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Development of PCR Primer Systems for Amplification of Nitrite Reductase Genes (*nirK* and *nirS*) To Detect Denitrifying Bacteria in Environmental Samples

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A system was developed for the detection of denitrifying bacteria by the amplification of specific nitrite reductase gene fragments with PCR. Primer sequences were found for the amplification of fragments from both nitrite reductase genes (*nirK* and *nirS*) after comparative sequence analysis. Whenever amplification was tried with these primers, the known *nir* type of denitrifying laboratory cultures could be confirmed. Likewise, the method allowed a determination of the *nir* type of five laboratory strains. The *nirK* gene could be amplified from *Blastobacter denitrificans*, *Alcaligenes xylosoxidans*, and *Alcaligenes* sp. (DSM 30128); the *nirS* gene was amplified from *Alcaligenes eutrophus* DSM 530 and from the denitrifying isolate IFAM 3698. For each of the two genes, at least one primer combination amplified successfully for all of the test strains. Specific amplification products were not obtained with nondenitrifying bacteria or with strains of the other *nir* type. The specificity of the amplified products was confirmed by subsequent sequencing. These results suggest the suitability of the method for the qualitative detection of denitrifying bacteria in environmental samples. This was shown by applying one generally amplifying primer combination for each *nir* gene developed in this study to total DNA preparations from aquatic habitats.

Denitrification is a dissimilatory process of bacteria in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production. The gaseous end products NO, N_2O, and N_2 are released concomitantly. In the environment, denitrification is responsible for the release of fixed nitrogen into the atmosphere in form of N_2 (13). It causes major nitrogen losses in agricultural soils to which fertilizers are applied. Accumulation of the greenhouse gases NO and N_2O leads to the destruction of the ozone layer (3, 13). Also, denitrifying bacteria cause the removal of nitrogen compounds from waste water, where denitrification is coupled to the nitrification process (13). Bioremediation of environmental pollutants can be achieved under denitrifying conditions (5, 10, 33).

Denitrifying bacteria are phylogenetically diverse. They belong to all major physiological groups except for the *Enterobacteriaceae*, obligate anaerobes, and gram-positive bacteria other than *Bacillus* spp. (34). Defined as a physiological group, these facultative anaerobes can switch from oxygen to nitrogen oxides as terminal electron acceptors when kept under anoxic conditions. Nitrite reductase is the key enzyme in the dissimilatory denitrification process. The reduction of nitrite to NO can be catalyzed by the products of two different nitrite reductase genes: one product contains copper (the *nirK* product), and the other contains cytochrome cd_1 (the *nirS* product). The two genes seem to occur mutually exclusively in a given strain, but both types have been found in different strains of the same species (4). Although structurally different, both enzyme types are functionally and physiologically equivalent (9, 35). *nirS* is more widely distributed; *nirK* is found in only 30% of the denitrifiers studied so far. However, *nirK* is found in a wider range of physiological groups (4). Several different approaches were used to determine the type of nitrite reductase in laboratory pure cultures. Diethyldithiocarbamate has been used to identify *nirK*-containing denitrifiers (21). Very specific detection, mostly at the strain level, could be achieved with antisera against dissimilatory nitrite reductase (dNirS [4, 24, 29] and dNirK [4, 17]). Another approach was the use of gene probes for *nirK* (12, 32) or *nirS* (12, 15, 24, 29), which were generally specific for the strains investigated. Weak reactivity also occurred for the *nirK* gene probe with DNA from some of the other *nir*-type denitrifiers (32); the *nirS* probe, on the other hand, hybridized with a more limited variety of strains (24, 30). A PCR method with one primer pair to target the *nirS* nitrite reductase gene showed higher specificity than hybridization experiments (30).

In the present study, we report on the application of new primer systems for both types of nitrite reductase genes. We used several different primer pairs to determine the *nir* type of denitrifying strains. Using samples from aquatic habitats, we amplified *nir* fragments and used the most reliable primer pairs for *nirK* or *nirS*, respectively, to successfully detect, in these aquatic samples, different populations of denitrifying bacteria.

MATERIALS AND METHODS

Bacteria and growth conditions. A variety of denitrifying and nondenitrifying bacterial strains (see Tables 2 and 3) were used to evaluate the specificity of designed PCR primers. All strains were grown aerobically at 27°C. For genomic DNA isolation, *Pseudomonas*, *Alcaligenes*, *Ochrobactrum*, *Paracoccus*, and *Azospirillum* strains and the denitrifying isolate IFAM 3698 were grown on nutrient broth (NB; Merck, Darmstadt, Germany). *Rhizobium* strains were grown on yeast extract medium (YEM [27]). *Hyphomicrobium zavarzinii* IFAM ZV-622^T was grown on 337-B1 medium (7) with 0.5% (vol/vol) methanol. *Rhodobacter sphaeroides* f. sp. *denitrificans* was grown on trypticase soy broth (TSB; Difco Laboratories, Detroit, Mich.), *Roseobacter denitrificans* was grown on oligotrophic medium (PYGV [25]) supplemented with 25‰ artificial seawater (16), and *Blastobacter denitrificans* was grown on peptone yeast extract glucose medium, i.e., PYGV without vitamins. Nondenitrifying strains of the *Enterobacteriaceae* were grown on Luria broth (LB [19]).

Extraction of genomic DNA. Genomic DNA was obtained from pure cultures by lysozyme-proteinase K-sodium dodecyl sulfate (SDS) treatment followed by

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phenol-chloroform extraction and subsequent ethanol precipitation (8). The purity and concentration of the DNA preparations were determined spectrophotometrically.

Preparation of total DNA from an enrichment culture and four environmental samples. DNA was prepared from five samples. (i) A 500-ml volume of medium 337-B1 with 0.5% (wt/vol) KNO_3 for the enrichment of denitrifying methylotrophic bacteria was inoculated with 100 μl of activated sludge from a sewage treatment plant near Plön (Schleswig-Holstein, Germany). After 4 weeks at 28°C under anaerobic conditions, 500 μl of the enrichment was again inoculated and kept under the same growth conditions. Six months later, cells were harvested by centrifugation (6,000 × *g* for 60 min at 4°C) and resuspended in 400 μl of double-distilled water. The DNA was extracted with Chelex 100 (28).

(ii) A 1.5-ml volume of activated sludge from a sewage treatment plant in Plön (Schleswig-Holstein, Germany) was pelleted (13600 × *g* for 10 min at 4°C), and the pellet was air dried and resuspended in 0.85% NaCl solution. DNA extraction (8) was followed by an additional hexadecyltrimethylammonium bromide (CTAB; Sigma Aldrich, Steinheim, Germany) precipitation step (1) to remove humic acids and carbohydrates.

(iii) Surface water (30 liters) from Lake Kleiner Plöner See (Schleswig-Holstein, Germany; collected in April 1996) was filtered through a cellulose filter (Sartorius, Göttingen, Germany) to remove particles larger than 100 μm and then through a fiberglass filter (pore size, 3 μm; Millipore, Bedford, Mass.), and cells were collected on a Durapore filter (pore size, 0.22 μm; Millipore). Bacterial cells were removed from the filter by shaking it (100 rpm for 5 h at 4°C) in 100 ml of filtered lake water (pore size, 0.22 μm) containing 0.1 mM EDTA. The cells were then harvested by centrifugation (8,000 × *g* for 45 min at 4°C). The air-dried pellet was resuspended in 10 ml of SET buffer (5% sucrose, 50 mM EDTA, 50 mM Tris-HCl [pH 7.6]). The cells were lysed by the method of Smalla (22) with modifications suggested by Gliesche et al. (8). The suspension was frozen (20 min at −20°C) and thawed (5 min at 30°C) and kept on ice with 1 volume of chilled acetone for 30 min. The pellet (after centrifugation at 4000 × *g* for 10 min) was dried, resuspended in 5 ml of SET buffer containing 5 mg of lysozyme, and incubated at 37°C for 1 h. DNA extraction and purification were performed by the method of Gliesche et al. (8).

(iv) Cells from 10 liters of lake water (Lake Plussee, Schleswig-Holstein, Germany; collected at a depth of 9 m in August 1996) were concentrated by tangential-flow filtration (31). To 100 μl of the cell suspension was added 200 μl of MilliQ water; DNA was extracted with Chelex 100 (28) and further purified with CTAB (1).

(v) DNA from sediment (Lake Kleiner Plöner See; collected in April 1996) was isolated by the method of van Elsas and Smalla (26) with an additional proteinase K treatment (50 μl of a 20-mg ml^{-1} solution) after the incubation with SDS.

PCR amplification of the *nir* genes. PCR amplifications from pure cultures and environmental samples were performed in a total volume of 50 μl containing 5 μl of 10× PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris-HCl [pH 8.4], 0.1% Triton X-100), 200 μM each deoxyribonucleoside triphosphate, 1.0 U of *Taq* polymerase (5 U μl^{-1}; Appligene Oncor, Illkirch, France), 25 pmol (for genomic DNA) or 35 pmol (for total DNA from environmental samples) of both primers (5 pmol μl^{-1} each), and DNA (10 to 100 ng). After a denaturation step of 5 min at 95°C, a "touchdown" PCR was performed (Thermocycler 2400; Perkin-Elmer, Branchburg, N.J.). This consisted of a denaturation step of 30 s at 95°C, a primer-annealing step of 40 s, and an extension step of 40 s at 72°C. After 30 cycles, a final 7-min incubation at 72°C was performed. During the first 10 cycles, the annealing temperature was decreased by 0.5°C every cycle, starting at 45°C until it reached a touchdown at 40°C. The additional 20 cycles were performed at an annealing temperature of 43°C. The amplification products were analyzed by electrophoresis on 2% (wt/vol) agarose gels (Boehringer, Ingelheim, Germany) followed by a 15-min staining with ethidium bromide (0.5 mg $liter^{-1}$).

Sequencing of amplified *nir* products. For DNA sequencing, amplified PCR products from pure cultures were purified with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) as specified by the manufacturer. DNA sequences were determined by direct sequencing of purified PCR products with the cycle-sequencing kit (GATC, Konstanz, Germany) and Thermosequenase 2.0 (Amersham, Braunschweig, Germany) as specified by the manufacturers. Labeling was performed by terminating the polymerization with biotin-labeled dideoxynucleoside triphosphates. After a denaturing step of 4 min at 94°C, 30 cycles of denaturation for 30 s at 94°C and primer annealing and extension for 30 s at 55°C were performed, followed by an additional extension step of 6 min at 60°C. The sequencing products were blotted with a direct blotting apparatus (GATC) onto a nylon membrane. The separated products were visualized by an enzyme-linked streptavidin-biotin coupling assay with a streptavidin-alkaline phosphatase conjugate (GATC) and NBT/X-phosphate (Boehringer, Mannheim, Germany) as specified by the manufacturers. The sequences obtained were compared with published *nirK* and *nirS* sequences in the EMBL Nucleotide Sequence Database by FASTA analysis of the HUSAR program package based on the Genetics Computer Group sequence analysis package (6).

Hybridization analysis of *nir* products from total DNA of environmental samples. Approximately 100 ng (pure cultures) or 250 ng (environmental samples) of PCR product was analyzed on an agarose gel (2%, wt/vol). After electrophoresis, the DNA was transferred onto a positively charged nylon membrane (QIAbrane Nylon Plus; Qiagen) by capillary transfer (24). The DNA was cross-linked to the membrane with UV light (45 s at 302 nm).

TABLE 1. Primer sequences and positions used to amplify fragments from *nirK* and *nirS* nitrite reductase

Primer[a]	Position[b]	Primer sequence (5′-3′)
nirK1F	526–542	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA
nirK2F	565–581	GC(C/G)(C/A)T(C/G)ATGGT(C/G)CTGCC
nirK3R	898–918	GAACTTGCCGGT(A/C/G)G(C/T)CCAGAC
nirK4R	942–959	GG(A/G)AT(A/G)A(A/G)CCAGGTTTCC
nirK5R	1023–1040	GCCTCGATCAG(A/G)TT(A/G)TGG
nirS1F	763–780	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T
nirS2F	855–874	TACCACCC(C/G)GA(A/G)CCGCGCGT
nirS3F	1002–1019	TTCCT(C/G/T)CA(C/T)GACGGCGGC
nirS4F	1317–1336	TTC(A/G)TCAAGAC(C/G)CA(C/T)CCGAA
nirS3R	1002–1019	GCCGCCGTC(A/G)TG(A/C/G)AGGAA
nirS5R	1494–1514	CTTGTTG(A/T)ACTCG(C/G)(C/G)CTGCAC
nirS6R	1638–1653	CGTTGAACTT(A/G)CCGGT

[a] The primers are indicated by nirK for the *nirK* gene and nirS for the *nirS* gene; forward and reverse primers are indicated by the last letters F and R, respectively.

[b] Positions in the *nirK* gene of *Alcaligenes faecalis* S-6 EMBL D13155 and in the *nirS* gene of *Pseudomonas stutzeri* ZoBell EMBL X56813.

Products generated with the primer combination nirK1F-nirK5R from genomic DNA from *Alcaligenes xylosoxidans* NCIMB 11015 and with the combination nirS1F-nirS6R from genomic DNA from *Pseudomonas stutzeri* ATCC 14405 were used as probes for *nirK* and *nirS*, respectively, to determine the specificity of *nir* products amplified from total environmental DNA. The *nir* products were purified by eluting the bands from an agarose gel by using the QIAquick gel extraction kit (Qiagen) as specified by the manufacturer. The probes were labeled randomly with digoxigenin by using the digoxigenin DNA labeling and detection kit (Boehringer) as specified by the manufacturer.

The membrane was prehybridized in 20 ml of DIG-Easy Hyb solution (Boehringer) for 2 h at 42°C. Hybridization was performed with 10 ml of DIG-Easy Hyb solution containing the specific probe (25 ng ml^{-1}) and by incubation overnight at 42°C. After hybridization, the membrane was washed twice for 5 min at room temperature in 100 ml of a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) SDS and twice at 45°C for *nirK* and 46°C for *nirS* for 15 min with 100 ml of a solution containing 0.5 × SSC and 0.1% (wt/vol) SDS. Subsequently, the hybridization of the digoxigenin-labeled probe was detected by an enzyme-linked immunoassay with nitroblue tetrazolium/X-phosphate as the substrate as specified by the manufacturer (Boehringer).

Nucleotide sequence accession numbers. The *nirK* and *nirS* sequences have been deposited in the EMBL nucleotide sequence databases under accession no. AJ224902 through AJ224913.

RESULTS

Design of PCR primers. For the *nirK* gene, six sequences were available from the EMBL database, i.e., from *Alcaligenes faecalis* S-6 (D13155), *Achromobacter cycloclastes* (Z48635), *Pseudomonas aureofaciens* (Z21945), *Pseudomonas* sp. strain G-179 (M97294), *Rhodobacter sphaeroides* (U62291), and *Rhizobium "hedysari"* (U65658). For the *nirS* gene, six sequences were available, i.e., from *Pseudomonas stutzeri* JM300 (M80653), *Pseudomonas stutzeri* (X56813), *Pseudomonas aeruginosa* (X16452), *Paracoccus denitrificans* Pd1222 (U05002), *Paracoccus denitrificans* LMD29.63 (U75413), and *Alcaligenes eutrophus* H16 (X91394). For each gene, the available sequences were aligned by using the MULTIALIGN program (6). Five conserved regions for *nirK* and six conserved regions for *nirS* were chosen to design the primers used in this study. The sequences of the primers are shown in Table 1, and their locations within the *nir* genes are shown in Table 1 and Fig. 1. Comparison of the chosen primer sequences to all stored sequences in the EMBL database by using the BLASTN program (6) revealed significant similarity only to sequences of the *nirK* or the *nirS* gene.

Amplification of *nirK* and *nirS* fragments from pure cultures. The selected primers were used to amplify *nirK* or *nirS*

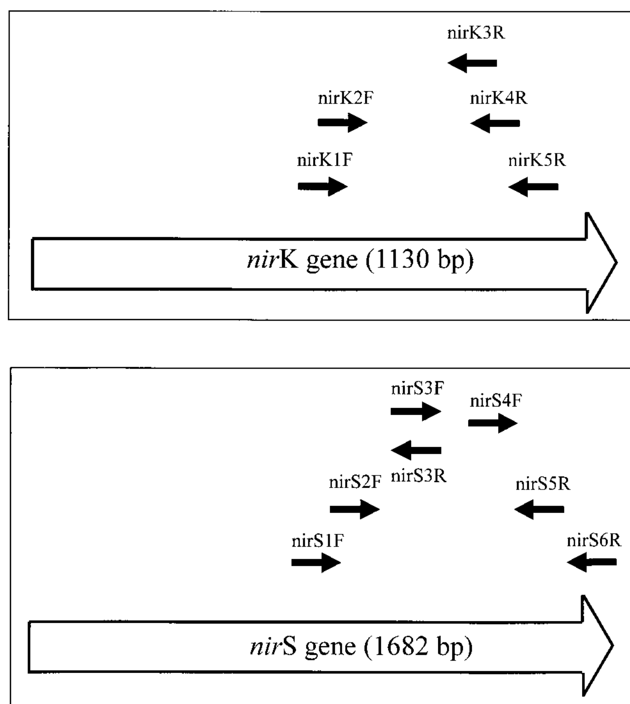

FIG. 1. Diagram showing the positions of primers used to amplify *nir* fragments: the positions for the nirK primers are correlated with the sequence of the *nirK* gene from *Alcaligenes faecalis* S-6 (top), and the positions for the nirS primers are correlated with the sequence of the *nirS* gene from *Pseudomonas stutzeri* ZoBell (bottom).

fragments from bacterial pure cultures known to denitrify and to contain either *nirK* or *nirS*. For *nirK*, amplification products were obtained with the primer combinations nirK1F-nirK3R (392 bp), nirK1F-nirK4R (433 bp), nirK1F-nirK5R (514 bp), nirK2F-nirK3R (353 bp), nirK2F-nirK4R (394 bp), and nirK2F-nirK5R (475 bp). For *nirS*, amplification products were obtained with the primer combinations nirS1F-nirS3R (256 bp), nirS1F-nirS5R (751 bp), nirS1F-nirS6R (890 bp), nirS2F-nirS3R (164 bp), nirS2F-nirS5R (659 bp), nirS2F-nirS6R (798 bp), nirS3F-nirS5R (512 bp), nirS3F-nirS6R (651 bp), nirS4F-nirS5R (197 bp), and nirS4F-nirS6R (336 bp). Products of the expected size could be obtained from all selected primer combinations for amplification of *nirK* fragments with genomic DNA from *Ochrobactrum anthropi* and for amplification of *nirS* fragments with genomic DNA from *Pseudomonas stutzeri*. With the other pure cultures used in this study, not all but several primer combinations generated products of the expected sizes when used in PCR amplification experiments (Tables 2 and 3).

At least one primer combination for each gene which reacted with all pure cultures known to contain either *nirK* or *nirS* was found. For *nirK*, the primer combinations nirK1F-nirK3R and nirK1F-nirK5R amplified with all tested *nirK*-containing strains. For *nirS*, amplification with the combination nirS1F-nirS6R generated products of the expected size from all *nirS*-containing strains.

Whenever weaker products of nonspecific sizes occurred besides the products of the expected sizes (Table 2 and 3), application of a higher temperature for primer annealing eliminated the nonspecific products. By using the selected primer combinations for *nirK* with DNA from pure cultures of denitrifying strains known to contain the coding sequence for

TABLE 2. Results of PCR amplifications with the different sets of primers for *nirK*[a]

Species or strain[b]	Source or reference[b]	Denitrification[c]	*nirK*[c]	PCR products[d] determined with primers:					
				nirK1F-nirK3R	nirK1F-nirK4R	nirK1F-nirK5R	nirK2F-nirK3R	nirK2F-nirK4R	nirK2F-nirK5R
Alcaligenes xylosoxidans NCIMB 11015	NCIMB	+	+	+	+	+	−	−	−
Hyphomicrobium zavarzinii IFAM ZV-622[T] (ATCC 27496)	ATCC	+	+	+	−	+	−	−	−
Ochrobactrum anthropi LMG 2136	LMG	+	+	+	+	+	+	+	+
Rhizobium meliloti Rm1021	C. Elmerich, Institut Pasteur, Paris, France	+	+	+	−	+	+	−	+
Rhizobium meliloti 20115	C. Elmerich, Institut Pasteur, Paris, France	+	+	+	−	+	+	−	+
Rhodobacter sphaeroides f. sp. *denitrificans*	Satoh et al. (20)	+	+	+	−	+	+[e]	−	+
Alcaligenes sp. strain (DSM 30128)	DSM	+	ND[g]	+	+	+	+[e]	−	+
Alcaligenes xylosoxidans subsp. *denitrificans* DSM 30026	DSM	+	ND	+	+	+	−	−	−
Blastobacter denitrificans IFAM 1005 (DSM 1113)	DSM	+	ND	+	−	+	−	−	+
Enterobacter agglomerans 339	W. Klingmüller, Universität Bayreuth, Bayreuth, Germany	−	−	−[f]	−	−	−[f]	−[f]	−[f]
Enterobacter cloacae NCIMB 11463	NCIMB	−	−	−	−	−	−[f]	−	−
Escherichia coli K-12 (DSM 498)	DSM	−	−	−[f]	−	−	−[f]	−[f]	−[f]
Klebsiella pneumoniae subsp. *pneumoniae* DSM 681	DSM	−	−	−[f]	−	−	−[f]	−	−

[a] Touchdown reaction from 45 to 40°C used.
[b] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; IFAM, Institut für Allgemeine Mikrobiologie, Universität Kiel, Kiel, Germany. LMG, Laboratorium voor Mikrobiologie, Universiteit Gent, Ghent, Belgium; NCIMB, National Collections of Industrial and Marine Bacteria, Aberdeen, United Kingdom.
[c] Data from literature.
[d] +, PCR product of the expected size; −, no PCR product.
[e] Expected PCR products and weaker products of unexpected and nonspecific size.
[f] No expected PCR products, only products of unexpected and nonspecific size.
[g] ND, not determined.

TABLE 3. Results of PCR amplifications with the different sets of primers for *nirS*[a]

Strain[b]	Source or reference[b]	Denitrification[c]	*nirS*[c]	PCR products[d] determined with primers:									
				nirS1F-nirS3R	nirS1F-nirS5R	nirS1F-nirS6R	nirS2F-nirS3R	nirS2F-nirS5R	nirS2F-nirS6R	nirS3F-nirS5R	nirS3F-nirS6R	nirS4F-nirS5R	nirS4F-nirS6R
Alcaligenes eutropus H16 (DSM 428)	DSM	+	+	+	+	+	+	−	+	−	−	−	−
Alcaligenes faecalis A15	H. Bothe, Universität Köln, Cologne, Germany	+	+	+	−	+	+	−	+	+	+	−	+
Azospirillum brasilense Sp7 (DSM 1690)	DSM	+	+	−	−	+	−	−	+	−	+	−	+
Paracoccus denitrificans ATCC 19367	ATCC	+	+	+	−	+	+	+	+	+	−	+	+
Pseudomonas aeruginosa DSM 6195	DSM	+	+	−	−	+[e]	−	+	−	−	−	−	+
Pseudomonas stutzeri ATCC 14405	ATCC	+	+	+	+	+	+	+	+	+	+	+	+
Roseobacter denitrificans ATCC 33942[T]	ATCC	+	+	+	+	+	+	+	+[e]	+	+	−	−
Alcaligenes eutrophus DSM 530	DSM	+	ND[g]	+	+	+	+	−	+	−	−	−	−
Denitrifying isolate IFAM 3698	IFAM	+	ND	−	−	+	+	+	+[e]	−	+	−	+
Escherichia coli K-12 (DSM 498)	DSM	−	−	−	−[f]	−	−[f]	−[f]	−[f]	−[f]	−[f]	−	−
Enterobacter agglomerans 339	W. Klingmüller, Universität Bayreuth, Bayreuth, Germany	−	−	−	−	−	−	−[f]	−[f]	−	−[f]	−	−
Enterobacter cloacae NCIMB 11463	NCIMB	−	−	−	−	−	−	−	−[f]	−[f]	−	−	−
Klebsiella pneumoniae subsp. *pneumoniae* DSM 681	DSM	−	−	−	−	−	−	−	−	−	−	−	−

[a] Touchdown reaction from 45 to 40°C used.
[b] See Table 2, footnote b.
[c] Data from literature.
[d] +, PCR product of the expected size; −, no PCR product.
[e] Expected PCR products and weaker products of unexpected and nonspecific size.
[f] No expected PCR products, only products of unexpected and nonspecific size.
[g] ND, not determined.

nirS and vice versa, no PCR products were obtained (data not shown). In addition, no products were obtained when applying the *nirK* or *nirS* primer combinations to DNA from nondenitrifying strains (Tables 2 and 3). Amplification experiments with eubacterial primers for 16S rRNA genes confirmed the ability to amplify gene fragments from the DNA of the pure cultures (data not shown). The results of the *nirK* and *nirS* nitrite reductase PCR experiments are summarized in Tables 2 and 3.

Determination of the type of *nir* gene of denitrifying bacteria. Five strains of denitrifying bacteria were tested to determine their type of nitrite reductase. Genomic DNA preparations from these strains reacted with either the *nirK* or the *nirS* primer combination in PCR amplification experiments. *Blastobacter denitrificans*, *Alcaligenes xylosoxidans*, and *Alcaligenes* sp. were determined to have the coding sequence for the *nirK* nitrite reductase as deduced from their amplification products with the *nirK* primer combinations (Table 2). The coding sequence for the *nirS* nitrite reductase was identified within genomic DNA from *Alcaligenes eutrophus* DSM 530 and the denitrifying isolate IFAM 3698 as deduced from their amplification products with the *nirS* primer combinations (Table 3).

Sequencing of *nir* gene fragments. To determine the specificity of the amplified fragments, the PCR products obtained with the primer combinations nirK1F-nirK5R and nirS1F-nirS6R from the pure cultures were partially sequenced. A total of 425 bp of the amplified fragments from *nirK* were sequenced; the amplified and sequenced regions for *nirS* were between 774 and 792 bp long. Comparison to all sequences in the EMBL database by using the FASTA program revealed that all sequences obtained were parts of the *nirK* or the *nirS* gene, respectively. The levels of homology of the fragments compared to the sequences of the *nirK* and *nirS* genes from the EMBL database are summarized in Tables 4 and 5, respectively. The calculations were done with the GAP program for the nucleic acid similarity and the TREE program for the deduced amino acid similarity.

Amplification of nitrite reductase genes from environmental samples. The development of a system of generally amplifying primer combinations for each gene permitted the qualitative detection of denitrifying bacteria in environmental samples. For these experiments, nirK1F-nirK5R or nirS1F-nirS6R were applied to total DNA preparations from an enrichment culture for denitrifying methylotrophic bacteria, from activated sludge, from the water of Lake Kleiner Plöner See, and Lake Plussee, and from sediment of Lake Kleiner Plöner See. For *nirK*, a weak product of the expected size (514 bp), as well as a smear of nonspecific products, was obtained from the DNA preparations from the water of Lake Kleiner Plöner See and Lake Plussee and from sediment of Lake Kleiner Plöner See. The weak band of 514 bp was extracted and reamplified at annealing temperatures of 65 to 60°C by applying the same primer combination. As a result, distinct bands of 514 bp were obtained (Fig. 2, lanes 2 to 6). For *nirS*, products of the expected size (about 890 bp) were generated (Fig. 3, lanes 2 to 6). The sizes of the products obtained from the environmental samples were the same as those of the positive controls for *nirK* of *Alcaligenes xylosoxidans* (Fig. 2, lane 7) and for *nirS* of *Pseudomonas stutzeri* (Fig. 3, lane 7).

Hybridization analysis with probes for *nirK* and *nirS*. To confirm the specificity of these amplified *nirK* and *nirS* fragments, hybridization experiments were performed. The DIG-labelled PCR product from *Alcaligenes xylosoxidans* (*nirK*), obtained with the primer combination nirK1F-nirK5R, and from *Pseudomonas stutzeri* (*nirS*), obtained with the primer combination nirS1F-nirS6R, were used as probes. All the amplified

TABLE 4. Nucleic acid and amino acid similarities of *nirK* gene fragments from denitrifying bacteria

Organism (EMBL accession no.)	% Similarity[a] to:														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Alcaligenes faecalis* S-6 (D13155)		84.4	64.5	78.7	62.4	79.4	66.0	67.4	66.7	82.3	84.4	84.4	68.8	100.0	71.6
2. *Achromobacter cycloclastes* (Z48635)	79.8		63.8	80.8	67.4	78.0	68.8	69.5	67.4	81.6	80.8	80.8	68.8	84.4	69.5
3. *Pseudomonas aureofaciens* (Z21945)	67.3	68.7		65.2	66.0	65.2	82.3	80.8	78.7	68.1	63.1	63.1	70.9	64.5	68.1
4. *Pseudomonas* sp. strain G-179 (M97294)	75.5	78.6	67.5		65.2	76.6	66.7	68.8	66.7	81.6	80.1	80.1	64.5	78.7	72.3
5. *Rhodobacter sphaeroides* 2.4.3 (U62291)	67.3	72.5	71.5	68.5		66.7	70.2	70.2	68.1	68.1	64.5	64.5	83.0	62.4	68.1
6. *Rhizobium "hedysari"* HCNT1 (U65658)	74.6	73.2	64.7	73.9	66.3		66.7	66.7	66.0	81.6	80.1	80.1	67.4	79.4	73.8
7. *Alcaligenes xylosoxidans* NCIMB 11015 (AJ224905)	68.0	74.8	80.9	70.1	75.8	66.6		95.7	92.2	69.5	65.2	65.2	73.0	66.0	69.5
8. *Alcaligenes xylosoxidans* subsp. *denitrificans* DSM 30026 (AJ224904)	69.6	73.9	77.2	70.1	75.5	65.6	91.3		95.0	71.6	67.4	67.4	73.0	67.4	71.6
9. *Alcaligenes* sp. strain DSM 30128 (AJ224903)	68.7	73.4	79.3	68.2	75.1	65.6	89.9	89.9		70.9	66.7	66.7	70.9	66.7	71.6
10. *Hyphomicrobium zavarzinii* IFAM-622^T (AJ224902)	76.9	79.8	68.7	76.5	70.6	73.9	70.6	70.8	69.9		85.1	85.1	70.2	82.3	71.6
11. *Rhizobium meliloti* 20115	78.3	80.9	66.8	76.2	71.7	75.8	73.6	72.5	72.7	81.4		100.0	68.1	84.4	72.3
12. *Rhizobium meliloti* Rm1021 (AJ224909)	78.3	80.9	66.8	76.2	71.7	75.8	73.6	72.5	72.7	81.4	100.0		68.1	84.4	72.3
13. *Rhodobacter sphaeroides* f. sp. *denitrificans* (AJ224908)	68.5	72.2	73.4	68.0	84.7	67.8	76.5	75.3	73.6	70.6	73.5	73.5		68.8	67.4
14. *Ochrobactrum anthropi* LMG 2136 (AJ2249057)	99.5	80.0	67.5	75.8	67.5	74.6	68.2	69.9	68.7	77.4	78.1	78.1	68.5		71.6
15. *Blastobacter denitrificans* IFAM 1005 (AJ224906)	73.2	73.6	70.3	70.3	70.3	68.7	76.0	76.7	74.6	74.6	74.1	74.1	70.6	73.4	

[a] Values in the upper right are levels of amino acid similarity, and those in the lower left are levels of nucleic acid similarity. Values are calculated from data obtained from the EMBL nucleotide sequence database by using the GAP program for the similarity of nucleic acids and by using the TREE program for the similarity of deduced amino acids.

fragments hybridized, and the products of the positive controls reacted with the specific probe (Fig. 2 and 3). Previous results had shown that the probes did not hybridize with negative controls (PCR without template), with nonspecific PCR products, or nonlabelled DNA standard (data not shown).

DISCUSSION

The genetic diversity of denitrifying bacteria in environmental samples can be investigated by different molecular methods. We describe herein the first steps required to detect denitrifying bacteria in aquatic habitats by the use of two distinct PCR systems for the nitrite reductase genes, *nirK* and *nirS*. Since denitrifiers are not defined by close phylogenetic relationship, an approach involving 16S rRNA molecules is not suitable for general detection of this physiological group in the environment. The use of rRNA-targeted probes has been successfully applied so far only for strains and specific groups to explore the denitrifying community of activated sludge (18).

A more general approach to the detection of all denitrifying bacteria in environmental samples could be the use of a physiological gene or of an enzyme as a molecular marker. For this purpose, nitrite reductase and its genes have been used by several authors, since this is the key enzyme in the denitrification process. Antisera against the dissimilatory nitrite reductase (dNir) from *Pseudomonas stutzeri* ATCC 14405 were highly specific and reacted with the immunizing strain and few other closely related bacteria (14, 29). Less specific reactions could be obtained with combinations of antisera against heme-type dNirs from *P. stutzeri* JM300 and *P. aeruginosa* (4). When a variety of approximately 150 denitrifying strains of uncharacterized dNir type were screened with this combination and an antiserum against Cu dNir from *Alcaligenes cycloclastes*, 90% of the strains could be identified as possessing either the heme-type or Cu dNir (4). Due to the inducible nature of the enzyme, antisera could be useful in detecting conditions of active denitrification (29). In contrast, approaches targeting the *nirK* or *nirS* gene would detect the denitrifiers irrespective of the denitrifying conditions. Compared to the antisera, a broader response to different pure cultures possessing the *nirS* gene was achieved by use of the specific gene probes (15, 24). By using hybridization with a gene probe for *nirK*, this type of nitrite reductase could be always confirmed in pure cultures (32). In enrichment cultures, different populations of denitri-

TABLE 5. Nucleic acid and amino acid similarities of *nirS* gene fragments from denitrifying bacteria

Organism (EMBL accession no.)	% Similarity[a] to:												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Pseudomonas stutzeri* JM300 (M80653)		93.5	55.5	53.9	53.1	58.4	58.4	55.5	53.9	87.4	71.9	49.6	87.0
2. *Pseudomonas stutzeri* ZoBell (X56813)	92.6		57.8	56.6	56.2	61.9	61.9	57.8	56.6	92.4	73.2	51.6	92.4
3. *Pseudomonas aeruginosa* (X16452)	67.3	67.2		67.3	68.5	70.7	70.7	100.0	67.3	56.2	60.5	61.1	55.9
4. *Paracoccus denitrificans* Pd1222 (U05002)	66.1	66.1	72.1		97.7	64.4	64.4	67.3	100.0	54.7	59.0	72.0	55.1
5. *Paracoccus denitrificans* LMD29.63 (U75413)	64.5	64.3	71.6	94.9		64.4	64.4	68.5	97.7	54.3	59.4	72.8	54.7
6. *Alcaligenes eutrophus* H16 (X91341)	69.7	70.8	76.0	73.2	71.5		100.0	70.7	64.4	60.7	64.6	62.5	60.3
7. *Alcaligenes eutrophus* DSM 530	69.7	70.8	76.0	73.2	71.5	100.0		70.7	64.4	60.7	64.6	62.5	60.3
8. *Pseudomonas aeruginosa* DSM 6195	67.3	67.2	100.0	72.1	71.6	76.0	76.0		67.3	56.2	60.5	61.1	55.9
9. *Paracoccus denitrificans* ATCC 19367	66.1	66.1	72.1	100.0	94.9	73.2	73.2	72.1		54.7	59.0	72.9	55.1
10. *Alcaligenes faecalis* A15 (AJ224913)	87.5	89.4	68.5	67.4	65.8	71.8	71.8	68.5	67.4		72.9	49.6	97.3
11. *Azospirillum brasilense* Sp7 DSM 1690 (AJ224912)	72.3	72.6	68.6	68.2	66.8	71.9	71.9	68.6	68.2	72.7		57.0	72.9
12. *Roseobacter denitrificans* ATCC 33942^T (AJ224911)	59.5	61.8	64.2	74.2	73.7	66.9	66.9	64.2	74.2	61.1	63.9		49.6
13. Denitrifying isolate IFAM 3698 (AJ224910)	86.1	89.4	68.2	67.1	66.0	70.8	70.8	68.2	67.1	94.4	74.9	61.3	

[a] Values in the upper right are levels or amino acid similarity, and those in the lower left are levels or nucleic acid similarity. Values are calculated from data obtained from the EMBL nucleotide database by using the GAP program for the similarity of nucleic acids and by using the TREE program for the similarity of the deduced amino acids.

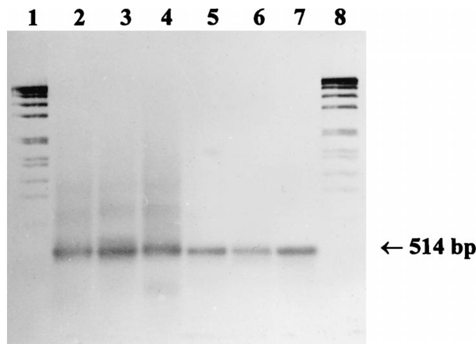

FIG. 2. Southern blot hybridization of *nirK* fragments obtained from environmental samples with the primer combination nirK1F-nirK5R to the digoxigenin-labeled fragment from *Alcaligenes xylosoxidans*. Lanes: 1 and 8, digoxigenin-labeled DNA size standard VII (Boehringer); 2, sediment from Lake Kleiner Plöner See (5 μl of PCR product), 3, water from Lake Plussee (7 μl); 4, water from Lake Kleiner Plöner See (7 μl); 5, activated sludge from the sewage treatment plant at Plön (10 μl); 6, enrichment culture for denitrifying methylotrophic bacteria (12 μl); 7, *A. xylosoxidans* positive control (5 μl).

fiers could be detected by restriction enzyme-digested preparations (*Hin*dIII) of total DNA with a probe for *nirS* (23).

PCR amplification systems, on the other hand, are limited neither to actively denitrifying cells nor to cultivated strains. Application to pure cultures of a primer pair derived from three *nirS* sequences flanking a conserved central region of the *nirS* gene revealed a reactivity broader than that with the use of antisera but not as satisfactory as that with the use of gene probes (30). A strain-specific reaction such as that obtained with the use of antisera was not achieved, although amplification failed with some *nirS*-containing strains as detected by hybridization. This may be because for PCR amplification only the homology of the primer hybridization region is decisive whereas hybridization of gene probes can be detected if any region of the probe shows sufficient homology. In the present study, a PCR system based on six sequences each for *nirK* or *nirS* available from the database promised an even more general approach. Regions that are conserved for both genes could not be found, because the enzymes are structurally different. When the sequences for each of the genes were aligned separately, conserved regions for each became evident. Degenerated primers that flanked regions at the more highly conserved C terminus of the two genes were designed. Primers were not positioned within the *nirS* region that codes for heme binding, because this is homologous to highly conserved regions in other heme-binding proteins (11). By using different combinations of primers and low-temperature stringency conditions in the PCR assays, amplification of *nir* fragments from both genes was possible for all denitrifying strains tested. The specificity of the PCR procedure was confirmed, since specific products were not obtained when the primer combinations were used with nondenitrifying strains that could perform assimilatory respiration of NO_3^- or with strains possessing the gene coding for the other *nir* type. This is consistent with the finding that the two genes are mutually exclusive in a given strain (4). Combinations containing nirK4R or those containing nirS5R resulted in the smallest number of specific products; this may be due to the degree of conservation in the region where the primer should hybridize. From every pure culture tested, at least two different primer combinations were applied successfully for *nirK* and at least three were applied successfully for *nirS*. The specificity of amplifications was confirmed by sequencing the largest products. Generally, sequencing revealed that the products from the pure cultures with the primer pairs nirK1F-nirK5R and nirS1F-nirS6R were specific fragments of the genes coding for copper-containing and cytochrome cd_1-containing nitrite reductase, respectively. Calculation of the homology revealed that with these primer pairs, PCR products could be obtained from genes showing homology as low as 65.5% for *nirK* and 59.5% for *nirS* to the sequences available for the design of the primers. This indicates that these PCR systems could be reasonable means for a more general detection of denitrifying bacteria.

The fragments of *nirS* investigated here were more heterogeneous than were those of *nirK*. In-frame deletions or insertions of up to 18 bp could be observed within the sequenced region of *nirS*. Since these results are not in agreement with the findings that the Cu dNirs were more heterogeneous than the cd_1 dNirs (4), the different molecular weights of the Cu dNir subunits may be a result of processing of the enzyme.

The degrees of conservation of the *nir* genes are very variable. The *nirK* fragments from eight strains of *Ochrobactrum anthropi* were 98.8 to 100.0% homologous (data not shown). On the other hand, the sequence of the fragment from two of these strains was 100% homologous to that of the same fragment from *Alcaligenes faecalis* S-6 (data not shown). However, both types of *nir* genes are distributed among closely related *Pseudomonas* (RNA group I [35]) and *Alcaligenes* (4) species. Even among strains of *Alcaligenes faecalis*, both types could be detected. The distribution of *nir* genes among denitrifying bacteria could be explained in different ways. The occurrence of the same *nir* type among phylogenetically different groups (34) might be caused by a common denitrifying ancestor. During evolution, the ability to denitrify may have been lost in some branches, resulting in closely related nondenitrifying and denitrifying strains (2). The occurrence of different *nir* types among the same species could be an indication of a horizontal gene transfer (4).

The PCR systems for the *nirK* and the *nirS* genes for nitrite reductase, using one generally amplifying primer combination for each *nir* gene, could be applied successfully to detect populations of denitrifying bacteria in aquatic systems. Due to higher cell densities, detection of such populations from soils by using these PCR systems should be possible as well.

nirS fragments could be amplified directly from DNA preparations of environmental samples, whereas for *nirK* a reamplification step was necessary when total DNA from water or

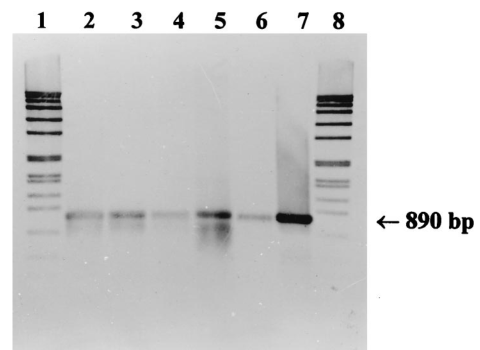

FIG. 3. Southern blot hybridization of *nirS* fragments obtained from environmental samples with the primer combination nirS1F-nirS6R to the digoxigenin-labeled fragment from *Pseudomonas stutzeri* ZoBell. Lanes: 1 and 8, digoxigenin-labeled DNA size standard VII (Boehringer); 2, sediment from Lake Kleiner Plöner See (15 μl), 3, water from Lake Plussee (12 μl); 4, water from Lake Kleiner Plöner See (20 μl); 5, activated sludge from the sewage treatment plant at Plön (10 μl); 6, enrichment culture for denitrifying methylotrophic bacteria (20 μl); 7, *P. stutzeri* ZoBell positive control (1 μl).

sediment of lakes was the target for amplification. This might be in agreement with a numerical preponderance of heme type nitrite reductase, as reported for the distribution of *nir* types in numerically dominant isolates from soils (4). Further investigations will be necessary to determine the abundance of *nirK* and *nirS* type denitrifiers and to obtain more information on the genetic diversity of denitrifying bacteria in the environment.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kinston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1991. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
2. **Betlach, M. R.** 1982. Evolution of bacterial denitrification and denitrifier diversity. Antonie Leeuwenhoek **48:**585–607.
3. **Conrad, R.** 1996. Soil organisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). Microbiol. Rev. **60:**609–640.
4. **Coyne, M. S., A. Arunakumari, B. A. Averill, and J. M. Tiedje.** 1989. Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductases in denitrifying bacteria. Appl. Environ. Microbiol. **55:**2924–2931.
5. **Fries, M. R., J. Zhou, J. Chee-Sanford, and J. M. Tiedje.** 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60:**2802–2810.
6. **Genetics Computer Group.** 1994. GCG program manual. Genetics Computer Group, Madison, Wis.
7. **Gliesche, C. G., and P. Hirsch.** 1992. Mutagenesis and chromosome mobilization in *Hyphomicrobium facilis* B-522. Can. J. Microbiol. **38:**1167–1174.
8. **Gliesche, C. G., M. Menzel, and A. Fesefeldt.** 1997. A rapid method for creating species-specific gene probes for methylotrophic bacteria. J. Microbiol. Methods **28:**25–34.
9. **Glockner, A. B., A. Jüngst, and W. G. Zumft.** 1993. Copper-containing nitrite reductase from *Pseudomonas aureofaciens* is functional in a mutationally cytochrome cd_1-free background ($NirS^-$) of *Pseudomonas stutzeri*. Arch. Microbiol. **160:**18–26.
10. **Hess, A., B. Zarda, D. Hahn, A. Häner, D. Stax, P. Höhener, and J. Zeyer.** 1997. In situ analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. **63:**2136–2141.
11. **Jüngst, A., S. Wakabayashi, H. Matsubara, and W. G. Zumft.** 1991. The *nirSTBM* region coding for cytochrome cd_1-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins. FEBS Lett. **279:**205–209.
12. **Kloos, K., A. Fesefeldt, C. G. Gliesche, and H. Bothe.** 1995. DNA-probing indicates the occurrence of denitrification and nitrogen fixation genes in *Hyphomicrobium*. Distribution of denitrifying and nitrogen fixing isolates of *Hyphomicrobium* in a sewage treatment plant. FEMS Microbiol. Ecol. **18:**205–213.
13. **Knowles, R.** 1982. Denitrification. Microbiol. Rev. **46:**43–70.
14. **Körner, H., and W. G. Zumft.** 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl. Environ. Microbiol. **55:**1670–1676.
15. **Linne Von Berg, K.-H., and H. Bothe.** 1992. The distribution of denitrifying bacteria in soils monitored by DNA-probing. FEMS Microbiol. Ecol. **86:**331–340.
16. **Lyman, J., and R. H. Fleming.** 1940. Composition of sea water. J. Mar. Res. **3:**134–167.
17. **Michalski, W. P., and D. J. D. Nicholas.** 1988. Immunological patterns of distribution of bacterial denitrifying enzymes. Phytochemistry **27:**2451–2456.
18. **Neef, A., A. Zaglauer, H. Meier, R. Amann, H. Lemmer, and K.-H. Schleifer.** 1996. Population analysis in a denitrifying sand filter: conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. Appl. Environ. Microbiol. **62:**4329–4339.
19. **Sambrook, J. E., F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., vol. 1 to 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Satoh, T., Y. Hoshino, and H. Kitamura.** 1976. *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. Arch. Microbiol. **108:**265–269.
21. **Shapleigh, J. P., and W. J. Payne.** 1985. Differentiation of cd_1 cytochrome and copper nitrite reductase production in denitrifiers. FEMS Microbiol. Lett. **26:**275–279.
22. **Smalla, K.** 1995. Extraction of microbial DNA from sewage and manure slurries, p. 1.1.3. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
23. **Smith, G. B., and J. M. Tiedje.** 1992. Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl. Environ. Microbiol. **58:**376–384.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
25. **Staley, J. T.** 1968. *Prosthecomicrobium* and *Ancalomicrobium*, new prosthecate fresh water bacteria. J. Bacteriol. **95:**1921–1944.
26. **Van Elsas, J. D., and K. Smalla.** 1995. Extraction of microbial community DNA from soils, p. 1.3.3. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
27. **Vincent, J. M.** 1971. A manual for the practical study of root-nodule bacteria. Burgess and Son LTB, Oxford, United Kingdom.
28. **Walsh, P. S., D. A. Metzger, and R. Higuchi.** 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. BioTechniques **10:**506–513.
29. **Ward, B. B., A. R. Cockcroft, and K. A. Kilpatrick.** 1993. Antibody and DNA probes for detection of nitrite reductase in seawater. J. Gen. Microbiol. **139:**2285–2293.
30. **Ward, B. B.** 1995. Diversity of culturable denitrifying bacteria. Limits of rDNA RFLP analysis and probes for the functional gene, nitrite reductase. Arch. Microbiol. **163:**167–175.
31. **Ward, B. B., M. A. Voytek, and K.-P. Witzel.** 1997. Phylogenetic diversity of natural populations of ammonia oxidizers investigated by specific PCR amplification. Microb. Ecol. **33:**87–96.
32. **Ye, R. W., M. R. Fries, S. G. Bezborodnikov, B. A. Averill, and J. M. Tiedje.** 1992. Characterization of the structural gene encoding a copper-containing nitrite reductase and homology of this gene to DNA of other denitrifiers. Appl. Environ. Microbiol. **59:**250–254.
33. **Zhou, J., A. Palumbo, and J. M. Tiedje.** 1997. Sensitive detection of a novel class of toluene-degrading denitrifiers, *Azoarcus tolulyticus*, with small-subunit rRNA primers and probes. Appl. Environ. Microbiol. **63:**2384–2390.
34. **Zumft, W. G.** 1992. The denitrifying procaryotes, p. 554–582. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The procaryotes, vol. 1. Springer-Verlag, New York, N.Y.
35. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.