to infer similar metabolism (5). This is especially true for denitrifying methanotrophs, because only a single enrichment culture has been described so far (7, 27).

The objective of the present study was to generalize the previous finding that NC10 bacteria were associated with an-

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TABLE 1. Primers and PCR conditions for the 16S rRNA gene libraries

Clone library	Forward primer		Reverse primer		Annealing temp (°C)	Amplicon positions[d]
	Designation	Sequence (5′–3′)	Designation	Sequence (5′–3′)		
Ino-nFR	202F[a]	GAC CAA AGG GGG CGA GCG	1043R[a]	TCT CCA CGC TCC CTT GCG	69	202–1060
Ino-Ra	8F[b]	AGA GTT TGA TYM TGG CTC AG	1043R[a]	TCT CCA CGC TCC CTT GCG	57	8–1060
Ino-Rb	8F[b]	AGA GTT TGA TYM TGG CTC AG	1043R*b*	TCT CCA CGT TCC CTT GCG	57	8–1060
Ino-F	202F[a]	GAC CAA AGG GGG CGA GCG	1492R[c]	GGT TAC CTT GTT ACG ACT T	57	202–1529
Enr-Ra	8F[b]	AGA GTT TGA TYM TGG CTC AG	1043R[a]	TCT CCA CGC TCC CTT GCG	59	8–1060
Enr-Rb	8F[b]	AGA GTT TGA TYM TGG CTC AG	1043R*b*	TCT CCA CGT TCC CTT GCG	57	8–1060
Enr-F	202F[a]	GAC CAA AGG GGG CGA GCG	1492R[c]	GGT TAC CTT GTT ACG ACT T	60	202–1529

[a] Based on previously described FISH probes (27).
[b] See reference 12.
[c] See reference 21.
[d] The positions are based on the gap-free sequence of the NC10 bacterial clone D-BACT (GenBank accession no. DQ369742), starting at *E. coli* position 8.

aerobic methane oxidation, a necessary step forward in addressing the significance of this poorly understood process as a methane sink in freshwater habitats.

MATERIALS AND METHODS

Sampling and enrichment. Sediment samples (upper 5 cm) were obtained in July 2006 from four ditches draining agricultural land in the Ooijpolder (51°50′40″N, 5°54′44″E), a floodplain of the River Rhine in The Netherlands. The samples were transported to the lab within 1 h and mixed with ambient water to obtain a homogeneous slurry (2 liters) used for inoculation.

The sediment was incubated in a 16-liter glass bioreactor (Applikon, Schiedam, The Netherlands), which was operated aseptically in a sequencing-batch mode (34) to prevent loss of biomass. Initially, a cycle consisted of 3 to 7 days of continuous supply of medium, a settling period of 1 to 2 h, and 30 to 60 min to draw off liquid from above the settled sediment and biomass. As activity increased, after 6 months the cycle was shortened to 22.5 h of supply of medium, 1 h of settling, and 30 min of drawing off of liquid. During the supply period, the culture was stirred gently at 100 rpm, sparged with CH_4-CO_2 (95:5, vol/vol; purity, >99.995%; flow rate, 10 ml/min; Air Liquide, France), and supplied with mineral medium (flow rate, 0.5 to 2 liters day^{-1}). The medium was continuously sparged with Ar-CO_2 (95:5, vol/vol) to maintain anoxic conditions and contained the following components (per liter): $KHCO_3$, 0.5 to 1.5 g (see Results); KH_2PO_4, 0.05 g; $CaCl_2 \cdot 2H_2O$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $NaNO_3$, 0.425 g (5 mM); $NaNO_2$, 0.0345 to 1.38 g (0.5 to 20 mM) (see Results); an acidic trace element solution, 0.5 ml; and an alkaline trace element solution, 0.2 ml. The acidic (100 mM HCl) trace element solution contained (per liter) 2.085 g $FeSO_4 \cdot 7H_2O$, 0.068 g $ZnSO_4 \cdot 7H_2O$, 0.12 g $CoCl_2 \cdot 6H_2O$, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.32 g $CuSO_4$, 0.095 g $NiCl_2 \cdot 6H_2O$, and 0.014 g H_3BO_3. The alkaline (10 mM NaOH) trace element solution contained (per liter) 0.067 g SeO_2, 0.050 g $Na_2WO_4 \cdot 2H_2O$, and 0.242 g Na_2MoO_4. All medium components were sterilized, either by 0.2-μm filtration (trace metal solution) or by autoclaving, and mixed aseptically.

The minimum liquid volume of the enrichment culture was kept at 10 liters by a level controller, and the maximum volume at the end of a filling period was 13 liters. A gas buffer (~13 l liters) filled with the exhaust gas and additionally flushed with Ar (100 ml min^{-1}) prevented entry of air during drawing off. The oxygen concentration was continuously monitored with a Clark-type oxygen electrode. The culture vessel was wrapped in black foil, and black tubing with low oxygen permeability (Viton and Norprene; Cole Parmer, United States) was used. The nitrite concentration in the bioreactor effluent was estimated daily, and the medium flow was adjusted if the concentration was <0.2 or >1.5 mM. Also, the $KHCO_3$ and $NaNO_2$ contents of the medium were varied depending on the denitrifying activity of the culture (see Results), and together with the CO_2 in the gas supplied, they resulted in a pH of the culture liquid between 6.9 and 7.5, as monitored with a pH electrode. The temperature was controlled at 30°C.

Molecular analysis. (i) DNA isolation. Samples (2 ml) for quantitative PCR (qPCR) were taken every 2 to 4 weeks in triplicate. For the clone libraries, samples were taken from the slurry used for inoculation and from the enrichment culture after 6 months and stored at −20°C until DNA isolation. After bead beating in 120 mM sodium phosphate buffer, DNA was extracted and purified as described by Kowalchuk et al. (18) (sodium dodecyl sulfate lysis, DNA precipitation with NaCl-polyethylene glycol 6000, protein precipitation with ammonium acetate, DNA precipitation with ethanol). The DNA was dissolved in water. DNA quality was checked by agarose gel electrophoresis, and the DNA concentrations of samples for qPCR were measured at 260 nm with a Smartspec 3000 spectrophotometer (Bio-Rad).

(ii) Phylogenetic analysis. To assess the diversity of bacteria affiliated with the NC10 phylum, 16S rRNA gene libraries were constructed after PCR amplification of DNA isolated from the inoculum (library Ino) and the enrichment culture (library Enr) after 6 months of incubation. One library (library Ino-nFR) was obtained using a nested PCR approach: a PCR with general bacterial primers 8F and 1545R (12) was followed by a second amplification using a specific primer pair and a high annealing temperature. The other libraries were obtained with combinations of a specific primer with a general primer and relatively low annealing temperatures (Table 1). PCR products were amplified in *Escherichia coli* with the pGEM-T Easy cloning vector (Promega, United States). Plasmids were isolated from 10 to 15 randomly selected clones per library using a GeneJet miniprep kit (Fermentas, Lithuania). Sequencing was performed at the DNA Diagnostics Center of Nijmegen University Medical Center using both the M13 forward and reverse primers. The quality of sequences was checked with the FinchTV program, and a BLAST search was performed to obtain related sequences (>90% similarity) from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/ [accessed October 2008]). Additionally, all sequences assigned to the NC10 phylum by the taxonomies of Hugenholtz and Pace (http://greengenes.lbl.gov [August 2008]) were added. To cover the full NC10 phylum, short sequences (minimum length, 548 bp) were also included. Three species of *Acidobacteria*, a closely related phylum (8), served as an outgroup. The sequences were aligned with the MUSCLE algorithm (6) and imported into the MEGA4 software (36), where the alignment was manually checked and trimmed. Phylogenetic trees were calculated on basis of 1,394 and 800 aligned positions (positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons) with MEGA's neighbor-joining, maximum parsimony, and minimum evolution (Fig. 1) methods, which yielded the same overall topology. The robustness of tree topology was also tested by bootstrap analysis (1,000 replicates). Evolutionary distances were computed with the Tajima-Nei correction. The phylum was divided into four well-supported groups (groups *a* to *d*).

(iii) qPCR. Based on the 16S rRNA gene sequences obtained from the enrichment culture, the sequence of a previously enriched bacterium (27), clone D-BACT (GenBank accession no. DQ369742), and closely related sequences (DQ837241, DQ837250, and AF351217), two primers pairs for qPCR were designed; qP1F (5′-GGG CTT GAC ATC CCA CGA ACC TG-3′) and qP1R (5′-CGC CTT CCT CCA GCT TGA CGC-3′) amplify positions 1001 to 1201, and qP2F (5′-GGG GAA CTG CCA GCG TCA AG-3′) and qP2R (5′-CTC AGC GAC TTC GAG TAC AG-3′) amplify positions 1169 to 1460. The primers and their optimal annealing temperature were tested with the enrichment culture (7), and PCR products obtained with both primer pairs were cloned and sequenced as described above. All of the sequences retrieved were the correct length and were very similar (97.6 to 98.5% identity) to the reference sequence (accession no. DQ369742). As a negative control, DNA from different mixed cultures dominated by anammox bacteria (15, 38) was used as a template, and it did not yield a PCR product. For each primer pair, standard curves (for 70 to 7×10^8 copies) were constructed, taking into account the molecular mass of the plasmid including the amplicon (Mongo Oligo mass calculator, version 2.06 [http://library.med.utah.edu/masspec/mongo.htm]) and the plasmid concentration determined by spectrophotometric measurement with a Smartspec 3000 (Bio-Rad) at 260 nm. qPCR for the standard curves and the samples was performed with the Bio-Rad IQTM 5 cycler and real-time detection system using

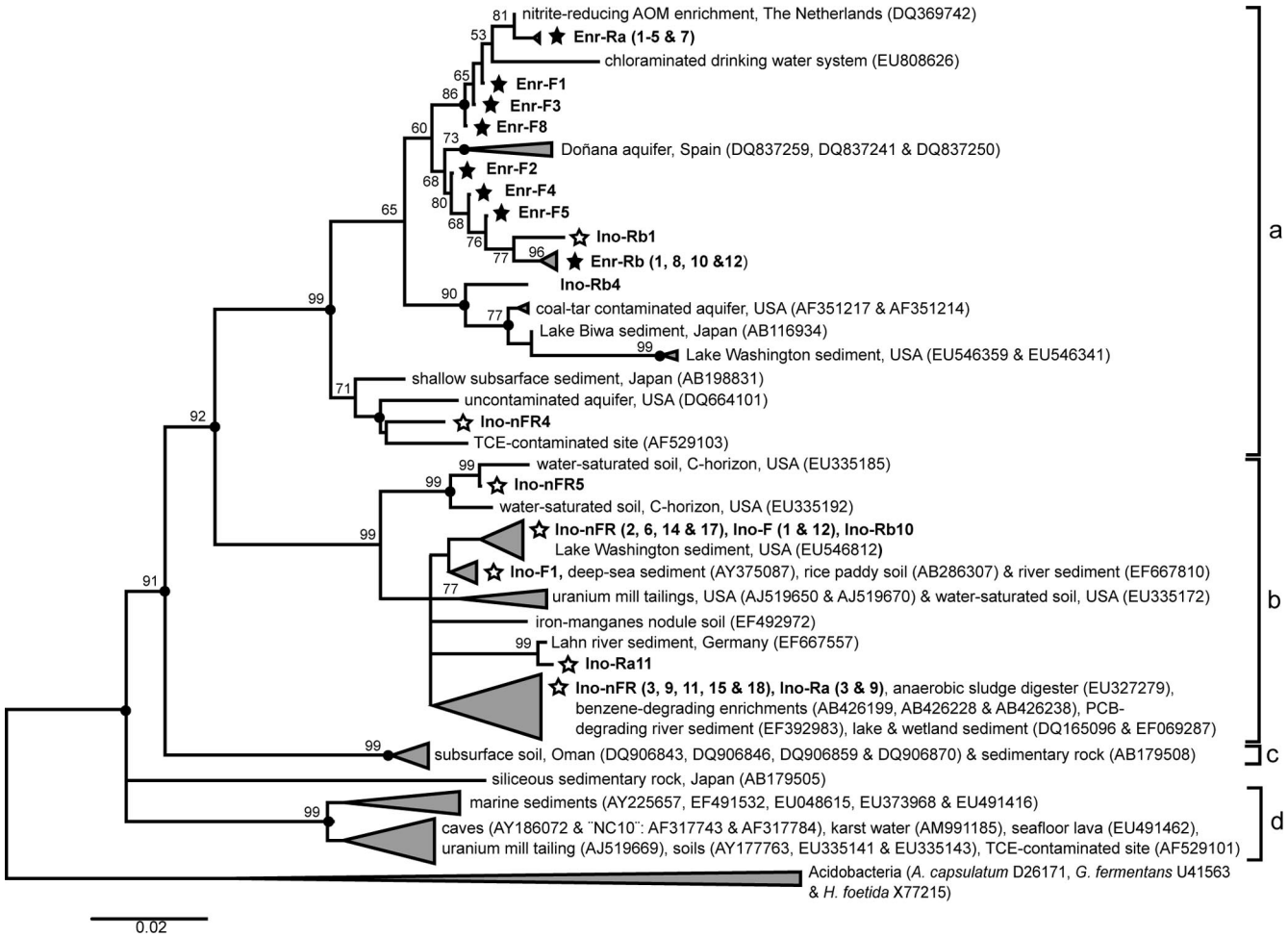

FIG. 1. Phylogenetic tree of the NC10 phylum with *Acidobacteria* as the outgroup. The sequences obtained from the inoculum of the enrichment culture (libraries Ino-nFR, Ino-F, Ino-Ra, and Ino-Rb) are indicated by an open star, and the sequences obtained from the enrichment culture after 6 months (libraries Enr-F, Enr-Ra, and Enr-Rb) are indicated by a filled star. The tree was calculated using the minimum evolution method and the Tajima-Nei correction. Bootstrap support values greater than 50% (n = 1,000) are indicated at the nodes, and the branches supported by all treeing methods (see Materials and Methods) are indicated by a circle.

IQTM SYBR green Supermix (Bio-Rad, United States), 800 nM forward primer, and 800 nM reverse primer. An initial denaturing step of 95°C for 3 min was followed by 40 cycles of 95°C for 1 min, 63°C for 1 min, and 72°C for 1 min. After a final extension for 5 min at 72°C, a melting curve analysis was carried out at temperatures from 60°C to 95°C, increasing at a rate of 0.5°C/30 s. The calculated efficiency was 100% for both primer pairs. The copy numbers in samples were calculated based on comparison with the threshold cycle values of the standard curve, taking into account the dilution and the liquid volume of the bioreactor during sampling.

(iv) FISH. Biomass from the enrichment culture was harvested monthly, fixed, stored, and subjected to fluorescence in situ hybridization (FISH) as described previously (7), using a concentration of 40% formamide. The following probes were used: S-*-DBACT-0193-a-A-18 and S-*-DBACT-1027-a-A-18 for dominant bacteria affiliated with the NC10 phylum (27), a mixture of EUB I-III and EUB V for most bacteria (4), and S-D-Arch-0915-a-A-20 for most archaea (33). The percentages of phylum NC10 bacteria were estimated based on visual inspection of at least one complete well.

Activity measurements. For batch incubation, biomass was transferred aerobically to glass bottles (volume, 8, 40, or 60 ml). Nitrite and 3-morpholinopropanesulfonic acid (MOPS) buffer (pH 7.3) were added to final concentrations of 1 to 2 and 10 mM, respectively. The bottles were capped with either black (Rubber B.V., Hilversum, The Netherlands), gray (Helvoet Pharma, Alken, Belgium), or red (Terumo, Leuven, Belgium) butyl rubber stoppers, and anaerobic conditions were created by six cycles of vacuuming and subsequent gassing with Ar-CO_2 (95:5), followed by 5 min of flushing with Ar-CO_2, leaving an overpressure of 0.5×10^5 Pa. Methane was added to final headspace concentrations of 2.5 to 10%. Samples were incubated on a magnetic stirrer at 30°C. To assess formation of gaseous N compounds, twofold-concentrated samples were preincubated anaerobically with methane until the residual nitrite and nitrate were exhausted, and [^{15}N]nitrite (99.6% ^{15}N; Isotec, United States) was used as the sole electron acceptor.

Analysis of nitrogen compounds, methane, and protein. The nitrite concentration in the enrichment culture was estimated daily with Merckoquant test strips (0 to 80 mg/liter; Merck, Germany). For batch experiments, ammonium and nitrite were measured colorimetrically as described previously (14). Methane was quantified by gas chromatography, and the total protein content was determined by the bicinchoninic acid assay (Pierce, United States) using a bovine serum albumin standard (Thermo Scientific, United States) as described previously (7). For quantification of gaseous nitrogen compounds, 50-μl samples were withdrawn with a gas-tight glass syringe and injected into a gas chromatograph while the inlet area was flushed with helium to prevent entry of air. Additionally, use of ^{15}N-labeled nitrite in the incubations enabled distinction between contaminating ^{14}N-N_2 (air) and ^{15}N-N_2 produced in the experiment. Gases were separated by gas chromatography (6890 series; Agilent, United States) using a Porapak Q column at 80°C (5 min) with helium as the carrier gas (flow rate, 24 ml min^{-1}). The gas chromatograph was coupled to a mass spectrometer (Agilent 5975C inert MSD; Agilent, United States) to quantify the molecular masses 28 to 30 Da (N_2) and 44 to 46 Da (N_2O). Data were analyzed using the software

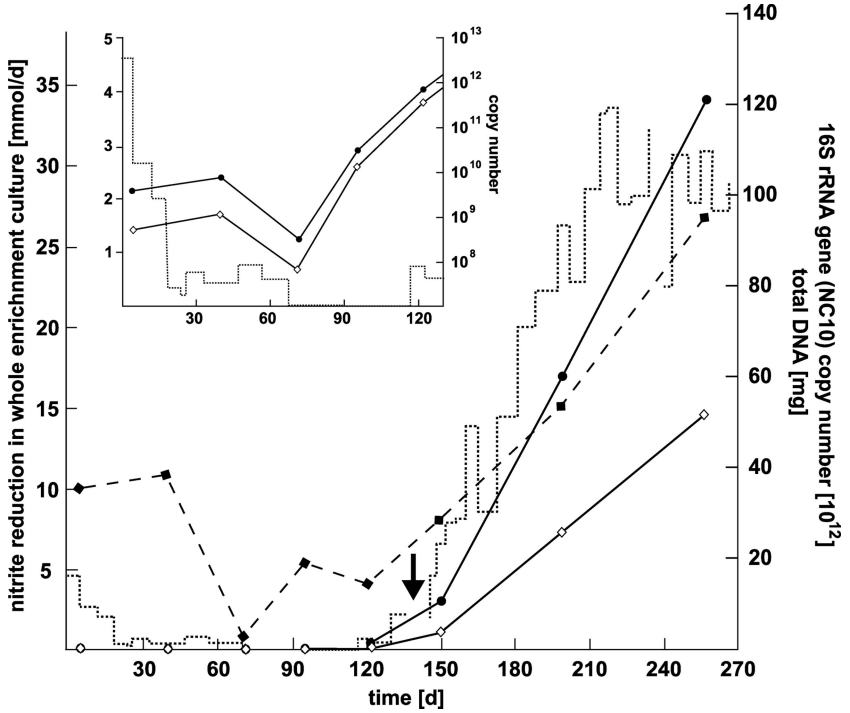

FIG. 2. Nitrite reduction (dotted line), DNA concentration (dashed line), and abundance of 16S rRNA genes of NC10 bacteria (solid lines) in the enrichment culture. The copy number of 16S rRNA genes of NC10 bacteria was assessed with the primer pairs qP1F/qP1R (filled circles) and qP2F/qP2R (open diamonds). The liquid volume of the culture was 10 to 13 liters. From day 136 to day 142 (arrow) the bioreactor did not receive medium and gas to assess methane oxidation activity. The inset shows data for the first 4 months, demonstrating the greater sensitivity of the qPCR approach than of activity measurements. At the end of the enrichment period (day 243) the culture contained 4.75 ± 0.8 g protein.

Enhanced MSD Chem Station (version E.02.00.493; Agilent). Calibration was performed with standards consisting of $^{28}N_2$ in helium. Additionally, the initial and final concentrations of N_2 and N_2O were verified by gas chromatography as described previously (7).

Nucleotide sequence accession numbers. The sequences of 16S rRNA genes have been deposited in the GenBank database under accession no. FJ621531 to FJ621562.

RESULTS AND DISCUSSION

Using previously described FISH probes (27) as primers in a nested PCR approach, we screened several freshwater sediments for the presence of phylum NC10 sequences. Of those, sediments from a ditch draining agricultural land in a floodplain of the River Rhine yielded an amplicon of the correct length. The sequences obtained after cloning (library Ino-nFR) (Table 1 and Fig. 1) were related to the bacterium dominating an enrichment culture performing anaerobic methane oxidation coupled to denitrification (27) and fell into two distinct groups (groups *a* and *b*) of the NC10 phylum. However, these bacteria were not detected in the sediment with FISH using the same probes. Because of the strong background fluorescence of sediment particles, only a few bacteria were visible, and no archaea could be detected.

The sediments were subsequently used to inoculate an anaerobic continuous culture bioreactor. The culture was sparged with CH_4-CO_2 as the sole carbon sources and supplied with mineral medium containing nitrite and nitrate as electron acceptors. In the first 3 weeks, the nitrite-reducing activity in the culture decreased from 4.6 mmol day^{-1} to zero (Fig. 2). After a 110-day period without measurable activity, nitrite reduction resumed, and methane oxidation activity, although still low, could be measured in a batch experiment with the whole culture from day 136 to day 142 (Fig. 2) (methane oxidation activity, 0.18 nmol min^{-1} mg protein^{-1}, as determined using previously described methods [7]). Until day 217, nitrite reduction increased in a roughly linear fashion up to a maximum of 33.5 mmol day^{-1} (Fig. 2). During this period, the nitrite concentration in the medium could be increased from 0.5 to 20 mM with nearly complete consumption by the culture. The $KHCO_3$ concentration was decreased from 15 to 0.5 mM to compensate for H^+ consumption associated with denitrification. The stagnation of activity at values between 27 and 32 mmol day^{-1} starting around day 230 may have been caused by the removal of about 2.3 liters of culture liquid with biomass (corresponding to ca. 1.1 g protein) for experiments over a 2-month period. In this period, the culture contained 4.75 ± 0.8 g protein, and the specific activity was 3.4 to 5.6 nmol NO_2^- min^{-1} mg protein^{-1}.

Methane oxidation activity and its coupling to denitrification was further demonstrated in different batch experiments (Fig. 3). Initially, all attempts to detect activity in serum bottles failed, which was also observed in a previous study (7). This was apparently due to the use of black butyl rubber stoppers, which caused total inhibition of the activity (Fig. 3A). Anoxic handling of the biomass, in contrast, was not crucial; exposure to oxygen during transfer to the bottles did not lead to lower activity. Repeated boiling of black stoppers in water and diluted HCl did not eliminate the inhibition. It is generally

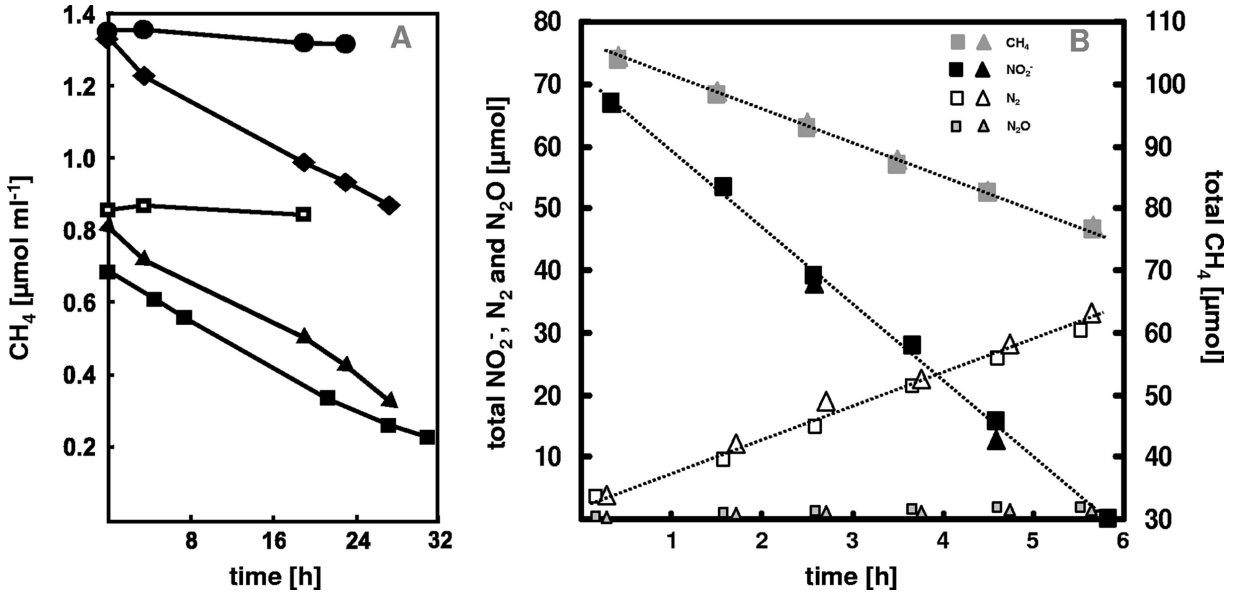

FIG. 3. (A) Influence of stopper material on methane oxidation activity in the presence of nitrite and control for methane loss through alternative stopper materials. Filled circles, black butyl rubber; filled squares, red butyl rubber; filled diamonds, gray butyl rubber with 40 ml biomass; filled triangles; gray butyl rubber with 8 ml biomass; open squares, gray butyl rubber with water. The errors of triplicate measurements are within the symbols. The experiments were carried out in duplicate, and essentially the same results were obtained. (B) Methane oxidation and nitrite reduction to dinitrogen gas in duplicate batch incubations (40 ml, twofold-concentrated biomass, 40 ± 4 mg protein, gray butyl rubber stoppers). Filled black symbols, [^{15}N]nitrite (average of triplicate measurements; the error bars are within the symbols); large gray symbols, methane (average of duplicate measurements); open symbols, [^{15}N]dinitrogen gas (single measurement); small gray symbols, [^{15}N]nitrous oxide (single measurement).

known that some aerobic methanotrophs can be inhibited by such stoppers, but for anaerobic methanotrophs this was completely unexpected. This observation makes it clear that in future studies of denitrification coupled to methane oxidation it is essential to choose a suitable stopper material. Using thick (~0.8 mm) red butyl rubber stoppers manufactured for blood collection tubes or gray butyl rubber stoppers, we obtained specific activities of 1.6 to 2.2 nmol CH_4 min^{-1} mg $protein^{-1}$ and 4.7 to 5.1 nmol NO_2^- min^{-1} mg $protein^{-1}$ in small-batch incubations, which were indistinguishable from the values for the continuous culture (3.4 to 5.6 nmol NO_2^- min^{-1} mg $protein^{-1}$). The specific activities observed here also compare well with previously described activities of similar enrichment cultures (3.7 nmol NO_2^- min^{-1} mg $protein^{-1}$ [7, 27]) and cultures of anaerobic, archaeal methanotrophs (1.7 nmol CH_4 min^{-1} mg $protein^{-1}$ [24]). The observed stoichiometry of nitrite consumption versus methane consumption (8:3.5) was similar to the theoretical value, 8:3 (7, 27). No ammonium formation was detected (detection limit, 20 μM).

After 5 months of enrichment, FISH was successful using the previously described probes targeting denitrifying methanotrophic bacteria of the NC10 phylum. The amount of bacterial biomass relative to the amount of sediment particles had strongly increased, decreasing the autofluorescence. Individual NC10 cells were visible in a matrix of other bacteria. One month later, the NC10 bacteria had become the dominant bacteria, and the level of enrichment was about 70% after 7 months. In the small rods (ca. 0.8 to 1 by 0.3 to 0.5 μm) the 4′,6′-diamidino-2-phenylindole (DAPI) signal was concentrated in the center of each cell, and the cells occurred both as aggregates and as single cells (Fig. 4). Archaea were not de-

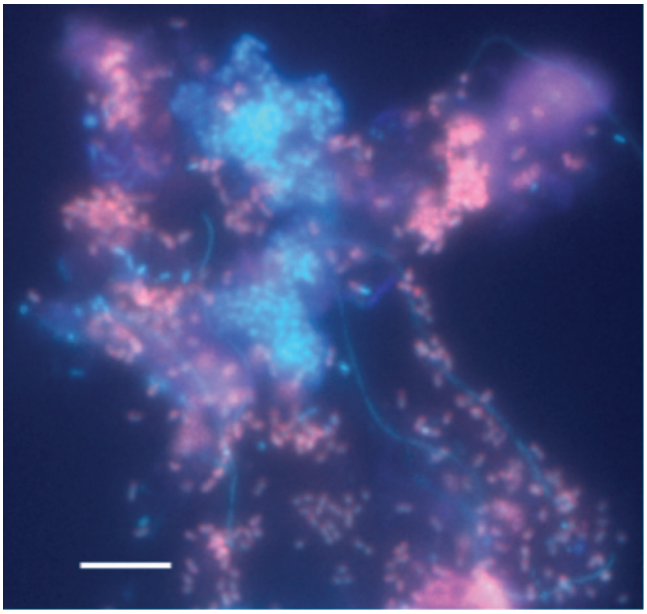

FIG. 4. FISH of biomass from the enrichment culture after 7 months. The cells were hybridized with probe S-*-DBACT-1027-a-A-18 (Cy3) (red) specific for NC10 bacteria (group a and part of group b); a mixture of probes EUB I-III and EUB V (Fluos) (dark blue), which detected nearly all eubacteria; and the DNA stain DAPI (cyan). NC10 bacteria appear pink due to double hybridization with the specific (red) and general (dark blue) probes. The DAPI signal is concentrated in the center of the cells. Essentially identical results were obtained with probe S-*-DBACT-0193-a-A-18. Scale bar = 5 μm.

tected at any time, confirming that they are dispensable for the nitrite-reducing, anaerobic oxidation of methane (7).

Even though NC10 phylum bacteria already accounted for more than 50% of the population in the enrichment culture, they were not detected in a clone library (31 clones) obtained after 6 months with general 16S rRNA gene primers (primers 8F and 1545R [12]). Instead, this library was dominated by uncultured *Acidobacteria* (11 clones) and *Chloroflexi* (10 clones) (data not shown). In order to explore the diversity of NC10 bacteria, we used combinations of general bacterial 16S rRNA gene primers and specific primers (Table 1) with DNA extracted from the inoculum and the enrichment culture. With this approach, most sequences obtained were affiliated with the NC10 phylum. As expected, their diversity in the inoculum was much greater than that in the enrichment culture after 6 months. The sequences amplified from the inoculum represented two groups, *a* and *b* (Fig. 1), but after 6 months only group *a* sequences were retrieved. This group also includes the strain previously enriched from the Twentekanaal (27) and several sequences from other freshwater environments (e.g., denitrifying zones of Lake Biwa sediments [17] and the Doñana aquifer [23] and methane-bearing sediment from Lake Washington [13]) and seems to be associated with anaerobic methane oxidation. Whether this is also the case for group *b*, many members of which were also found in the inoculum used, remains to be determined. Still, all sequences of the NC10 phylum known to date (Fig. 1) come from aquatic, potentially anoxic, methane-bearing environments. Very likely, these bacteria are underrepresented in biodiversity surveys performed with general primers, since they were not detected with 16S rRNA gene clone libraries even when they made up the majority of the microbial community. The cause of this strong negative bias is unclear. Our results indicate that the general primers used did not have mismatches with the targeted sequences. Also, there was no overrepresentation of the nucleotides A and T at the priming sites binding to the wobble bases of the degenerate forward primer, another factor shown to decrease amplification efficiency (26).

For the enriched group *a*, two primer pairs for qPCR were developed and used with samples collected throughout the enrichment procedure (Fig. 2). In negative controls, the cycle threshold value was more than 40 cycles, whereas the cycle threshold value of all samples was less than 29. The melting curve for the PCR products from the first 2 months had several peaks, indicating that there was formation of multiple products. This can be attributed to the high concentration of nontarget DNA in these samples. The first two measurements, therefore, are upper estimates and are less reliable than quantifications after day 71, when only one peak was observed. Nevertheless, the reproducible, twofold difference between the two primer sets remains difficult to explain. Both primer sets had no mismatches with the template 16S rRNA gene sequence, and the PCR efficiencies of the dilution curves were identical (100%). Other authors have found differences of up to 26% even with the same primer set (31), illustrating the limits of the qPCR approach. Multiple primer sets, like those used in the present study, do not necessarily generate more reliable results, but they can provide a more realistic view of the uncertainties of this technique and underline the need to confirm the findings with other non-PCR-based methods. However, the growth of the target population could be demonstrated by qPCR approximately 1 month before its activity became detectable (Fig. 2, inset).

Assuming one or two copies of the 16S rRNA gene per cell (a realistic estimate for slowly growing bacteria [16]), the specific per-cell activity was approximately 0.09 fmol CH_4 day^{-1} $cell^{-1}$ (for one copy) or 0.18 fmol CH_4 day^{-1} $cell^{-1}$ (for two copies) assessed with qPCR primer pair qP1. With primer pair qP2, this value was 0.20 fmol CH_4 day^{-1} $cell^{-1}$ (for one copy) or 0.40 fmol CH_4 day^{-1} $cell^{-1}$ (for two copies). These values are low, but given the tiny cell size (volume of roughly 0.05 to 0.2 μm^3), they are still on the same order of magnitude as the values for other slowly growing, anaerobic bacteria (e.g., 2.6 to 6.36 fmol NH_4 day^{-1} $cell^{-1}$ for anaerobic ammonium-oxidizing bacteria [20]). We also assessed the agreement between the number of cells and the protein content of the culture using the estimates of cell volume (0.05 to 0.2 μm^3) to determine the approximate protein content using the formula of Norland et al. (for the relationship between cell volume and dry weight [25]). If we multiplied the dry weight by the cell number obtained by qPCR (see above) and assumed that protein accounts for 50% of the dry weight, we obtained a theoretical content of 0.3 to 4.5 g for the enrichment culture. This matches the measured protein content, 4.75 ± 0.8 g, well, especially when the presence of other nontargeted bacteria is considered.

The steady increase in copy number from day 71 onward coincides with an increase in nitrite-reducing activity and biomass. Together, these results support the hypotheses that the denitrification observed in the first month of enrichment can be attributed to the oxidation of endogenous electron donors (decaying biomass, also indicated by the decreasing DNA content [Fig. 2]) present in the sediment, whereas the increase in biomass and nitrite-reducing activity observed from month 4 onward was predominantly due to the growth of bacteria belonging to NC10 group *a* using methane as an electron donor. Together with the results of previous studies (7, 27), these findings strongly support a general role for these organisms in the biochemically enigmatic process of anaerobic methane oxidation coupled to denitrification.

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