APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2003, p. 3476–3483 Vol. 69, No. 6
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.6.3476–3483.2003

Nitric Oxide Reductase (*norB*) Genes from Pure Cultures and Environmental Samples

Gesche Braker* and James M. Tiedje

Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824

Received 6 January 2003/Accepted 24 February 2003

A PCR-based approach was developed to recover nitric oxide (NO) reductase (*norB*) genes as a functional marker gene for denitrifying bacteria. *norB* database sequences grouped in two very distinct branches. One encodes the quinol-oxidizing single-subunit class (qNorB), while the other class is a cytochrome *bc*-type complex (cNorB). The latter oxidizes cytochrome *c*, and the gene is localized adjacent to *norC*. While both *norB* types occur in denitrifying strains, the *qnorB* type was also found in a variety of nondenitrifying strains, suggesting a function in detoxifying NO. Branch-specific degenerate primer sets detected the two *norB* types in our denitrifier cultures. Specificity was confirmed by sequence analysis of the *norB* amplicons and failure to amplify *norB* from nondenitrifying strains. These primer sets also specifically amplified *norB* from freshwater and marine sediments. Pairwise comparison of amplified *norB* sequences indicated minimum levels of amino acid identity of 43.9% for *qnorB* and 38% for *cnorB*. Phylogenetic analysis confirmed the existence of two classes of *norB* genes, which clustered according to the respective primer set. Within the *qnorB* cluster, the majority of genes from isolates and a few environmental clones formed a separate subcluster. Most environmental *qnorB* clones originating from both habitats clustered into two distinct subclusters of novel sequences from presumably as yet uncultivated organisms. *cnorB* clones were located on separate branches within subclusters of genes from known organisms, suggesting an origin from similar organisms.

Nitric oxide (NO) is produced by prokaryotes as an intermediate during respiratory denitrification, when oxidized nitrogen compounds are used as alternative electron acceptors under oxygen-limited conditions (50). The ability to denitrify is widespread among a variety of phylogenetically unrelated organisms and was presumably acquired through horizontal gene transfer. Thus, molecular studies based on functional marker genes coding for key enzymes of the denitrification process have the potential to reflect structures of denitrifying communities in environmental samples. Gene-specific primer sets were developed previously to detect *narG* (16), *narH* (26), *nirK* and *nirS* (3, 17), and *nosZ* (34), but a gene-specific primer set to target nitric oxide reductase genes was lacking. Nitric oxide reductase is encoded by the *norB* gene and catalyzes the reduction of NO to N_2O, which represents an unusual reaction in biology, the formation of an N-N bond. The existence of a separate enzyme, apart from nitrite reductase, that catalyzes NO reduction was demonstrated by mutational disruption of nitrite reductase genes (*nirK* and *nirS*) and thereby resolved the complete denitrification pathway in vivo (46, 53).

Genes coding for both the small and large subunits of nitric oxide reductase, *norC* and *norB*, respectively, were retrieved from denitrifying bacteria, including *Paracoccus halodenitrificans* (30), *Pseudomonas* spp. (1, 52), *Alcaligenes faecalis* (20), and *Rhodobacter sphaeroides* (2). The mature protein accepts electrons from cytochrome *c* and therefore was designated cNor. For the denitrifier *Ralstonia eutropha* harboring a different but homologous class of nitric oxide reductases, however, the adjacent *norC*-encoded subunit was lacking, and the predicted protein showed instead an N-terminal extension with homology to *norC* (9). This single-component type of *norB* gene was designated qNor because electrons are transferred from quinol. Both gene types are localized on the genome. Interestingly, *Ralstonia eutropha* H16 has a second very similar copy of this gene on a megaplasmid (10). Genes of the *qnorB* type were also discovered during genome annotation in a variety of nondenitrifying, mostly pathogenic organisms such as *Neisseria meningitidis* and *Mycobacterium avium*. Complementation studies of an NO reductase-deficient mutant of *Ralstonia eutropha* with the *norB* gene of a *Synechocystis* sp. also demonstrated the presence of a potentially functional *norB* gene within the nondenitrifying cyanobacterium (5). Detoxification of NO produced by the accompanying microflora or by macrophages during the host defense was proposed as the function of nitric oxide reductases in nondenitrifying organisms (29).

In the present study, we report on the development of distinct *norB*-specific primer sets to detect the two types of nitric oxide reductase genes and explore the genetic diversity of these gene types in pure cultures and environmental samples.

MATERIALS AND METHODS

Bacterial strains. To evaluate the specificity of the newly designed *norB* primers, denitrifying strains and nondenitrifying controls (see Table 2) were grown aerobically at 27°C in nutrient broth (Merck, Darmstadt, Germany) with the following exceptions: the *Rhizobium* sp. was grown on yeast extract medium (YEM [43]), *Hyphomicrobium zavarzinii* IFAM ZV-622^T was grown on 337-B1 medium (14) with 0.5% methanol, *Roseobacter denitrificans* was grown on oligotrophic medium (PYGV [38]) supplemented with 25‰ artificial seawater (21), and *Blastobacter denitrificans* was grown on peptone yeast extract glucose medium (PYGV without vitamins). Nondenitrifying strains were grown on Luria broth (31).

* Corresponding author. Present address: Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse, D-35043 Marburg, Germany. Phone: 49 6421 178810. Fax: 49 6421 178999. E-mail: braker@staff.uni-marburg.de.

Primer design. *norB* nucleotide sequences available from the EMBL sequence database and from annotated genes of genome sequencing projects were translated to amino acids, and deduced amino acids were aligned automatically with the ClustalW function (http://www.ebi.ac.uk/clustalw/). Additionally, amino acid alignments were edited manually, and nucleotide sequences were aligned accordingly. Degenerate primers were designed to target conserved regions of the two groups of *norB* genes separately and were designated qnorB and cnorB.

DNA extractions and PCR conditions. Genomic DNA from pure cultures was extracted as described by Gliesche et al. (15). DNA extracts from *Azoarcus* sp. strains and from *Thauera aromatica* K172 were kindly provided by Sabine A. Rech (San José State University, San José, Calif.) and John K. Davis (Michigan State University, East Lansing Mich.), respectively. Total DNA from a freshwater sediment sample from the Red Cedar River (East Lansing, Mich.) was extracted according to the method of Purdy et al. (28) immediately after sampling. A marine sediment sample from the Washington margin of the Pacific Ocean (water depth, 1,936 m; sediment core section from a depth of 0.5 to 1.0 cm) was kept frozen at −20°C until total DNA was extracted with the freeze-thaw procedure of van Elsas and Smalla (42). In addition, a proteinase K treatment (50 μl of a 20-mg/ml solution) was performed after incubation with sodium dodecyl sulfate. DNA was quantified and analyzed spectrophotometrically by taking measurements at 230, 260, and 280 nm. The purity of the DNA was high, as judged by the ratios of absorption at 260 nm and 280 nm of 1.71 to 1.84 for the Red Cedar River samples ($n = 5$) and 1.68 and 1.82 for the Washington margin samples ($n = 2$).

Amplification of *norB* fragments from 50 ng of pure culture DNA extract or 100 ng of environmental DNA extract was performed in 50-μl reactions containing 50 pmol of each primer, 200 μM each deoxynucleoside triphosphate (Gibco-BRL, Gaithersburg, Md.), 400 ng of bovine serum albumin (Roche Molecular Biochemicals, Indianapolis, Ind.) μl^{-1}, 1.5 mM $MgCl_2$, and 1 U of *Taq* polymerase (Sigma Chemical Co., St. Louis, Mo.) in 1× reaction buffer, provided with the enzyme. After 5 min of denaturation, 40 PCR cycles were done, including 10 initial cycles of 30 s of denaturation at 95°C, 40 s of primer annealing with a touch down from 57 to 52.5°C, primer extension of 1 min at 72°C, and an additional 30 cycles with a constant annealing temperature of 55°C. PCR products (10 μl) were analyzed on 2% (wt/vol) agarose gels (Gibco-BRL) and visualized by UV excitation after staining with ethidium bromide (0.5 mg $liter^{-1}$).

Cloning and screening of environmental clones. PCR products of the expected size from environmental DNA extracts obtained with primer sets qnorB2F-7R and cnorB2F-6R were eluted from agarose gels with the QIAquick gel extraction kit (Qiagen, Chatsworth, Calif.). Eluted PCR products were ligated into the pCR2.1 vector, and subsequently cells provided with the TA cloning kit (Invitrogen, San Diego, Calif.) were transformed according to the manufacturer's instructions. Inserts of the proper size were detected by PCR performed with a small amount of cells from 100 randomly chosen white colonies and the appropriate *norB* primer set under the conditions described previously. Clones (30 to 35) containing an insert of the proper size were screened by restriction fragment length polymorphism (RFLP). PCR products (5 μl) were hydrolyzed in two separate reactions with 3 U each of restriction endonucleases *Msp*I and *Rsa*I for the qnorB primer set and *Hha*I and *Msp*I for the cnorB primer set. Restriction fragments were separated on 3.5% Metaphor agarose gels as described previously (49). Subsequently, RFLPs were compared with the GelCompar software (Applied Maths, Kortrijk, Belgium) by applying the unweighted pair group method with arithmetic averages and the Jaccard algorithm. The resulting clusters were additionally compared by eye.

Sequencing. *norB* PCR products from pure cultures and inserts from clones amplified with vector-specific primers were purified with the QIAquick PCR purification kit (Qiagen, Chatsworth, Calif.). Both strands were sequenced directly from 70 ng of PCR product with primers used for PCR amplification for pure cultures and vector-specific primers (M13 reverse and T7 Promoter) for clones and the ABI Big Dye terminator kit version 2.0 (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. Subsequently, excess primers and dye terminators were removed with Autoseq G-50 columns (Amersham-Pharmacia Biotech, Freiburg, Germany), and cycle sequencing reactions were analyzed with an ABI 377 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Forward and reverse strand *norB* sequences were assembled with the Seqman II software (DNAstar Inc. Madison, Wis.) and aligned to sequences from the EMBL database with the ARB Fast aligner feature (http://www.arb-home.de). For phylogenetic analysis, a filter was applied, including 100 deduced amino acid positions with a minimum identity of 0% and a maximum identity of 100% but omitting insertions or deletions (indels) due to ambiguous positional homology. Phylogenetic analyses were performed with ARB and the Phylip software package version 3.6a2.1 (12). Trees were constructed with the distance matrix-based methods, neighbor joining (ARB and PHYLIP), and FITCH (PHYLIP), parsimony PROTPARS (PHYLIP), and maximum likelihood MOLPHY (Institute Pasteur, Paris, France; http://bioweb.pasteur.fr/seqanal/interfaces/prot_nucml.html). Statistical evaluation of tree topologies was performed by bootstrap analysis (PHYLIP) with 1,000 resamplings for neighbor joining (Jones-Taylor-Thornton amino acid replacement model) and parsimony. After comparison of trees generated with different methods, a consensus tree was constructed by introducing multifurcations where the topology was not resolved.

TABLE 1. Selected primers used for amplification of *norB* genes

Primer[a]	Position[b] (nt)	Primer sequence[c] (5′–3′)
qnorB2F	1204–1220	GGN CAY CAR GGN TAY GA
qnorB5R	1466–1444	ACC CAN AGR TGN ACN ACC CAC CA
qnorB7R	1841–1822	GGN GGR TTD ATC ADG AAN CC
cnorB1F	364–380	GAR TTY CTN GAR CAR CC
cnorB2F	553–571	GAC AAG NNN TAC TGG TGG T
cnorB6R	942–925	GAA NCC CCA NAC NCC NGC
cnorB7R	1007–991	TGN CCR TGN GCN GCN GT

[a] Primers are named by *qnorB*-targeting genes for quinol-oxidizing nitric oxide reductase and by *cnorB*-targeting genes for cytochrome *c*-oxidizing nitric oxide reductase; forward and reverse primers are indicated by F and R as the last letter, respectively.
[b] Positions correspond to the *qnorB* gene of *Ralstonia eutropha* H16 (AF002661) and the *cnorB* gene of *Paracoccus denitrificans* Pd1222 (U28078). nt, nucleotide.
[c] N = A, C, G, or T; Y = C or T; R = A or G; D = G, A, or T.

Nucleotide sequence accession numbers. *norB* gene sequences from pure cultures and sediment samples have been deposited in the EMBL nucleotide sequence database under accession numbers AJ507329 through AJ507380.

RESULTS

Primer design. Alignment and phylogenetic analysis of *norB* sequences that were available from the databases revealed two distantly related clusters of *norB* sequences. The first cluster comprised five *norB* genes of the denitrifier *Ralstonia eutropha* and nondenitrifying strains of *Synechocystis* sp. and *Neisseria meningitis* spp. which exhibited an N-terminal extension and for which quinol served as an electron donor for the protein. The *norB* genes of the denitrifiers *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Paracoccus denitrificans*, *Halomonas halodenitrificans*, *Rhodobacter sphaeroides*, *Bradyrhizobium japonicum*, *Pseudomonas* sp., and *Alcaligenes faecalis* S-6 grouped in a second cluster of *norB* genes with an adjacent *norC* gene and with cytochrome *c* as an electron donor. The sequence identities of all pairwise compared *norB* sequences (100 amino acids) that were available from the database ranged from 22.1 to 100%. Within clusters, sequence identity was higher, from 41.3 to 98.3% for genes with the N-terminal extension and from 53.0 to 100% for genes with an adjacent *norC* gene. Therefore, primers were designed for each cluster separately and designated qnorB and cnorB (Table 1). Degeneracies were introduced representing all wobble positions observed among the aligned sequences. Comparison of the chosen primer sequences to all sequences stored in the sequence databases with the FastA program (http://www.ebi.ac.uk/fasta33/) indicated significant sequence similarity only to *norB* genes.

Amplification of *norB* genes from pure cultures and environmental samples. Primers were evaluated with all possible combinations, i.e., eight combinations for *qnorB* and 17 combinations for *cnorB* in PCR amplification of *norB* from a variety of pure culture DNA extracts from denitrifying and

TABLE 2. Amplification of *norB* genes from denitrifying strains and sediment samples with selected primer sets qnorB and cnorB

Strain	Source or reference[a]	Denitrification[b]	PCR products obtained with primer set[c]:					Database sequence	Amplification with primer set:
			qnorB 2F-5R (262 bp)	qnorB 2F-7R (637 bp)	cnorB 1F-6R (578 bp)	cnorB 2F-6R (389 bp)	cnorB 2F-7R (454 bp)		
Alcaligenes sp.	DSM 30128	+	+	+	−	−	−	−	qnorB
Alcaligenes faecalis	DSM 30030	+	+	+	−	−	−	−	qnorB
Alcaligenes xylosoxidans	NCIMB 11015	+	+	+	−	−	−	−	qnorB
Ralstonia eutropha H16	DSM 428	+	+	+	−	−	−	+	qnorB
Synechocystis sp. strain PCC6803	R. Cramm, Humboldt Universität, Berlin, Germany	−	+	+	−	−	−	+	qnorB
Alcaligenes faecalis A15	H. Bothe, Universität, Köln, Cologne, Germany	+	−	−	−	+	+	−	cnorB
Azoarcus tolulyticus Td-3	Fries et al. (13)	+	−	−	+[+]	+	+	−	cnorB
Azoarcus tolulyticus Tol-4	Zhou et al. (48)	+	−	−	+[+]	+	+	−	cnorB
Azoarcus tolulyticus Td-17	Fries et al. (13)	+	−	−	+[+]	+	+	−	cnorB
Azospirillum brasilense Sp7	DSM 1690	+	−	−	+[+]	+	+	−	cnorB
Blastobacter denitrificans	DSM 1113	+	−	−	+[+]	(+)[+]	+	−	cnorB
"*Corynebacterium nephridii*"	ATCC 11425	+	−	−	+	+	+	−	cnorB
Denitrifier	IFAM 3698	+	−	−	+[+]	+	+	−	cnorB
Hyphomicrobium zavarzinii	ATCC 27496	+	−	−	+	+	+	−	cnorB
Ochrobactrum anthropi GSF M26	M. Schloter, GSF, Neuherberg, Germany	+	−	−	+	+	+	−	cnorB
Paracoccus denitrificans Pd1222	ATCC 19367	+	−	−	(+)	+[+]	+	+	cnorB
Paracoccus halodenitrificans	IFO 14912	+	−	−	+[+]	+	+	−	cnorB
Pseudomonas aeruginosa	NCTC 6750	+	−	−	−	+	+	−	cnorB
Pseudomonas fluorescens AK	Michotey et al. (24)	+	−	−	+	+	+	−	cnorB
Pseudomonas sp. strain G-179	Ye et al. (47)	+	−	−	+	+	+	+	cnorB
Pseudomonas stutzeri	ATCC 14405	+	−	−	+[+]	+	+	−	cnorB
Pseudomonas stutzeri JM300	Coyne et al. (8)	+	−	−	+[+]	+	+	−	cnorB
Rhodobacter sphaeroides	Satoh et al. (33)	+	−	−	−	(+)[+]	+	+	cnorB
Roseobacter denitrificans	ATCC 33942	+	−	−	+[+]	+	+	+	cnorB
Thauera aromatica K172	DSM 6984	+	−	−	+	+[+]	+[+]	−	cnorB
Pantoea aerogenes	DSM 3493	−	−	−	−	−	−	−	qnorB/cnorB
Enterobacter cloacae	NCIMB 11463	−	−	−	−	−	−	−	qnorB/cnorB
Escherichia coli	DSM 498	−	−	−	−	−	−	−	qnorB/cnorB
Freshwater sediment	Red Cedar River, Mich.	ND	+	+	−	+	+	−	qnorB/cnorB
Marine sediment	Washington margin, Wash.	ND	+	+	−	+	+[+]	−	qnorB/cnorB

[a] ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; GSF, National Research Center for Environment and Health; IFO, Institute for Fermentation, Osaka, Japan; NCIMB, National Collection of Industrial and Marine Bacteria; NCTC, National Collection of Type Cultures.

[b] Data from the literature.

[c] +, PCR product of expected size; −, no amplification; +[+], PCR product of expected size and extra band of unexpected size; (+), weak PCR product; (+)[+], weak PCR product of expected size and extra band of unexpected size. The expected size of the PCR product is shown in parentheses for each primer set. ND, not determined.

nondenitrifying strains (Table 2). Generally, for a given denitrifying strain, amplification was accomplished with primer sets specific for either the *qnorB* or the *cnorB* cluster. Two combinations, qnorB2F-5R and qnorB2F-7R, yielded the expected amplification products (262 and 637 bp) for *qnorB* from the nondenitrifying *Synechocystis* sp. strain PCC6803 and denitrifying *Ralstonia eutropha* H16 and additionally detected the *qnorB* type from three denitrifying *Alcaligenes* strains.

All other denitrifying strains representing different genera within the *Proteobacteria* were targeted by primer combinations cnorB2F-6R (389 bp) and cnorB2F-7R (454 bp) specific for *cnorB*. Amplification with primer combination cnorB1F-6R (578 bp) was also successful for the majority of these denitrifying strains except for *Alcaligenes faecalis* A15, *Pseudomonas aeruginosa,* and *Rhodobacter sphaeroides*. Other primer sets targeting *qnorB* and *cnorB* yielded specific PCR products only from a subset of the strains tested (data not shown). The gene specificity of the primers was confirmed by amplification of a PCR product of the expected size and by the failure of these primers to amplify any DNA fragment from nondenitrifying bacteria. Whenever a PCR product of unexpected size occurred, the PCR conditions were adjusted to higher stringency by applying higher annealing temperatures until amplification yielded a single band of the expected size.

When these primer sets were applied to amplify *norB* gene fragments from total DNA extracts from sediments of the Red Cedar River and the Washington margin, both primer combinations for *qnorB* yielded a distinct band of the expected size. For *cnorB*, however, PCR products of the expected size were obtained from primer set cnorB2F-6R, but primer combination cnorB2F-7R yielded additional bands of unexpected sizes and the combination cnorB1F-6R failed to amplify *norB* genes from these environmental samples.

RFLP analysis. Cloned *norB* PCR products (130 clones) from sediment samples generated with primer combinations qnorB2F-7R and cnorB2F-6R were screened by restriction fragment polymorphism (RFLP) analysis (Fig. 1). For both *norB* types, a large number of restriction patterns of the clones screened for each clone library (34 and 35 *qnorB* clones and 31

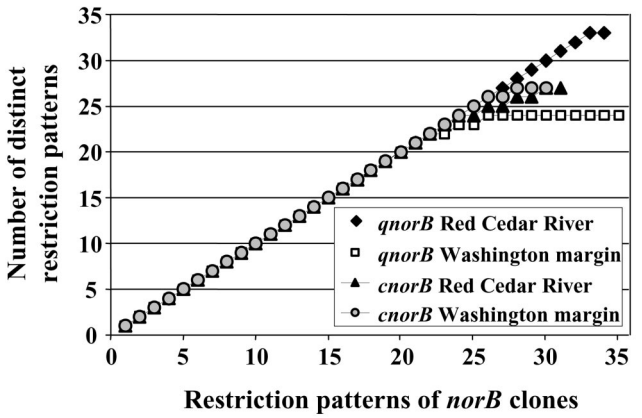


FIG. 1. Diversity of nitric oxide-reducing bacteria in environmental samples as evaluated by RFLP analysis of cloned nitric oxide (*norB*) genes from marine and freshwater sediments. *qnorB* clones were hydrolyzed with restriction enzymes *Msp*I and *Rsa*I, and *cnorB* clones were hydrolyzed with *Hha*I and *Msp*I.

and 30 *cnorB* clones from Red Cedar River and the Washington margin, respectively) were unique for each sediment sample, meaning that 63 to 94% of the clones were nonredundant. One very abundant group of *qnorB* clones from the Washington margin represented 28% of all clones, whereas *qnorB* clones from the Red Cedar River sediment showed the highest level of diversity, with only a single redundant clone. One *qnorB* clone was redundant between both environmental samples. Communities from both habitats based on *cnorB* genes had a similar intermediate level of diversity.

Sequence analysis. Analyses of primary sequences and deduced amino acids confirmed that PCR products of the expected sizes from denitrifying strains were indeed *norB* fragments, based on sequence similarity and the presence of heme and iron binding sites. Furthermore, all PCR products amplified from total environmental DNA extracts with the qnorB primer set were specific *norB* genes. All *cnorB* clones from the Red Cedar River sediments were identified as *norB* gene fragments, but two clones from the Washington margin sediment were not identified as *norB* sequences. One of these clones contained a PCR product which was slightly larger than expected.

Pairwise comparison of *qnorB* sequences showed that genes from four denitrifying strains (*Ralstonia eutropha* and three *Alcaligenes* strains) were significantly more similar (*t* test, $P < 0.05$) to each other, with an average identity of 81.6% (70.6 to 97.4%) than to nondenitrifying strains ($\bar{x} = 53.9\%$; range, 47.9 to 62.0%) or to environmental clones ($\bar{x} = 56.7\%$; range, 47.9 to 87.6%). The average pairwise similarity of cloned *qnorB* genes was 62.1% (47.6 to 92.8%), and clones from the two sampling sites were not significantly different (*t* test, $P < 0.05$) from each other, with an average pairwise identity of 63.6% and 60.8% for clones from the Red Cedar River and the Washington margin, respectively.

cnorB genes, based on a broader variety of denitrifying strains, were less identical by pairwise comparison, with an average identity of 73.9%, ranging from 26.6% identity (*Pyrobaculum aerophilum* compared to *Achromobacter cycloclastes*, *Pseudomonas* sp. strain G-179, and *Sinorhizobium meliloti*) to 100% (*Ochrobactrum anthropi* compared to "*Corynebacterium nephridii*"). Genes from pure cultures were significantly different from clones, with an average pairwise similarity of 65.8% (*t* test, $P < 0.05$), but clones from both habitats showed similar levels of identity, 62.3% and 62.8% for Red Cedar River and the Washington margin, respectively, and thus they were not statistically different.

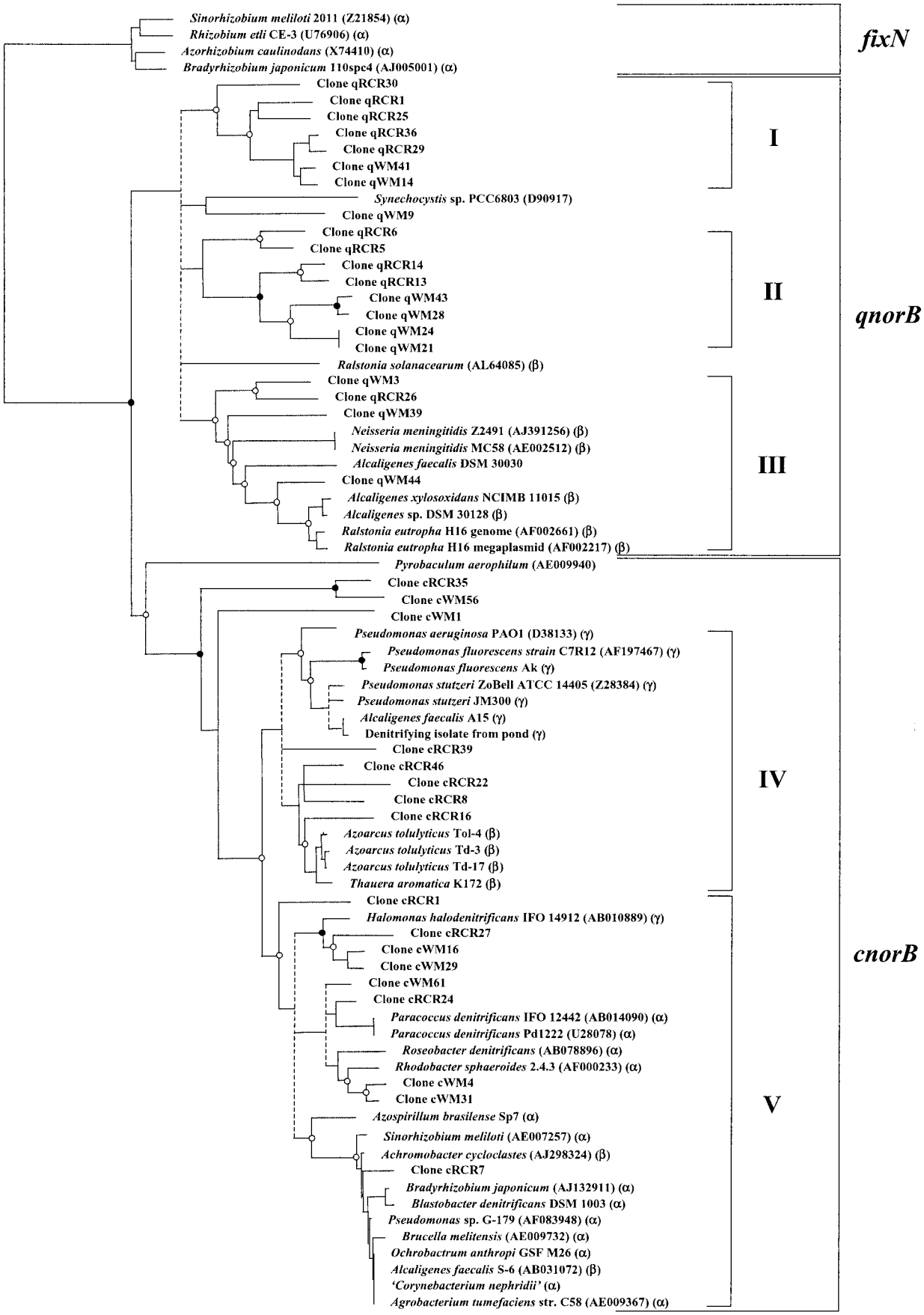
Phylogenetic analysis. Phylogenetic analyses consistently separated the *norB* genes into two major clusters, *qnorB* and *cnorB* genes (Fig. 2). The *qnorB* cluster consisted of sequences from denitrifying strains of the genus *Alcaligenes* and *Ralstonia eutropha* and several nondenitrifying strains. All denitrifying strains were found within one subcluster (subcluster III), but genes from two nondenitrifying strains of *Neisseria meningitidis* were closely related, belonging to the same subcluster. Genes from described species within this cluster originated from members of the β-proteobacteria. The majority of genes from environmental samples fell into subcluster I and subcluster II, which consisted of environmental clones exclusively. Clones from the Red Cedar River and the Washington margin sediments were found in both subclusters, thus not showing any habitat-specific clustering. A minority of clones belonged to subcluster III, which was dominated by the described species.

For *cnorB*, genes were grouped into two major subclusters (IV and V), with four distantly related sequences from environmental clones cRCR5, cRCR8, and cWM1 and *Pyrobaculum aerophilum* branching deeply outside these subclusters. Cluster IV consisted of *cnorB* sequences from the genera *Pseudomonas*, *Azoarcus,* and *Thauera*, and cluster V consisted of genes from a variety of denitrifying species belonging to the α-proteobacteria except for *Halomonas halodenitrificans* (γ-proteobacterium) and *Achromobacter cycloclastes* and *Alcaligenes faecalis* S-6 (β-proteobacteria), respectively. Environmental clones from both habitats clustered on separate branches within the radiation of *norB* sequences from denitrifying isolates belonging to the α-, β-, and γ-proteobacteria.

DISCUSSION

NO production occurs during respiratory denitrification but also during nitrate respiration of fungi (36, 37). However, nitric oxide reductase (cytochrome P450nor) from denitrifying fungi differs structurally and functionally from prokaryotic nitric oxide reductases (25) and is restricted to fungi (39, 40). Therefore, primer sets developed to detect each of the two classes of *norB* genes (*qnorB* and *cnorB*) in our PCR assays are specific for prokaryotic NO reductase genes. Furthermore, the primer sets were designed to exclude amplification of FixN, subunit I of cytochrome oxidases, which was proposed to be a NorB homologue (32, 41).

With these primers, a *norB* gene of either type was found within all of the denitrifiers tested. Genes of the *Ralstonia* type were detected in other denitrifying strains (*Alcaligenes faecalis* DSM 30030, *Alcaligenes xylosoxidans* NCIMB11015, and *Alcaligenes* sp. strain DSM 30128), indicating that this *norB* type is found in denitrifiers other than *Ralstonia eutropha. qnorB* genes from these denitrifiers and *Ralstonia eutropha* which are phylogenetically affiliated to the β-proteobacteria are more closely related to each other than to any *norB* gene from nondenitrifying species. However, in *Thauera aromatica* and *Azo-*


Sinorhizobium meliloti 2011 (Z21854) (α)
Rhizobium etli CE-3 (U76906) (α)
Azorhizobium caulinodans (X74410) (α)
Bradyrhizobium japonicum 110spc4 (AJ005001) (α)
Clone qRCR30
Clone qRCR1
Clone qRCR25
Clone qRCR36
Clone qRCR29
Clone qWM41
Clone qWM14
Synechocystis sp. PCC6803 (D90917)
Clone qWM9
Clone qRCR6
Clone qRCR5
Clone qRCR14
Clone qRCR13
Clone qWM43
Clone qWM28
Clone qWM24
Clone qWM21
Ralstonia solanacearum (AL64085) (β)
Clone qWM3
Clone qRCR26
Clone qWM39
Neisseria meningitidis Z2491 (AJ391256) (β)
Neisseria meningitidis MC58 (AE002512) (β)
Alcaligenes faecalis DSM 30030
Clone qWM44
Alcaligenes xylosoxidans NCIMB 11015 (β)
Alcaligenes sp. DSM 30128 (β)
Ralstonia eutropha H16 genome (AF002661) (β)
Ralstonia eutropha H16 megaplasmid (AF002217) (β)
Pyrobaculum aerophilum (AE009940)
Clone cRCR35
Clone cWM56
Clone cWM1
Pseudomonas aeruginosa PAO1 (D38133) (γ)
Pseudomonas fluorescens strain C7R12 (AF197467) (γ)
Pseudomonas fluorescens Ak (γ)
Pseudomonas stutzeri ZoBell ATCC 14405 (Z28384) (γ)
Pseudomonas stutzeri JM300 (γ)
Alcaligenes faecalis A15 (γ)
Denitrifying isolate from pond (γ)
Clone cRCR39
Clone cRCR46
Clone cRCR22
Clone cRCR8
Clone cRCR16
Azoarcus tolulyticus Tol-4 (β)
Azoarcus tolulyticus Td-3 (β)
Azoarcus tolulyticus Td-17 (β)
Thauera aromatica K172 (β)
Clone cRCR1
Halomonas halodenitrificans IFO 14912 (AB010889) (γ)
Clone cRCR27
Clone cWM16
Clone cWM29
Clone cWM61
Clone cRCR24
Paracoccus denitrificans IFO 12442 (AB014090) (α)
Paracoccus denitrificans Pd1222 (U28078) (α)
Roseobacter denitrificans (AB078896) (α)
Rhodobacter sphaeroides 2.4.3 (AF000233) (α)
Clone cWM4
Clone cWM31
Azospirillum brasilense Sp7 (α)
Sinorhizobium meliloti (AE007257) (α)
Achromobacter cycloclastes (AJ298324) (β)
Clone cRCR7
Bradyrhizobium japonicum (AJ132911) (α)
Blastobacter denitrificans DSM 1003 (α)
Pseudomonas sp. G-179 (AF083948) (α)
Brucella melitensis (AE009732) (α)
Ochrobactrum anthropi GSF M26 (α)
Alcaligenes faecalis S-6 (AB031072) (β)
'Corynebacterium nephridii' (α)
Agrobacterium tumefaciens str. C58 (AE009367) (α)
fixN
I
II
III
qnorB
IV
V
cnorB

arcus tolulyticus, also β-proteobacteria, *norB* genes of the *cnorB* type were detected instead. The presence of *cnorB* genes in a variety of denitrifying strains was confirmed by amplification and sequencing of these genes. Additionally, we demonstrated the *cnorB* type for a variety of denitrifying strains, including *Azospirillum brasilense*, *Blastobacter denitrificans*, "*Corynebacterium nephridii*," and *Ochrobactrum anthropi*. These organisms belong to the α-, β-, and γ-proteobacteria, respectively.

Primer specificity was also confirmed by cloning *norB* genes from a freshwater sediment and a marine sediment. Clones chosen for sequencing by screening through RFLP revealed a large number of unique *norB* genotypes in both habitats at a level similar to our observations of *nirK* and *nirS* gene diversity in marine sediment samples (4). Sequence analysis of two redundant and representative clones (qWM21 and qWM24) confirmed the specificity of the predominant group of *qnorB* genes from the Washington margin sample. Generally, the qnorB primer combination was highly specific because all clones sequenced were indeed *norB* gene fragments. For *cnorB*, two clones from the Washington margin sample were not identified as *norB* sequences. A third clone was a chimera and was excluded from further analysis. All deduced amino acid sequences of specific *norB* amplicons exhibited the three conserved histidine residues corresponding to positions 194, 245, and 246 of the *Paracoccus denitrificans* amino acid sequence, which are required for binding of two hemes and nonheme iron (Fe_B) (1, 10, 44). These residues are conserved in both types of NorB, which supports the fact that even the very unrelated *norB* sequences from environmental samples are indeed fragments of *norB* genes. Additionally, a glutamate residue (E_{198} in *Paracoccus denitrificans*) which is essential for the activity but not for the assembly of nitric oxide reductases (6) was recovered in all *norB* sequences. Conservation of structural motifs provides strong evidence for proper functionality of the predicted *norB* gene products, although they are rather dissimilar in their primary structure.

Phylogenetic analysis placed environmental *qnorB* genes in subclusters separate from those from cultivated organisms and environmental *cnorB* genes on separate branches within clusters, demonstrating the now typically observed discrepancy of genes from cultured organisms versus environmental sources (4, 35). Generally, the topology of the trees was stable with respect to the two main clusters of *qnorB* and *cnorB* genes and for the subclustering when *fixN* genes were included as an outgroup in tree calculation. However, the positions of subclusters within the main clusters depended on the method of tree calculation. Therefore, multifurcations were introduced when the topology was not resolved. Thus, three main clusters of *qnorB* genes were found, two of which consisted exclusively of environmental clones, suggesting that these genes originated from as yet uncultivated species with unknown physiological features, whereas the third contained mainly genes from described species.

The newly detected *qnorB* genes from *Alcaligenes* spp. also grouped into the latter subcluster with *qnorB* from *Ralstonia eutropha* and nondenitrifying strains such as the *Neisseria* sp. Other than in the photosynthetic cyanobacterium *Synechocystis* and *Ralstonia solanacearum, qnorB* was mainly detected in nondenitrifying pathogens, e.g., *Mycobacterium avium*, *Staphylococcus aureus*, and *Corynebacterium diphtheriae* (18), although these sequences were not included in our analysis due to uncertainties in the alignment. The recruitment of this single gene from the denitrification pathway by pathogens may provide a detoxification mechanism for NO produced by macrophages from the host defense system, e.g., qNorB of *Neisseria gonorrhoeae* was found to be active in its host (19). Nitric oxide has cytotoxic effects and inhibits a number of metalloproteins found in bacterial respiratory chains (44). Under environmental conditions, organisms face NO produced by bacteria such as nitrifiers (7) and actinomycetes (11), by cyanobacteria, green algae (22), and even higher plants (45). The function of a *norB* gene in the nondenitrifying *Synechocystis* sp. is probably for NO removal because it was demonstrated to be potentially functional (5).

The majority of known denitrifiers harbor *norB* genes of the *norCB* type, based on analysis of the genes from pure cultures obtained in this study and on database sequences. Environmental *norB* clones of this class with the exception of three deeply branching clones (cRCR35, cWM1, and cWM56) are found in subclusters with known denitrifiers belonging to the *Proteobacteria*. The distribution of *norB* sequences among known denitrifiers suggests that for the *cnorB* branch, most of the environmental sequences might be derived from *Proteobacteria*, which is consistent with our findings from a previous study exploring the diversity of nitrite reductase genes in environmental samples (4). More obscure is the origin of the very unrelated *cnorB* genes and genes of the *qnorB* subclusters I and II. Interestingly, *norB* genes from known denitrifiers did not cluster strictly according to the phylogeny of the organisms and their type of nitrite reductase genes (4), suggesting that denitrification genes were acquired through horizontal gene transfer. However, these inconsistencies in gene phylogeny were not surprising, considering that in *P. stutzeri*, denitrification genes are organized in gene clusters (51), in *Ralstonia eutropha* they exist on megaplasmids (10), and they may be dispersed over the chromosome, as in *Bradyrhizobium japonicum* (23). Further evidence for horizontal gene transfer is the fact that the *norB* genes from both marine and freshwater sediments were almost evenly distributed throughout all *norB* subclusters, which was in contrast to our previous study, which found nitrite reductase gene clusters to be strongly habitat specific (4, 27).

In summary, the development of these primer sets to target

FIG. 2. Phylogenetic analysis of *norB* genes. Neighbor joining tree (Jones-Taylor-Thornton model of amino acid exchange) based on partial *norB* amplicons (100 amino acids; accession numbers in parentheses). A consensus tree was constructed from distance methods (neighbor joining and FITCH), parsimony, and maximum likelihood by introducing multifurcations (dashed lines) where tree topology was not consistently resolved. Bootstrap values were generated from 1,000 replicates of neighbor joining and parsimony analysis. ●, bootstrap values >90%; ○, bootstrap values 50 to 90%; bootstrap values of <50% are omitted. Clones obtained from the Washington margin and Red Cedar River are designated WM and RCR, respectively, plus q and c for *qnorB* and *cnorB*, respectively. The phylogenetic positions of isolates are indicated by α, β, and γ for the subgroups of the *Proteobacteria*. Roman numbers indicate clusters of *norB* genes.

norB genes allows broad detection of the last denitrification gene for which a detection assay was lacking, nitric oxide reductase. Thus, molecular tools are available to explore all key steps in the denitrification process independently, for example, to study horizontal gene transfer or expression of denitrification genes.

ACKNOWLEDGMENTS

We gratefully acknowledge Ralf Conrad for allowing us to complete this work in his laboratory and Michael W. Friedrich for advice for constructing phylogenetic trees. Valérie Michotey and Rainer Cramm are acknowledged for kindly providing *Pseudomonas fluorescens* strain AK and *Synechocystis* sp. strain PCC6803; Sabine A. Rech and John K. Davis are acknowledged for kindly providing DNA extracts from *Azoarcus* sp. and *Thauera aromatica,* respectively; and Allan H. Devol is acknowledged for providing the Washington margin sediment core.

This research was funded by the Biotechnological Investigations-Ocean Margins Program, Biological and Environmental Research, U.S. Department of Energy (grant DE-FG02-98ER 62.535).

REFERENCES

1. **Arai, H., Y. Igarashi, and T. Kodama.** 1995. The structural genes for nitric oxide reductase from *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **1261:** 279–284.
2. **Bartnikas, T. B., I. E. Tosques, W. P. Laratta, J. Shi, and J. P. Shapleigh.** 1997. Characterization of the nitric oxide reductase-encoding region in *Rhodobacter sphaeroides* 2.4. J. Bacteriol. **179:**3534–3540.
3. **Braker, G., A. Fesefeldt, and K.-P. Witzel.** 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Appl. Environ. Microbiol. **64:**3769–3775.
4. **Braker, G., J. Zhou, L. Wu, A. H. Devol, and J. M. Tiedje.** 2000. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. Appl. Environ. Microbiol. **66:**2096–2104.
5. **Büsch, A., B. Friedrich, and R. Cramm.** 2002. Characterization of the *norB* gene, encoding nitric oxide reductase, in the nondenitrifying cyanobacterium *Synechocystis* sp. strain PCC6803. Appl. Environ. Microbiol. **68:**668–672.
6. **Butland, G., S. Spiro, N. J. Watmough, and D. J. Richardson.** 2001. Two conserved glutamates in the bacterial nitric oxide reductase are essential for activity but not assembly of the enzyme. J. Bacteriol. **183:**189–199.
7. **Conrad, R.** 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). Microbiol. Rev. **60:**609–640.
8. **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.
9. **Cramm, R., A. Pohlmann, and B. Friedrich.** 1999. Purification and characterization of the single-component nitric oxide reductase from *Ralstonia eutropha* H16. FEBS Lett. **460:**6–10.
10. **Cramm, R., R. A. Siddiqui, and B. Friedrich.** 1997. Two isofunctional nitric oxide reductases in *Alcaligenes eutrophus* H16. J. Bacteriol. **179:**6769–6777.
11. **Cutruzzolà, F.** 1999. Bacterial nitric oxide synthesis. Biochim. Biophys. Acta **1411:**231–249.
12. **Felsenstein, J.** 1993. PHYLIP: phylogeny inference package (version 3.5c). Department of Genetics, University of Washington, Seattle, Wash.
13. **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.
14. **Gliesche, C. G., and P. Hirsch.** 1992. Mutagenesis and chromosome mobilization in *Hyphomicrobium facilis* B-522. Can. J. Microbiol. **38:**1167–1174.
15. **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.
16. **Gregory, L. G., A. Karakas-Sen, D. J. Richardson, and S. Spiro.** 2000. Detection of genes for membrane-bound nitrate reductase in nitrate-respiring bacteria and in community DNA. FEMS Microbiol. Lett. **183:**275–279.
17. **Hallin, S., and P.-E. Lindgren.** 1999. PCR detection of genes encoding nitrite reductases in denitrifying bacteria. Appl. Environ. Microbiol. **65:** 1652–1657.
18. **Hendriks, J., A. Oubrie, J. Castresana, A. Urbani, S. Gemeinhardt, and M. Saraste.** 2000. Nitric oxide reductases in bacteria. Biochim. Biophys. Acta **1459:**266–273.
19. **Householder, T. C., E. M. Fozo, J. A. Cardinale, and V. L. Clark.** 2000. Gonococcal nitric oxide reductase is encoded by a single gene, *norB*, which is required for anaerobic growth and is induced by nitric oxide. Infect. Immun. **68:**5241–5246.
20. **Kukimoto, M., M. Nishiyama, M. Tanokura, and S. Horinouchi.** 2000. Gene organization for nitric oxide reduction in *Alcaligenes faecalis* S-6. Biosci. Biotechnol. Biochem. **64:**852–857.
21. **Lyman, J., and R. H. Fleming.** 1940. Composition of sea water. J. Mar. Res. **3:**134–167.
22. **Mallick, N., L. C. Rai, F. H. Mohn, and C. J. Soeder.** 1999. Studies on nitric oxide (NO) formation by the green alga *Scenedesmus obliquus* and the diazotrophic cyanobacterium *Anabaena doliolum*. Chemosphere **39:**1601–1610.
23. **Mesa, S., M. Göttfert, and E. J. Bedmar.** 2001. The *nir*, *nor*, and *nos* denitrification genes are dispersed over the *Bradyrhizobium japonicum* chromosome. Arch. Microbiol. **176:**136–142.
24. **Michotey, V., V. Méjean, and P. Bonin.** 2001. Comparison of methods for quantification of cytochrome cd_1-denitrifying bacteria in environmental marine samples. Appl. Environ. Microbiol. **66:**1564–1571.
25. **Okamoto, N., Y. Imai, H. Shoun, and Y. Shiro.** 1998. Site-directed mutagenesis of the conserved threonine (Thr243) of the distal helix of fungal cytochrome $P450_{nor}$. Biochemistry **37:**8839–8847.
26. **Petri, R., and J. Imhoff.** 2000. The relationship of nitrate reducing bacteria on the basis of *narH* gene sequences and comparison of *narH* and 16S rDNA based phylogeny. Syst. Appl. Microbiol. **23:**47–57.
27. **Priemé, A., G. Braker, and J. M. Tiedje.** 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. Appl. Environ. Microbiol. **68:**1893–1900.
28. **Purdy, K. J., T. M. Embley, S. Takii, and D. B. Nedwell.** 1996. Rapid extraction of DNA and rRNA from sediments by a novel hydroxyapatite spin-column method. Appl. Environ. Microbiol. **62:**3905–3907.
29. **Richardson, D. J.** 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology **146:**551–571.
30. **Sakurai, N., and T. Sakurai.** 1998. Genomic DNA cloning of the region encoding nitric oxide reductase in *Paracoccus halodenitrificans* and a structural model relevant to cytochrome oxidase. Biochem. Biophys. Res. Commun. **243:**400–406.
31. **Sambrook, J. E., F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor. N.Y.
32. **Saraste, M., and J. Castresana.** 1994. Cytochrome oxidase evolved by tinkering with denitrification enzymes. FEBS Lett. **341:**1–4.
33. **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.
34. **Scala, D. J., and L. J. Kerkhof.** 1998. Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol. Lett. **162:**61–68.
35. **Scala, D. J., and L. J. Kerkhof.** 1999. Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. Appl. Environ. Microbiol. **65:** 1681–1687.
36. **Shoun, H., D.-H. Kim, H. Uchiyama, and J. Sugiyama.** 1992. Denitrification by fungi. FEMS Microbiol. Lett. **94:**277–283.
37. **Shoun, H., and T. Tanimoto.** 1991. Denitrification by the fungus *Fusarium oxysporum* and involvement of cytochrome P-450 in the respiratory nitrite reduction. J. Biol. Chem. **17:**1078–1082.
38. **Staley, J. T.** 1968. *Prosthecomicrobium* and *Ancalomicrobium*, new prosthecate freshwater bacteria. J. Bacteriol. **95:**1921–1944.
39. **Takaya, N., S. Suzuki, S. Kuwazaki, H. Shoun, F. Maruo, M. Yamaguchi, and K. Takeo.** 1999. Cytochrome P450nor, a novel class of mitochondrial cytochrome P450 involved in nitrate respiration in the fungus *Fusarium oxysporum*. Arch. Biochem. Biophys. **372:**340–346.
40. **Tsuruta, S., N. Takaya, L. Zhang, H. Shoun, K. Kimura, M. Hamamoto, and T. Nakase.** 1998. Denitrification by yeasts and occurrence of cytochrome P450nor in *Trichosporon cutaneum*. FEMS Microbiol. Lett. **168:**105–110.
41. **van der Oost, J., A. P. de Boer, J. W. de Gier, W. G. Zumft, A. H. Stouthamer, and R. J. van Spanning.** 1994. The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. FEMS Microbiol. Lett. **121:**1–9.
42. **van Elsas, J. D., and K. Smalla.** 1995. Extraction of microbial community DNA from soils, p. 1.3.3/1–1.3.3/11. *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.
43. **Vincent, J. M.** 1971. A manual for the practical study of root-nodule bacteria. Burgess and Son LTB, Oxford, United Kingdom.
44. **Watmough, N. J., G. Butland, M. R. Cheesman, J. W. B. Moir, D. J. Richardson, and S. Spiro.** 1999. Nitric oxide in bacteria: synthesis and consumption. Biochim. Biophys. Acta **1411:**456–474.
45. **Yamasaki, H., and Y. Sakihama.** 2000. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Lett. **468:**89–92.
46. **Ye, R. W., B. A. Averill, and J. M. Tiedje.** 1992. Characterization of Tn*5* mutants deficient in dissimilatory nitrite reduction in *Pseudomonas* sp. strain G-179, which contains a copper nitrite reductase. J. Bacteriol. **174:**6653–6658.
47. **Ye, R. W., M. R. Fries, S. G. Bezborodnikov, B. A. Averill, and J. M. Tiedje.**

1993. 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.
48. **Zhou, J., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** 1995. Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth of toluene and description of *Azoarcus tolulyticus* sp. nov. Int. J. Syst. Bacteriol. **45:**500–506.
49. **Zhou, J., M. E. Davey, J. B. Figueras, E. Rivkina, D. Gilichinsky, and J. M. Tiedje.** 1997. Phylogenetic diversity of a bacterial commmunity determined from Siberian tundra soil DNA. Microbiology **143:**3913–3919.
50. **Zumft, W. G.** 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:**533–616.
51. **Zumft, W. G.** 1997. Enzyme diversity and mosaic gene organization in denitrification. Antonie van Leeuwenhoek **71:**43–58.
52. **Zumft, W. G., C. Braun, and H. Cypers.** 1994. Nitric oxide reductase from *Pseudomonas stutzeri*. Eur. J. Biochem. **219:**481–490.
53. **Zumft, W. G., H. Körner, S. Löchelt, A. Viebrock, and K. Frunzke.** 1988. Defects in cytochrome cd_1-dependent nitrite respiration of transposon Tn*5*-induced mutants from *Pseudomonas stutzeri*. Arch. Microbiol. **149:**492–498.