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Application of Recognition of Individual Genes-Fluorescence In Situ Hybridization (RING-FISH) To Detect Nitrite Reductase Genes (*nirK*) of Denitrifiers in Pure Cultures and Environmental Samples▽†

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Denitrification is an alternative type of anaerobic respiration in which nitrate is reduced to gaseous products via nitrite. The key step in this process is the reduction of nitrite to nitric oxide, which is catalyzed by two structurally different but functionally equivalent forms of nitrite reductase encoded by the *nirK* and *nirS* genes. Cultivation-independent studies based on these functional marker genes showed that in the environment there was a dominance of organisms with *nirK* and *nirS* genes presumably derived from organisms that have not been cultured yet. However, the phylogenetic affiliation of these organisms has not been resolved since the ability to denitrify is widespread in phylogenetically unrelated organisms. To unravel the phylogeny of the organisms from which the nitrite reductase (*nirK*) genes originated, one option is to use a special variant of whole-cell hybridization termed recognition of individual genes-fluorescence in situ hybridization (RING-FISH). In RING-FISH a multiply labeled transcript polynucleotide probe is used to detect a single gene on the bacterial chromosome during FISH. Here, RING-FISH was used with laboratory cultures and environmental samples, such as activated sludge. Furthermore, probe-based cell sorting using magnetic beads could also be carried out with mixtures of pure cultures, which led to effective depletion of the *nirK*-negative organism but capture of the *nirK*-positive organism, which was demonstrated by terminal restriction fragment length polymorphism analysis based on 16S rRNA genes. The results indicate that RING-FISH coupled with probe-based cell sorting could be used with environmental samples, which could provide a means for phylogenetic classification of *nirK*-type denitrifiers. Thus, the results of RING-FISH could increase our understanding of the phylogeny and function of denitrifying microorganisms in the environment.

Denitrification sensu stricto is the reduction of oxidized nitrogen compounds (nitrate and nitrite) with the production of nitrous oxide (N_2O) and/or dinitrogen (N_2). Nitrous oxide is a very potent greenhouse gas that is emitted into the atmosphere and has well-known climatic effects (10, 13). Denitrification and other nitrogen-converting processes in soils limit the availability of nitrate to plants by converting it to gaseous products and account for up to 70% of the total annual global N_2O emission (8, 21). On the other hand, the conversion to dinitrogen is beneficial in wastewater treatment because it removes nitrate and thus counteracts the eutrophication of receiving water bodies.

Most of the microorganisms responsible for denitrification are facultative anaerobes that use this process as an alternative anaerobic respiration pathway for energy conservation. This ability is widespread in a broad variety of phylogenetically unrelated organisms and has been found in more than 50 genera and 130 species belonging to the *Bacteria*, as well as the *Archaea* (33, 41). A common feature of denitrifiers is the conversion of nitrite to gaseous products that cannot be further assimilated by the organisms, which distinguishes true denitrifiers from nitrate reducers. Dissimilatory nitrite reduction is catalyzed by two structurally different but functionally equivalent enzymes (16), a copper-containing nitrite reductase and a cytochrome cd_1-containing nitrite reductase, which are encoded by the *nirK* and *nirS* genes, respectively. Due to the wide distribution of these genes in phylogenetically unrelated microorganisms and the resulting unsuitability of a 16S rRNA gene-based approach, nitrite reductase genes have been targeted frequently as functional marker genes for detection of denitrifiers in diverse environments. These environments included marine water columns (7, 29) and sediments (5, 25), sewage sludge (19), groundwater (39), and soil (6, 32), and the denitrifier communities were examined by using cultivation-independent PCR-based approaches. These studies revealed an enormous diversity of nitrite reductase genotypes that were only distantly related to the nitrite reductase genotypes of cultured denitrifiers. Some clusters in the *nir* gene trees are dominated by or consist exclusively of sequences from uncultured organisms. However, a limitation of this approach is the unresolved link between the functional gene diversity and the phylogenetic diversity of the organisms in the environment since at present it is not possible to infer phylogenetic relationships of the organisms based only on their nitrite reductase genes.

To unravel the phylogenetic affiliation of the key players in the denitrification process in the environment, two options are available. One option is to obtain isolates that are dominant in their habitats and thus relevant for nitrogen cycling in nature.

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However, the fraction of the environmental microbial fauna that can be cultured is generally very small (2). In addition, cultivation experiments are very labor-intensive and usually result in a limited number of isolates that are well adapted to the cultivation conditions but are often numerically and functionally irrelevant in their habitats. A different approach to obtain broader insight into the phylogenetic affiliation of members of denitrifier communities in the environment is to use a special variant of fluorescence in situ hybridization (FISH) called recognition of individual genes (RING)-FISH and to combine it with subsequent cell sorting. RING-FISH (or poly-FISH) involves using polyribonucleotide probes that are multiply labeled with several reporter molecules, and it is characterized by typical halo-shaped fluorescence signals in the periphery of the cells. These halo-shaped signals are hypothesized to occur due to folding of the single-stranded RNA-probe molecules into secondary structures (42), which results in the formation of a network of probes around the cells during whole-cell hybridization (38, 44). Polynucleotide probes were first used during in situ hybridization for identification of bacteria based on their 23S rRNA (38), but they also specifically hybridized to large-subunit rRNA in a variety of yeasts (44). Moreover, using multiply labeled 100-bp 16S rRNA-based polynucleotide probes allowed workers to identify and quantify archaeal and bacterial cells in marine plankton samples (12, 22, 31). Compared to the results obtained with conventional FISH using singly labeled oligonucleotide probes, the use of multiply labeled probes increased the intensity of the fluorescence signal and thus the sensitivity of RING-FISH between 10- and 50-fold (12, 38). Hence, the use of RING-FISH also allowed in situ detection of individual genes or gene fragments whose copy numbers in each cell were low, including genes or gene fragments in plasmids (10^1 to 10^3 copies) and even in chromosomal DNA (<10 copies) (46). For instance, the plasmid-carried beta-lactamase gene and the chromosomal glyceraldehyde-3-phosphate dehydrogenase gene in *Escherichia coli*, as well as the prepilin peptidase gene in *Xanthomonas campestris* (46), have been detected. Moreover, after hybridization of the probes to functional genes, cells can be specifically retrieved from cell mixtures (e.g., from enrichments or environmental samples) by polynucleotide probe-based cell sorting (35, 45). Retained cells can then be subjected to further analyses (e.g., amplification and cloning of their 16S rRNA genes) to determine the phylogenetic affiliations of the organisms.

In this study, we adapted the RING-FISH technique and used it for detection *nirK*-type nitrite reductase genes in denitrifier pure cultures, and we confirmed the specificity of the hybridization of polynucleotide probes to chromosomally located single-copy *nirK* genes by subsequent cell sorting using magnetic beads. The use of RING-FISH also allowed us to visualize *nirK*-containing microorganisms in environmental samples, such as activated sludge.

MATERIALS AND METHODS

Organisms. The strains used as *nirK*-positive control organisms were *Alcaligenes xylosoxidans* NCIMB 11015, *Hyphomicrobium aestuarii* DSM 1564, *Pseudomonas* sp. strain G-179 (40), and *Sinorhizobium meliloti* Rm20115. The following strains were used as *nirK*-negative controls: *Bacillus pumilus* DSM 27, *Desulfobacter postgatei* DSM 2034, *Hyphomicrobium zavarzinii* IFAM ZV-580 (20), and *Pseudomonas stutzeri* JM300 (9). Most strains were cultured aerobically at 25°C in the media recommended by the culture collections or by previous workers; the only exception was *D. postgatei*, which was grown anaerobically under an N_2-CO_2 (80:20, vol/vol) atmosphere at 30°C. Bacterial strains were grown to the exponential phase (optical density at 600 nm, 0.4 to 0.6) and then inoculated into fresh growth medium (5 to 10%) and incubated again. This procedure was repeated three or four times to obtain actively growing cells in similar growth phases.

Environmental samples and enrichments. Activated sludge was collected at the sewage treatment plant in Marburg-Cappel, Germany, in June 2007. Denitrifiers from 1 liter of activated sludge were enriched by adding (i) KNO_3 to a final concentration of 5 mM, (ii) KNO_3 and glucose to final concentrations of 5 mM and 5 μM, respectively, and (iii) KNO_3 and methanol to final concentrations of 5 mM and 25 μM, respectively. The enrichments were incubated anaerobically at room temperature in the dark for 4 weeks. Nitrate consumption was monitored weekly using the Merckoquant nitrate test (Merck, Darmstadt, Germany), and nitrate was replenished whenever it was consumed.

Cell fixation. Prior to cell fixation cells from pure cultures and activated sludge were treated as follows. Cells from pure cultures were collected in the exponential growth phase by centrifugation at 2,800 × *g* for 10 min, and each cell pellet was resuspended in phosphate-buffered saline (PBS) (137 mM NaCl, 10 mM Na_2HPO_4-KH_2PO_4, 2.7 mM KCl; pH 7.2). Cell aggregates from activated sludge (2 ml) were disrupted by pressing them through a fine needle (25 gauge; 0.5 by 25 mm) several times. Gram-negative cells and cells from environmental samples were fixed in paraformaldehyde, and gram-positive cells were fixed in 96% (vol/vol) ethanol as described previously (2).

DNA extraction and generation of polynucleotide transcript probes. Genomic DNA from pure cultures of *A. xylosoxidans*, *H. aestuarii*, *Pseudomonas* sp. strain G-179, and *S. meliloti* was extracted with phenol-chloroform (3). Templates for in vitro transcription were generated from the DNA extracts by PCR amplification of *nirK* gene fragments (515 bp) using PCR conditions and primers nirK1F and nirK5R described by Braker et al. (4) with the T3 promoter sequence attached to the reverse primer to allow initiation of transcription. Amplicons of the proper size were excised from agarose (1.5%, wt/vol) gels and purified using the Wizard SV gel and PCR clean up system (Promega, Mannheim, Germany). Polynucleotide transcript probes were generated by in vitro transcription and were simultaneously labeled by incorporating either biotin-16-UTP or digoxigenin-11-UTP (Roche Molecular Diagnostics) as described by Zwirglmaier et al. (44). The concentration of the polynucleotide probes was determined using a NanoDrop instrument (Thermo Fisher Scientific, Wilmington, DE).

In situ hybridization with polyribonucleotide transcript and oligonucleotide probes. Hybridizations were performed either with fixed cells immobilized on Teflon-coated glass slides (MAGV, Rabenau-Londorf, Germany) or with cells in solution. For hybridizations on glass slides, aliquots (5 to 10 μl) of fixed cells in suspension (approximately 5×10^4 cells), of fixed cells from enrichments, or of activated sludge were subjected to the hybridization procedure described by Zwirglmaier et al. (44). For hybridizations in solution, aliquots (30 μl) of fixed cells from pure cultures were treated as described by Zwirglmaier et al. (45). All hybridizations were performed for 24 h using a formamide concentration of 15% in the hybridization buffer and 4 and 5 μg of polynucleotide probe for hybridizations on glass slides and in solution, respectively. After hybridization in solution, cells were collected by centrifugation at 8,000 × *g* for 3 min, resuspended in 100 μl washing buffer (150 mM NaCl, 100 mM Tris-HCl [pH 8.0], 10% [wt/vol] sodium dodecyl sulfate; pH 7.4), and washed at 53°C for 30 min to remove the unbound polynucleotide probe. Cells were again collected by centrifugation at 8,000 × *g* for 3 min and resuspended in 15 μl PBS.

After hybridization with polynucleotide probes, the biotin or digoxigenin label of the polynucleotide probes was detected. Biotin-labeled probes were detected with a streptavidin-Cy3 conjugate (Sigma Aldrich), and digoxigenin-labeled probes were detected with anti-digoxigenin-Fab fragments (Roche Molecular Diagnostics) coupled to fluorescein according to the manufacturer's recommendations. All hybridization preparations were visualized by fluorescence microscopy (Axiophot; Carl Zeiss Microimaging GmbH, Göttingen, Germany) at wavelengths of 570 and 523 nm to detect the Cy3 and fluorescein labels, respectively.

In mixtures of *nirK*-positive and *nirK*-negative strains, cells were differentiated prior to detection by labeling the *nirK*-negative organisms by whole-cell hybridization with Cy3-labeled 16S rRNA-targeted oligonucleotide probes, as described previously (34). Cells from environmental samples were hybridized by performing whole-cell hybridization with 16S rRNA-targeted probes EUB338I to EUB338III (1, 11).

Cell sorting using magnetic beads. For cell sorting experiments approximately 6×10^7 cells of mixed *nirK*-positive and -negative strains were used for hybridization in solution with 8 μg polynucleotide probe in 30 μl hybridization buffer. Hybridizations with polynucleotide probes were performed under conditions

described above. Subsequently, cell sorting was performed in lieu of the detection procedure. For this, 100 μl of Dynabeads pan mouse immunoglobulin G with an antidigoxigenin epitope (4×10^8 beads ml^{-1}; Invitrogen, Karlsruhe, Germany) was used. First, the beads were washed with 1 ml PBS and 0.1% bovine serum albumin (BSA) in a 1.5-ml Eppendorf tube by placing it into a MagneSphere magnetic separation stand (Promega, Mannheim, Germany) and removing the supernatant after the beads were collected on one side of the tube. Then the beads were resuspended in 100 μl PBS–0.1% (vol/vol) BSA. To 15 μl of each of the pure-culture mixtures 600 μl PBS was added, and this suspension was added to the washed beads. Cells and beads were incubated for 30 min on a rotary shaker at 100 rpm at room temperature to allow binding of cells to the beads via the digoxigenin label of the polynucleotide probe that had hybridized. Then the beads with bound cells were collected by placing the Eppendorf tube in the magnetic separation stand for 1 min, and the supernatant containing cells to which the probe had not hybridized was removed. The beads were washed four times with 1.5 ml PBS–0.1% BSA and were finally resuspended in 20 μl PBS.

T-RFLP analysis. DNA was released from the cells bound to the beads by a freeze-thaw procedure. Then an aliquot (4 μl of a bead suspension) was subjected to PCR amplification of bacterial 16S rRNA genes using a protocol and primers described previously (3a). PCR products were purified using the Wizard SV gel and PCR clean up system (Promega). For terminal restriction fragment length polymorphism (T-RFLP) analysis aliquots (100 ng) of the purified PCR product were cleaved overnight at 37°C with 3 U of restriction endonuclease HhaI in the manufacturer's recommended reaction buffer using a reaction volume of 10 μl. After purification using Autoseq G-50 columns (Amersham Pharmacia Biotech Inc., Piscataway, NY), an aliquot (3 μl) was analyzed with a 3130 automated sequencer by comparing the sizes of the peaks to an internal standard (MapMarker 1000; 30 to 1,000 bp; BioVentures Inc., Murfreesboro, TN). The cell sorting efficiency was quantified based on the relative area of peaks indicative of a given *nirK*-negative pure culture by comparing the peak areas before and after cell sorting, as follows: ([relative peak area of nontarget cells before cell sorting − relative peak area of nontarget cells after cell sorting]/relative peak area of nontarget cells after cell sorting) × 100.

RESULTS

Optimization of hybridization conditions. Polynucleotide probes were generated by in vitro transcription of a 515-bp amplicon of the nitrite reductase (*nirK*) gene of four denitrifier strains, *A. xylosoxidans* NCIMB 11015, *H. aestuarii* DSM 1564, *Pseudomonas* sp. strain G-179, and *S. meliloti* Rm20115. For in situ hybridizations, these probes were used either individually or as equimolar mixtures of all four probes. Initially, the optimal hybridization conditions for use of each individual probe were evaluated. Therefore, the stringency of the hybridization was varied by using different formamide concentrations in the hybridization buffer (5, 10, 15, 20, and 25%) and by estimating the number of cells that showed the halo-shaped fluorescence signal typical of RING-FISH. For all probes and *nirK*-type denitrifier strains, the largest fraction of cells (98 to 100% of the cells spotted on the slide) was detected using the homologous probe for each pure culture with a formamide concentration of 15% (Fig. 1; see also Fig. S1 to S4A in the supplemental material). With concentrations less than and greater than this concentration lower fractions of halos (with 5% formamide, approximately 50%; with 10% formamide, approximately 80%; with 20% formamide, approximately 60%; and with 25% formamide, approximately 10%) were observed for all probes (see Fig. S1 to S4A in the supplemental material). In addition, with a formamide concentration of 15% in the hybridization buffer none of the negative controls (*B. pumilus* DSM 27, *D. postgatei* DSM 2034, *H. zavarzinii* IFAM ZV-580, and *P. stutzeri* JM300) showed any halo-shaped fluorescence signal (see Fig. S5 in the supplemental material [data for *D. postgatei* not shown]). The small point-shaped fluorescent signals that occurred in large aggregates of *H. zavarzinii* cells were nonspecific signals that were not removed by the washing procedure. It is noteworthy that in experiments with a given stringency *nirK*-positive and -negative cells were hybridized on the same slide using identical solutions and hybridization conditions. The same results (i.e., almost quantitative detection and specificity of the probes with 15% formamide) were obtained when a mixture of the homologous probe and three heterologous probes was used with *nirK*-positive and -negative control strains (see Fig. S1 to S4A in the supplemental material). As a consequence, a formamide concentration of 15% was used to adjust the stringency conditions for all further hybridization experiments with pure cultures, as well as with environmental samples.

Mixtures of *nirK*-positive and -negative strains. RING-FISH for detection of *nirK*-type denitrifiers was also used for hybridizations with mixtures of one *nirK*-positive strain and one *nirK*-negative strain by using the polynucleotide probes individually or equimolar mixtures of all four probes. This was done by mixing cells of morphologically distinct strains (e.g., *A. xylosoxidans* and *H. zavarzinii* strains or *H. aestuarii* and *P. stutzeri* strains) or, alternatively, by mixing cells of strains with similar morphologies. In the latter case nondenitrifiers were discriminated from *nirK*-type denitrifiers by using specific oligonucleotide probes (LGC0355 for *B. pumilus*, which is specific for *Firmicutes* [17]; Hypho-1241 for *H. zavarzinii* ZV-580, which is specific for *Hyphomicrobium* spp. [24]; and GAM42a for *P. stutzeri*, which is specific for *Gammaproteobacteria* [27]) to counterstain nondenitrifier cells. Most possible combinations of *nirK*-positive and -negative pure cultures were tested; the exceptions were combinations with *D. postgatei* due to the high background fluorescence of this strain. RING-FISH labeled the *nirK*-type denitrifiers exclusively, while hybridizations using oligonucleotide probes stained only the *nirK*-negative control strains. For instance, when mixtures of the *nirK*-type denitrifier *A. xylosoxidans* and the nondenitrifier *B. pumilus* or *H. zavarzinii* were used, the denitrifier showed the halo-shaped RING-FISH fluorescence signal, while the nondenitrifiers were detected by the specific oligonucleotide probes (Fig. 2). No difference in specificity was observed when the homologous probes were used individually or in combination with the three heterologous probes.

Cell sorting. Hybridization using *nirK* polynucleotide probes was used as the initial step for sorting cells in mixtures of *nirK*-positive and -negative strains. Generally, cell sorting is performed with cells in suspension, and it was done with a mixture of four probes in this case. Given that the specificity and efficiency were reproducible for hybridizations performed on glass slides, as well as in solution, hybridizations were carried out with mixtures of two cultures, a *nirK*-positive pure culture and a *nirK*-negative pure culture. The mixtures included most of the possible combinations of *nirK*-positive and -negative pure cultures used in this study. They were prepared with *nirK*-negative cells comprising a large fraction of the total cell count (between 75 and 91%) and the *nirK*-positive organism comprising the remaining fraction (Table 1 and Fig. 3). Cell sorting was achieved by magnetically capturing cells that had hybridized with digoxigenin-labeled polynucleotide probes and by removing the supernatant containing unbound cells and thereby supposedly depleting *nirK*-negative cells. Then 16S rRNA genes were amplified from cells coupled to the beads,

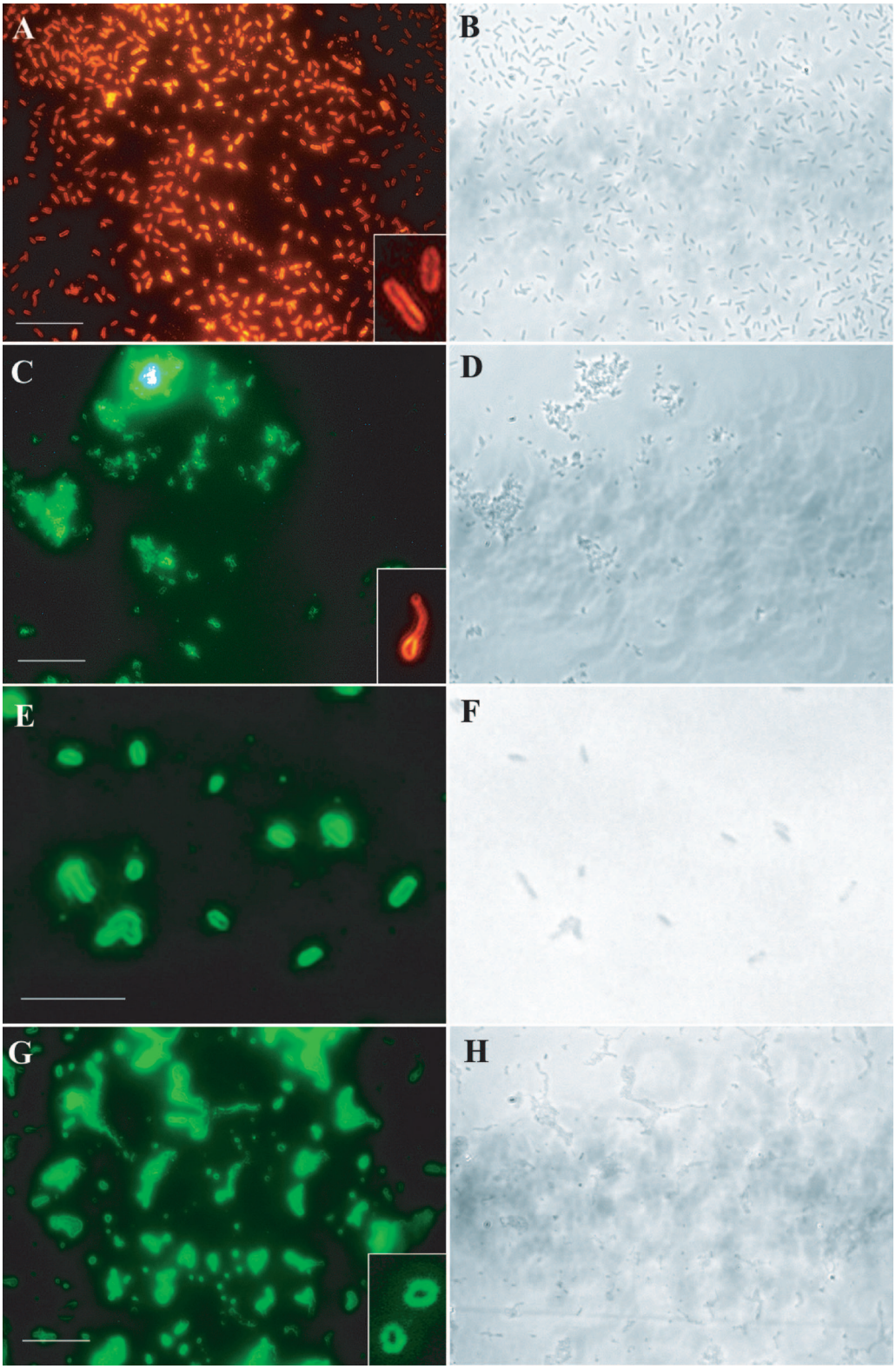

FIG. 1. Detection of nitrite reductase (*nirK*) genes in *nirK*-positive pure cultures by RING-FISH. Cells were hybridized with the homologous *nirK* polynucleotide probes and labeled with Cy3 (red fluorescence) or fluorescein (green fluorescence). (A and B) *A. xylosoxidans* NCIMB 11015. (C and D) *H. aestuarii* DSM 1564. (E and F) *Pseudomonas* sp. strain G-179. (G and H) *S. meliloti* Rm20115. The insets show the halo shape of the fluorescence signal. Panels B, D, F, and H are phase-contrast images. Bars = 10 μm.

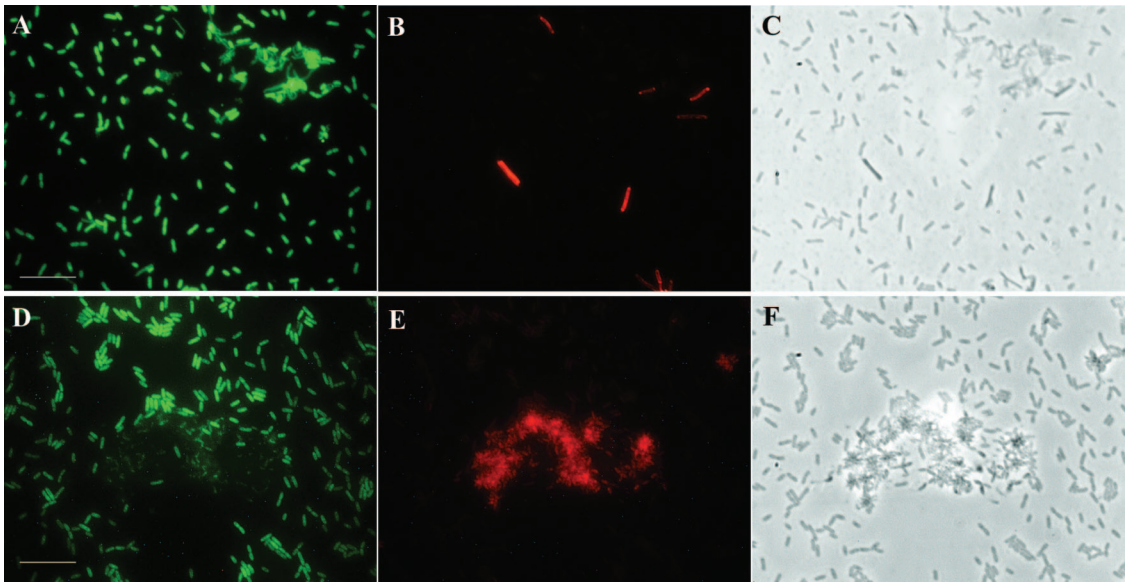

FIG. 2. Discrimination of *nirK*-type denitrifiers and nondenitrifiers in mixtures of pure cultures by RING-FISH. Denitrifier cells were hybridized with *nirK* polynucleotide probes, and nondenitrifiers were counterstained by FISH using Cy3-labeled oligonucleotide probes. (A to C) Mixture of the denitrifier *A. xylosoxidans* NCIMB 11015 and the nondenitrifier *B. pumilus* DSM 27. (D to F) Mixture of the denitrifier *A. xylosoxidans* NCIMB 11015 and the nondenitrifier *H. zavarzinii* ZV-580. The organisms were labeled with fluorescein after RING-FISH (A and D) and with Cy3 after FISH (B and E). Panels C and F are phase-contrast images. Bars = 10 μm.

and the amplicons were analyzed by T-RFLP analysis. For the T-RFLP analysis the restriction endonuclease HhaI was used, which resulted in terminal restriction fragments that were of distinct lengths for the *nirK*-positive and -negative pure cultures in the mixture. Terminal restriction fragment lengths (the theoretical lengths for *nirK*-positive organisms were 565 bp for *A. xylosoxidans*, 336 bp for *H. zavarzinii*, and 56 bp for *Pseudomonas* sp. and *S. meliloti*; the theoretical lengths for *nirK*-negative organisms were 240 bp for *B. pumilus*, 206 bp for *P. stutzeri*, and 97 bp for *D. postgatei*) were determined theoretically and experimentally. Some bacterial strains have different 16S rRNA gene copy numbers, which may result in different relative peak areas even if equal amounts of cells are used. Hence, the efficiency of cell sorting (the fraction in which the *nirK*-negative organism was depleted from the mixture) was estimated by calculating the ratio of the relative areas of the peaks specific for the organisms before and after cell sorting.

TABLE 1. Cell depletion efficiency of *nirK*-negative strains in mixtures of *nirK*-positive and -negative strains after cell sorting

nirK-positive strain	% *nirK*-negative cells in all cells/% depletion of *nirK*-negative strain with:			
	B. pumilus DSM 27	*D. postgatei* DSM 2034	*H. zavarzinii* IFAM-ZV580	*P. stutzeri* JM300
A. xylosoxidans NCIMB 11015	75/99.9	80/99.8	ND[a]	80/98.6
H. aestuarii DSM 1564	75/100	ND	ND	91/98.6
Pseudomonas sp. strain G-179	ND	83/100	75/100	83/92.5
S. meliloti Rm20115	ND	83/100	91/99.6	ND

[a] ND, not determined.

For all mixtures of strains used here effective cell sorting was obtained independent of the pure cultures chosen. The efficiency of depletion of the *nirK*-negative strain in the mixtures varied from 92 to 100% and thus was generally high. It was also largely independent of the ratios of *nirK*-positive and -negative cells, as observed, for instance, when 90, 75, 50, 25, and 10% *D. postgatei* was mixed with the *nirK*-type denitrifier *A. xylosoxidans* (Fig. 3). The efficiency of depletion for these mixtures was around 90% if the fraction of *nirK*-positive cells was higher than 50%. Thus, it was in a range (>92%) similar to the range observed for all other mixtures. However, in contrast to our initial attempts, in this experiment an exceptional lower effi-

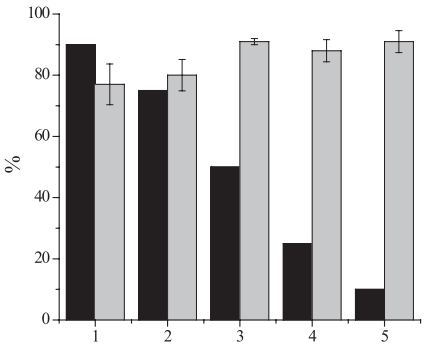

FIG. 3. Cell sorting efficiency for mixtures of the *nirK*-type denitrifier *A. xylosoxidans* NCIMB 11015 and the nondenitrifier *D. postgatei* DSM 2034. Depletion of *D. postgatei* was determined by T-RFLP analysis of PCR-amplified 16S rRNA genes of cells captured after hybridization with *nirK* polynucleotide probes. Black bars, fractions of the nondenitrifier in mixtures of denitrifier and nondenitrifier pure cultures; gray bars, fractions of the nondenitrifier depleted from mixtures. The error bars indicate standard deviations ($n = 3$).

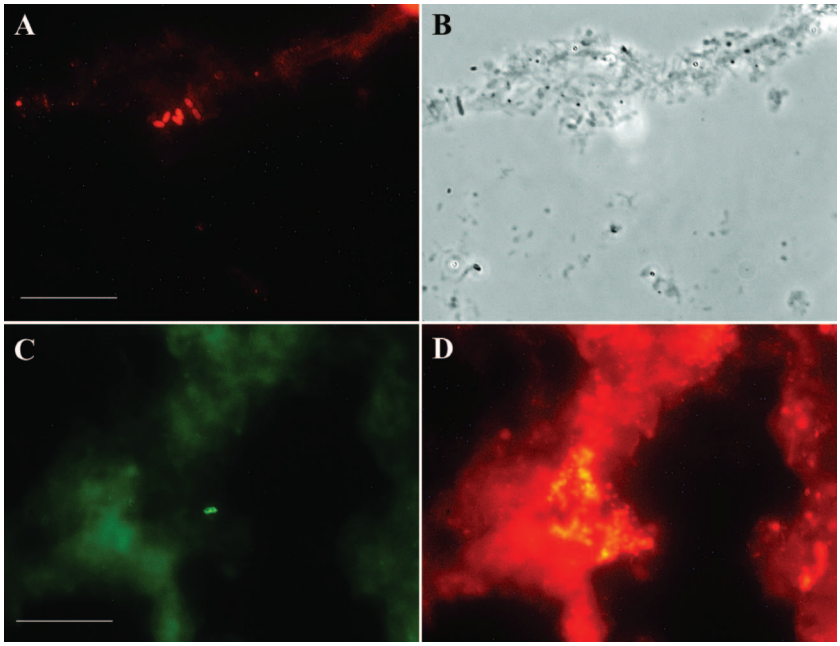

FIG. 4. Detection of *nirK*-type denitrifiers in activated sludge by using RING-FISH with *nirK* polynucleotide probes. (A and C) Halo-shaped fluorescence signals of Cy3-labeled denitrifiers. (B) Floc in phase-contrast micrograph. (D) Bacterial cells in sludge stained by oligonucleotide probes EUB338I to EUB338III. Bars = 10 μm.

ciency of depletion (approximately 80%) was observed for mixtures of these two strains when *nirK*-negative cells accounted for more than 50% of all cells.

Activated sludge. Denitrifiers were also detected in activated sludge by using RING-FISH to target *nirK* genes. Halo-shaped signals in the periphery of mainly rod-shaped organisms were detected for only few cells in the floc (Fig. 4). Additional evidence for successful use of RING-FISH to detect *nirK*-type denitrifiers in environmental samples was provided by the detection of increased numbers of halo-shaped signals after enrichment of denitrifiers in activated sludge. Enrichments were grown with nitrate as an electron acceptor either using the prevalent carbon sources or after addition of glucose or methanol as an additional carbon source. For RING-FISH with activated sludge and enriched sludge, a mixture of *nirK* polynucleotide probes for all four *nirK*-type denitrifiers was used. In fresh sludge (1 μl) an average of 54 halos were detected using either the digoxigenin–anti-digoxigenin antibody with the fluorescein label or the biotin-streptavidin conjugate labeled with Cy3 (Fig. 5). After 4 weeks of enrichment, the number of halos increased between five- and ninefold. A fivefold increase in the number of halos was observed for the enrichment with nitrate only, and a ninefold increase occurred in the enrichment supplemented with methanol.

DISCUSSION

Evaluation of optimum hybridization conditions. In this study, we adapted use of polyribonucleotide probes to specifically label and detect *nirK*-type denitrifiers based on their *nirK* genes. These genes are chromosomal single-copy genes coding for copper-containing nitrite reductase that are commonly used as functional marker genes to detect this type of denitrifier in environmental samples by cultivation-independent approaches (25, 29, 32, 36). Initially, the hybridization conditions were optimized using *nirK*-positive and -negative pure cultures to obtain specific and sensitive results. Polynucleotide probes were generated for four denitrifiers whose partial *nirK* sequences were 72 to 85% identical to each other and clustered with the sequences of other cultured denitrifiers in the *nirK* gene tree (see Fig. S6 in the supplemental material). The probes comprised a 515-bp fragment of the *nirK* gene, which was within the range of lengths (50 to 800 nucleotides) that were found to work for RING-FISH (44). In previous studies halo formation was observed only at high probe concentrations

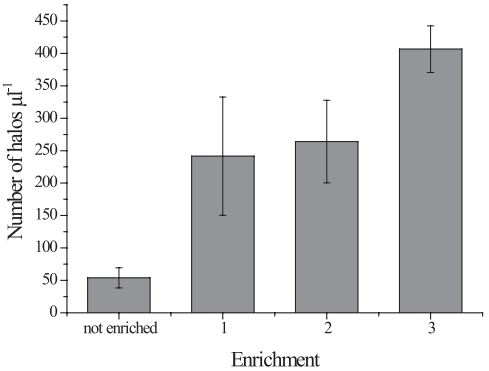

FIG. 5. Enrichment of *nirK*-type denitrifiers in activated sludge detected by RING-FISH using *nirK* polynucleotide probes. Denitrifiers were visualized in nonenriched sludge and in sludge enriched anaerobically for 4 weeks. Bar 1, sludge supplemented with 5 mM nitrate; bar 2, sludge supplemented with 5 mM KNO_3 and 5 μM glucose; bar 3, sludge supplemented 5 mM KNO_3 and 25 μM methanol. The error bars indicate standard deviations (n = 2).

(200 to 250 ng/μl) (44), which is also consistent with the concentrations used here (300 and 160 ng/μl for hybridization on glass slides and in solution, respectively).

The halo-shaped fluorescence signal is believed to result from a network of probes formed in the periphery of the cell that is anchored via hybridization of a single probe molecule to a gene on the chromosome (44). Network formation depends on the tendency of the single-stranded RNA probes to fold into secondary structures under certain hybridization conditions (44). As indicated by in silico analysis of the *nirK* polynucleotide probe sequences using RNAdraw V1.1b2 (www.rnadraw.com/), all probes had low energy values (the ΔG values were between −69 and −88 kcal/mol with 15% formamide) and thus had a strong tendency to form secondary structures (see Fig. S1 to S4 in the supplemental material). The optimum stringency conditions for specific hybridizations were evaluated empirically by varying the formamide concentration in the hybridization buffer. Highly efficient hybridization of the probes was observed when a hybridization temperature of 53°C and 15% formamide in the hybridization buffer were used. The hybridization efficiency was independent of the use of an individual homologous probe or an equimolar mixture of probes for all four strains. Consistently, quantitative hybridization was observed only for cells in the exponential growth phase, indicating that there may have been differences in the cell wall structure or DNA accessibility during different developmental stages for the individual cells (43, 46). However, only the basic RING-FISH protocol was used without any modifications to further permeabilize the cell walls of the organisms, such as treatments with lysozyme, lysostaphin, or mutanolysin, as suggested recently (14). Under stringency conditions that were lower and higher than the optimum conditions fewer *nirK*-positive cells were labeled. At least for *Pseudomonas* sp. and *S. meliloti* this finding agrees with the significant shift in the formation of specific secondary structures in silico when the formamide concentration was increased from 15 to 20% (see Fig. S1 to S4 in the supplemental material). Generally, the lowest hybridization efficiency with 25% formamide correlated with the most condensed secondary structures of the mRNA molecules, suggesting that these structures are not accessible for network formation.

Specificity of hybridizations. The specificity of the polynucleotide probes was confirmed by RING-FISH and *nirK* polynucleotide probe-based cell sorting of *nirK*-positive and -negative pure cultures. Under optimum hybridization conditions probes exclusively labeled the *nirK*-positive cells, but with lower formamide concentrations nonspecific fluorescence signals of *nirK*-negative cells were also detected during RING-FISH, although this was never observed for *B. pumilus*, probably due to impermeability of the gram-positive cell wall. However, nonspecific hybridizations with the probes did not result in the typical halo-shaped signal; rather, the signals resembled signals obtained by conventional oligo-FISH and thus stained the entire cell. This suggests that no probe network was formed in the periphery of the cells. The specificity of the signals was not affected by using either directly labeled fluorescent probes or the indirect method that included a separate step to detect digoxigenin and biotin reporter molecules with a fluorescently labeled anti-digoxigenin antibody and streptavidin conjugate, respectively. No signals were observed when the conjugates were used without prior hybridization of the probes, thus confirming that the signal was dependent on previous hybridization of the probes.

Since the indirect detection method and polynucleotide probe-based cell sorting share initial steps, we focused on using the latter approach. The results of cell sorting experiments using magnetic beads and subsequent analysis of the 16S rRNA genes of denitrifier-nondenitrifier mixtures also indicated the specificity of the hybridization. Generally, high efficiency (80 to 100%) was observed for depletion of the *nirK*-negative strain from mixtures, which was largely independent of the ratio of *nirK*-positive and -negative cells in the mixture, indicating that there was successful enrichment of denitrifiers based on their *nirK* genes. We initially attempted to sort cells based on the hybridization of the *nirK* polynucleotide probe network to DNA-coated microplates, as described previously by Zwirglmaier et al. (45). However, the specificity of cell separation was significantly lower using this technique compared to the specificity of the very specific depletion of *nirK*-negative cells using digoxigenin as a reporter molecule and magnetic beads to capture cells to whose DNA the probes had hybridized.

Finally, hybridization of sense polynucleotide probes to mRNA was not tested because cultures were kept under nondenitrifying conditions. With few exceptions (23), the denitrification process is triggered by the absence of oxygen and the presence of oxidized nitrogen compounds; thus, expression of denitrification genes is unlikely and also should not hamper the enrichment of *nirK*-type denitrifiers from environmental samples by probe-based cell capture.

Detection of *nirK*-positive organisms from environmental samples. There was effective and specific detection of *nirK* genes in pure cultures, as shown above, and *nirK*-type denitrifiers were also detected in environmental samples (activated sludge) by RING-FISH. RING-FISH was used directly with fixed activated sludge, and cells with the typical peripheral halo-shaped fluorescence signal surrounded by the vast majority of other (bacterial) cells not showing any fluorescence were detected in the flocs. The potential ability to detect *nirK*-type denitrifiers by RING-FISH was further shown by the results obtained with 25 *nirK* clones that were obtained from the activated sludge sample. The sequences clustered exclusively in the vicinity of the probe sequences in the *nirK* gene tree; hence, the results indicate that denitrifiers with similar genotypes were dominant in this habitat (see Fig. S6 in the supplemental material). Moreover, increased numbers of cells with halos after enrichment for denitrifiers by addition of the electron acceptor nitrate or nitrate and glucose or methanol as an additional carbon source provided further evidence that there was specific detection of *nirK*-type denitrifiers in the sludge. The highest numbers of halos were found in the methanol-fed enrichment. Methanol is frequently used in sewage treatment systems to enhance denitrification rates due to its low cost, and it has been found to cause shifts in the overall denitrifier community and, more specifically, in the *nirK*-type denitrifier community (15, 18). In contrast to previous cultivation-based work (28, 37), these more recent studies suggest that there is dominance of populations other than *Hyphomicrobium* spp. and *Paracoccus* spp. that are specialized to utilize this C_1 compound. On the other hand, in another study the workers re-

trieved *nirK* sequences related to the sequences of *H. zavarzinii* and *Rhizobium hedysari* from the active methanol-assimilating nitrate-reducing bacterial populations in activated sludge (30). However, it is important to state that based on separate analyses of *nirK* or 16S rRNA gene phylogeny it is premature to reach conclusions concerning the phylogenetic affiliation of denitrifiers or to infer that microorganisms are able to denitrify, respectively. Thus, hybridization using polynucleotide probes combined with conventional oligo-FISH or *nirK*-based cell sorting with subsequent 16S rRNA gene analysis has the potential to help resolve the unknown link between functional diversity and phylogenetic diversity. Polynucleotide probes are less specific than oligonucleotides and allow discrimination of prokaryotic groups separated by large evolutionary distances. For instance, archaeal and bacterial groups that are less than 75 and 70% related based on their 16 and 23S rRNA genes, respectively, could be discriminated (26). This agrees with our findings. Analyses with the *nirK* polynucleotide probes used in this study did not detect pure cultures with more distantly related *nirK* genes (<72%) by RING-FISH (unpublished data). This suggests that *nirK* polynucleotide probes specific for different clusters in the gene tree could be designed, allowing more focused analysis of the genetic background. However, two prerequisites for successful cell sorting for specifically retrieving microorganisms from environmental samples based on detection of functional low-copy-number genes are breaking up cell aggregates and improving the accessibility of the probes to target organisms (for instance, by using special pretreatment protocols) (14).

In summary, RING-FISH and polynucleotide probe-based cell sorting were successfully adapted to specifically and effectively detect *nirK*-type denitrifiers in pure cultures and also in environmental samples. Thus, a method using specific probes to selectively enrich these organisms is now available to obtain more insight into the phylogenetic affiliation of denitrifiers that are numerically dominant and thus relevant for denitrification in the environment. However, we believe that the entire procedure (RING-FISH combined with subsequent cell sorting) needs to be refined further for specific and more quantitative retrieval of denitrifiers from complex samples.

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