

## Nitrite Reductase Genes (*nirK* and *nirS*) as Functional Markers To Investigate Diversity of Denitrifying Bacteria in Pacific Northwest Marine Sediment Communities

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**Genetic heterogeneity of denitrifying bacteria in sediment samples from Puget Sound and two sites on the Washington continental margin was studied by PCR approaches amplifying *nirK* and *nirS* genes. These structurally different but functionally equivalent single-copy genes coding for nitrite reductases, a key enzyme of the denitrification process, were used as a molecular marker for denitrifying bacteria. *nirS* sequences could be amplified from samples of both sampling sites, whereas *nirK* sequences were detected only in samples from the Washington margin. To assess the underlying *nir* gene structure, PCR products of both genes were cloned and screened by restriction fragment length polymorphism (RFLP). Rarefaction analysis revealed a high level of diversity especially for *nirS* clones from Puget Sound and a slightly lower level of diversity for *nirK* and *nirS* clones from the Washington margin. One group dominated within *nirK* clones, but no dominance and only a few redundant clones were seen between sediment samples for *nirS* clones in both habitats. Hybridization and sequencing confirmed that all but one of the 228 putative *nirS* clones were *nirS* with levels of nucleotide identities as low as 45.3%. Phylogenetic analysis grouped *nirS* clones into three distinct subclusters within the *nirS* gene tree which corresponded to the two habitats from which they were obtained. These sequences had little relationship to any strain with known *nirS* sequences or to isolates (mostly close relatives of *Pseudomonas stutzeri*) from the Washington margin sediment samples. *nirK* clones were more closely related to each other than were the *nirS* clones, with 78.6% and higher nucleotide identities; clones showing only weak hybridization signals were not related to known *nirK* sequences. All *nirK* clones were also grouped into a distinct cluster which could not be placed with any strain with known *nirK* sequences. These findings show a very high diversity of *nir* sequences within small samples and that these novel *nir* clusters, some very divergent from known sequences, are not known in cultivated denitrifiers.**

Denitrification is a dissimilatory process where oxidized nitrogen compounds ( $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) are used as alternative electron acceptors for energy production. Nitrogen oxides are reduced stepwise to gaseous end products ( $\text{NO}$ ,  $\text{N}_2\text{O}$ , and  $\text{N}_2$ ) which are concomitantly released, leading to a loss of fixed nitrogen to the environment. In marine coastal sediments, 40 to 50% of external inputs of dissolved inorganic nitrogen are removed by denitrification (23), resulting in an unbalanced nitrogen budget in the ocean (5, 7). Accumulation of the intermediates  $\text{NO}$  and  $\text{N}_2\text{O}$  contributes to global warming and the destruction of the ozone layer (6). Thus, the underlying communities of denitrifying bacteria and their responses to environmental conditions need to be understood. Commonly, 16S rDNA molecules are tools to investigate microbial communities which avoid limitations of culturability (9, 11, 16). Lately, several functional genes were shown to be useful for the same purpose (17, 19, 28) because their phylogeny is congruent or very similar to phylogenetic relationships based on 16S rRNA gene (rDNA) analyses. In many cases, functional genes provide a resolution below species level because of higher

evolutionary rates of the less conserved functional molecules. Additionally, this approach may indicate functional diversity in the environment.

An approach involving 16S rDNA does not appear to be suitable to investigate communities of denitrifying bacteria, as denitrification is widespread among phylogenetically unrelated groups (33). Common metabolic functions have been used for detection and community analysis of denitrifying bacteria such as nitrite and nitrous oxide reduction (3, 12, 21, 22). These key steps of the denitrification process are catalyzed by nitrite and nitrous oxide reductase, respectively. Two structurally different but functionally equivalent enzymes catalyze nitrite reduction: a copper and a cytochrome *cd*<sub>1</sub>-containing nitrite reductase encoded by the genes *nirK* and *nirS*, respectively. The *nirK* and *nirS* genes were useful targets for PCR primers to detect communities of denitrifying bacteria in samples from freshwater environments and activated sludge (3, 12). In the present study, we successfully applied these PCR methods and subsequent clone analysis to detect denitrifying bacteria in sections of marine sediment cores from Puget Sound and from the Washington margin. A cultivation study was set up concomitantly with sediment samples from the Washington margin to isolate representative denitrifying bacteria from the same environment. Cultivation of denitrifiers with *nir* genes closely related to sequences from the cloning experiment would allow details of function of novel gene sequences to be explored.

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TABLE 1. Characteristics of sampling stations

Station	Latitude, longitude	Water depth (m)	Penetration depth into sediment (cm)		Carbon (%)	Bottom water O <sub>2</sub> concn (μM)	Isolate(s)	Clones	
			O <sub>2</sub>	NO <sub>3</sub> <sup>-</sup>				<i>nirK</i>	<i>nirS</i>
Washington margin									
301 (A)	46°48'60"N, 124°37'20"W	119	0.4	0.4	1.69	151	A3-5		
303 (B)	46°47'42"N, 125°21'80"W	1,936	0.9	0.9	2.34	75	B9-12	wB	wA-C
304 (C)	46°45'05"N, 126°00'58"W	2,530	2.2	6.0	1.62	93	C10-1	wC	wD-F
305 (D)	46°44'97"N, 127°59'96"W	2,664	3.4	21.0	1.18	112	D4-14, D7-6, D9-1		
306 (E)	46°29'60"N, 124°43'22"W	630	0.6	0.6	2.52	27	E4-2		
307 (F)	46°26'61"N, 124°47'22"W	997	0.7	0.8	3.05	23	F8-5, F9-2		
Puget Sound									
Carkeek	47°43'50"N, 122°23'90"W	182	0.4	0.4	~2	202			pA/pB/pC

### MATERIALS AND METHODS

**Sediment sampling.** Sediment cores investigated came from offshore Washington coast and Puget Sound, Washington. Sampling stations and their locations are described in Table 1. The Washington coast sediment cores were separated each into 10 sections of 0.5 or 1.0 cm. Sections were collected (November 1997) into sterile polypropylene bags and stored at 4°C until isolation of denitrifying bacteria and DNA extraction in Michigan. The Puget Sound sediment core was collected in March 1998, divided into three sections (1.0 to 1.5 cm, 1.5 to 2.0 cm, and 6.0 to 6.5 cm), and placed in sterile polypropylene bags. Samples were stored on ice, shipped to Michigan on dry ice, and stored at -20°C until DNA extraction.

**Isolation of denitrifying bacteria and growth conditions.** To isolate denitrifying bacteria from the Washington margin (Table 1), samples from 10 discrete depth intervals from six stations were diluted in 10-fold series, and each dilution was incubated anaerobically at room temperature in nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with artificial seawater (3% salt [14]) and KNO<sub>3</sub> (5 mM). After 2 weeks, denitrifying activity was monitored by nitrate disappearance using the Szechrome reagent (Polysciences, Inc., Warrington, Pa.). The highest dilution from each sample showing denitrifying activity was used for bacterial isolation on nutrient agar. At least four bacteria differing in colony morphology, color, and size were picked from each sample. Prior to sequencing, unique isolates were differentiated by repetitive extragenic palindromic-PCR fingerprint analysis (18). The 16S rDNA gene sequencing was performed as described previously (32), and the general taxonomic position was determined with the BLAST program from the Genetics Computer Group program package (10).

Denitrifying bacteria were also enriched from the Puget Sound core in 10-fold serial dilutions but in marine medium (Gibco BRL, Gaithersburg, Md.) supplemented with 5 mM KNO<sub>3</sub> and incubated anaerobically. Additionally, these enrichments were supplemented with either yeast extract (0.5 mg ml<sup>-1</sup>) or Casitone (0.5 mg ml<sup>-1</sup>). After incubation for 8 months at 22°C, the enrichments were tested microscopically for growth and qualitatively for the removal of nitrate and nitrite, using test sticks (Merck, Darmstadt, Germany).

**Extraction and purification of DNA.** DNA was prepared from pure cultures, environmental samples, and enrichments. (i) Genomic DNA from pure cultures was obtained by lysozyme-proteinase K-sodium dodecyl sulfate (SDS) treatment. DNA was purified by precipitation with hexamethylammonium bromide (Sigma Chemical Co., St. Louis, Mo.), phenol-chloroform extraction, and subsequent precipitation with ethanol (1). (ii) Total DNA was extracted from sediment sections of Washington margin sediment cores 303 and 304 (sections 0.5 to 1.0 cm [1,936 m] and 0.0 to 0.5 cm [2,530 m]) and from the three sections from Puget Sound (Table 1). DNA from 5 g of sediment each was extracted by the method of van Elsas and Smalla (29), using the freeze-thaw procedure. The method was modified by using an additional proteinase K treatment (50 μl of a 20-mg ml<sup>-1</sup> solution) after incubation with SDS. (iii) Total DNA was extracted from 5 ml of liquid enrichment culture of the 10<sup>-2</sup> dilution. Cells were harvested by sequential centrifugation (13,600 × g, 15 min, 22°C) in the same 2-ml tube, resuspended in 1.5 ml of 120 mM sodium phosphate buffer (pH 8.0). DNA was extracted as described for step ii. DNA was quantified and analyzed spectrophotometrically at 230, 260, and 280 nm.

**PCR amplification of *nir* fragments.** Fragments of the *nirK* and *nirS* gene were amplified using primer pairs *nirK1F-nirK5R* for *nirK* and *nirS1F-nirS6R* for *nirS*, developed by Braker et al. (3). Touchdown PCR was done in a model 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) using conditions as described elsewhere (3) except that the annealing temperature during the first 10 cycles was shifted to 56 to 51 and to 54°C during the following 25 cycles. Additionally, the amount of primer was doubled to 1 μM each primer, and to increase specificity of the reactions, bovine serum albumin (400 ng μl<sup>-1</sup>; Boehringer Mannheim) was added. Aliquots of 10 μl of the reactions were analyzed by

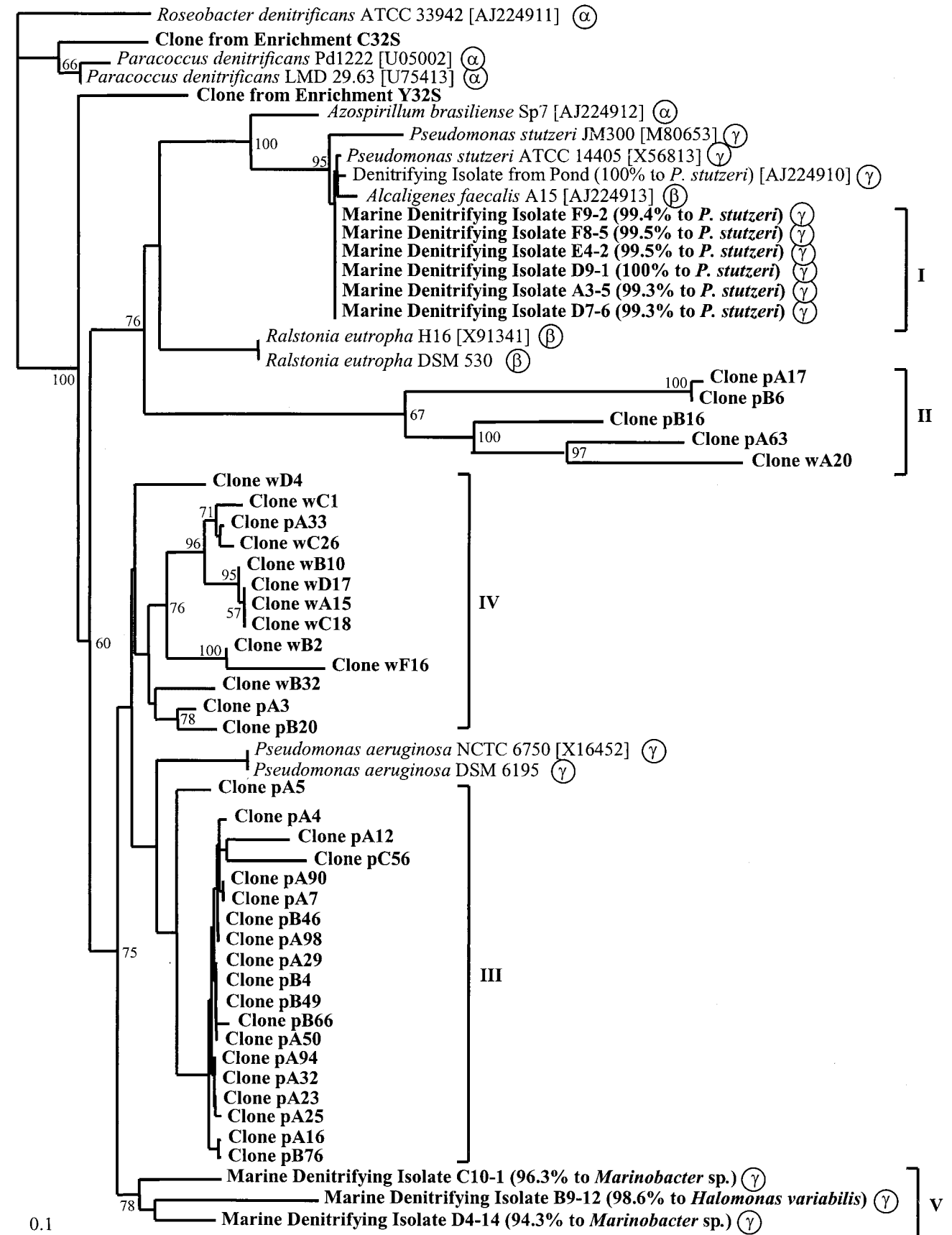
electrophoresis on 2% (wt/vol) agarose gels (Gibco BRL) followed by 15 min of staining with ethidium bromide (0.5 mg liter<sup>-1</sup>). Bands were visualized by UV excitation.

**Cloning of *nirK* and *nirS* PCR products from sediment samples and enrichments.** For *nirK*, PCR products from sediment samples were purified by eluting the bands from an agarose gel (2% [wt/vol]) using the QIAquick gel extraction kit (Qiagen, Chatsworth, Calif.) as specified by the manufacturer. Eluted *nirK* PCR (3 μl) products or amplified *nirS* PCR products (3 μl) were cloned using the TA cloning kit (Invitrogen, San Diego, Calif.). One hundred clones for each sediment sample were randomly selected for further analysis. *nirK* or *nirS* PCR products were cloned from enrichments, and eight clones each were analyzed. A small amount of cell material picked up with a toothpick was resuspended in a preprepared 25-μl PCR mix, and inserts were amplified as described elsewhere (31). Aliquots (2.5 μl) were analyzed on 2% (wt/vol) agarose gels. PCR product (0.2 μl) from clones containing inserts of the estimated size were further used for a 25-μl nested PCR amplification. In this step, the *nirK* and *nirS* primer pairs and conditions were used as described for the initial PCR. Aliquots of the PCR were analyzed on 2% (wt/vol) agarose gels.

**RFLP screening of *nirK* and *nirS* clone libraries.** PCR products were first screened by restriction fragment length polymorphism (RFLP). For *nirK* clones, PCR products were digested in two separate reactions with 0.3 U of restriction enzymes *Hae*III and *Msp*I (Gibco BRL) at 37°C overnight. For *nirS* clones, PCR products were digested in a single reaction with 0.3 U of each restriction enzyme *Hha*I and *Msp*I (Gibco BRL) at 37°C overnight. The digested products were separated by gel electrophoresis in 3.5% (wt/vol) Metaphor agarose (FMC Bio-products, Rockland, Maine) in freshly prepared 1× Tris-borate-EDTA buffer at 4°C with 7 V cm<sup>-1</sup> for 4 h. The RFLP patterns were analyzed and clustered with the GelCompar software (Applied Maths, Kortrijk, Belgium) applying the unweighted pair group method using arithmetic averages (UPGMA) and the Jaccard algorithm. The resulting clusters were additionally compared by eye.

**Dot blot hybridization.** One microliter, or 1 μl of a dilution of up to 1:3, of each nested PCR product from the clones was spotted onto a positively charged nylon membrane (Zeta-Probe; BioRad, Hercules, Calif.) and fixed by UV cross-linking for 45 s at 302 nm. Four different probes for each gene were used to hybridize with cloned PCR products: for *nirK*, PCR products obtained with primer pair *nirK1F-nirK5R* from *Pseudomonas* sp. strain G-179 (30), *Rhodobacter sphaeroides* f. sp. *denitrificans* (20), *Blastobacter denitrificans* DSM 1113, and *Alcaligenes* sp. strain DSM 30128. For *nirS*, PCR products were generated with primer pair *nirS1F-nirS6R* from *Pseudomonas stutzeri* JM300, *Pseudomonas aeruginosa* NCTC 6750, *Roseobacter denitrificans* ATCC 33942, and two marine denitrifying isolates, B9-12 and D4-14 (this study). Probes generated from *P. stutzeri* JM300 and *P. aeruginosa* were used in a mixture of equal amounts (25). PCR products were purified by elution from an agarose gel (2% [wt/vol]) by using the QIAquick gel extraction kit (Qiagen) as specified by the manufacturer. The probes were randomly labeled by using the digoxigenin DNA labeling and detection kit (Boehringer Mannheim) as specified by the manufacturer. After prehybridization of the membranes at 42°C for 6 h in DIG Easy Hyb solution (Boehringer Mannheim), hybridization was performed at 42°C overnight in a DIG Easy Hyb solution containing the specific probe (25 ng ml<sup>-1</sup>). After hybridization, the membrane was washed twice for 5 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% (wt/vol) SDS and twice at 56°C for *nirK* and at 58°C for *nirS* for 15 min in 0.5× SSC-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 Mannheim). Strength of hybridization signals was assigned visually as 0 (no hybridization signal) to 6 (strong hybridization signal).

**Southern blot hybridization.** Aliquots of *nirK* PCR products (10 μl) from Puget Sound DNA extracts and from one Washington coast (0.5 to 1.0 cm, 1,936



m) DNA extract and a *nirK* PCR product from *Alcaligenes* sp. strain DSM 30128 were separated in an 2% (wt/vol) agarose gel. The DNA was transferred onto a positively charged nylon membrane (Bio-Rad) by capillary transfer (26) and UV cross-linked to the membrane with UV light (45 s at 302 nm). Hybridization with the probes for *nirK* was performed as described above.

**Sequencing of *nir* products and phylogenetic analysis.** For DNA sequencing, amplified products were purified with the QIAquick PCR purification kit (Qiagen) as specified. DNA sequences were determined by direct sequencing with a model 373A DNA sequencer (Applied Biosystems Inc., Foster City, Calif.) by using dye terminator chemistry. The primers used for sequencing were *nirK5R* for *nirK* and *nirS1F* and *nirS6R* for *nirS*.

Nucleotide sequences and deduced amino acid sequences were aligned with sequences from the EMBL database, using the CLUSTAL program from the Genetics Computer Group program package (10), and dendrograms were constructed by using the programs SEQBOOT, DNADIST, DNAPARS, PROTDIST, PROTPARS, and CONSENSUS from the PHYLIP version 3.5c package (8).

**Nucleotide sequence accession numbers.** *nirK* and *nirS* gene sequences from marine sediments and enrichment cultures have been deposited in the EMBL nucleotide sequence database under accession no. AJ248392 through AJ248448.

## RESULTS

**Nitrite reductase genes of marine denitrifying isolates.** Nine denitrifying isolates shown to be distinct by REP-PCR (data not shown) were investigated for the presence of the *nirK* or the *nirS* gene. All of these isolates showed amplification only with the *nirS*-specific primer combination resulting in one PCR product of the expected size (890 bp), and none reacted with the *nirK*-specific primer pair. Identity of the *nirS* products was confirmed by sequencing. The general taxonomic position of the isolates was determined by sequencing of the 16S rDNA genes and by comparison to the EMBL database. All isolates were members of the  $\gamma$ -*Proteobacteria*. Six of them were close relatives of *P. stutzeri*, two were related to *Marinobacter* sp., and one was related to *Halomonas variabilis*. The branching within the *nirS* gene tree and the general taxonomic position are shown in Fig. 1.

**Amplification of *nir* genes from sediment samples and enrichments.** DNA of high molecular mass was extracted from all sediment samples, with 1 g of wet sediment yielding up to 12.5  $\mu$ g of purified DNA. To confirm the suitability of DNA extracts for PCR amplification, 16S rDNA genes were successfully amplified from total DNA extracts from all sediments. Amplification using the *nirS*-specific primer pair was successful for sediment samples from Puget Sound and the Washington coast, resulting in a single PCR product of the predicted size (approximately 890 bp). PCR products of the copper-containing nitrite reductase using the *nirK*-specific primer pair were obtained only from the Washington coast sediment DNA extracts, not from the Puget Sound sediments. Failure to amplify *nirK* fragments from these sediment samples also occurred when the DNA extraction and PCR procedure were repeated. PCR products of the predicted size (514 bp) were weak and showed one additional unspecific band for the DNA extract from the 2,530-m Washington coast sample. Therefore, bands of the predicted size were eluted from an agarose gel and concentrated before cloning. A Southern blot confirmed that no amplification was achieved with the *nirK*-specific primers from Puget Sound samples, while *nirK* products were detected from a Washington coast sample (1,936 m) as a positive control (data not shown).

*nirK* PCR products were obtained only from the enrichment supplemented with yeast extract, but the *nirS*-specific primers amplified fragments from both enrichments supplemented with yeast extract or Casitone. The PCR-amplified regions of the *nirK* and *nirS* genes were cloned successfully from both the Washington coast and Puget Sound sediment samples and from the enrichments.

**RFLP analysis of *nirK* and *nirS* clones.** A total of 185 *nirK* clones (93 from the Washington coast 1,936 m and 92 from 2,530 m) and 229 *nirS* clones (77 from Puget Sound 1.0 to 1.5 cm, 63 from 1.5 to 2.0 cm, 75 from 6.0 to 6.5 cm; 56 from the Washington coast 1,936 m and 58 from 2,530 m) were screened by RFLP. For *nirK* clones, the analysis resulted in one dominant group of clones occurring in both samples from the Washington coast comprising 29 and 37% of all *nirK* clones. Several minor groups were also detected, but 34 and 40% of *nirK* clones were unique to one sediment sample. For *nirS* clones, no dominant group was found. Groups consisted of no more than five redundant clones, and the amount of clones unique to one sediment sample ranged from 55 to 70% (Puget Sound 1.0 to 1.5 cm, 55%; 1.5 to 2.0 cm, 61%; 6.0 to 6.5 cm, 59%; Washington coast 1,936 m, 70%; Washington coast 2,530 m, 67%). Rarefaction analysis showed a similar level of diversity for *nirK* and *nirS* sequences from the Washington coast samples, whereas it indicated a higher level of diversity of *nirS* sequences from the Puget Sound sediment samples (Fig. 2). Comparison of RFLP patterns from *nirS* clones did not indicate any congruence to those from marine denitrifying isolates and other denitrifying strains (Fig. 1).

Analysis of clones from enrichments showed one dominant group of five *nirS* clones and three unique RFLP patterns from the enrichment supplemented with Casitone and one single pattern each for *nirK* and *nirS* clones from the enrichment supplemented with yeast extract. None of these patterns matched those of clones from the sediment samples or from marine denitrifying isolates or denitrifying strains.

**Dot blot hybridization of *nirK* and *nirS* clones.** To determine the specificity of cloned *nirK* and *nirS* PCR products, all clones were hybridized to four probes for each gene. The probes were selected to show identity levels of 68.2 to 74.6% for *nirK* and 61.0 to 76.6% for *nirS* to each other. The stringency conditions were set to detect >70% identity to achieve a broad reactivity to unrelated clones. With a few exceptions, hybridization signals of all four probes for a specific clone did not vary more than three strengths. The same differences were found for clones with the same RFLP pattern due to uneven amounts of the DNA spotted. For *nirK* clones, a visible hybridization signal (signal strength 1 or stronger) to all probes was detected. *nirK* clones most frequently showed hybridization signals of 4 and stronger, with a predominance of signal strength 4 according to the dominant group of *nirK* clones in these sediment samples (Fig. 3A).

Except for two *nirS* clones which did not hybridize to any of the probes, all clones hybridized to at least one of the probes. *nirS* clones showing signal strength of 4 and stronger (Fig. 3B) were dominant in all sediment samples investigated. Thus, *nirS* clones in these sediments show dominance of conserved sequences in the amplified sample based on hybridization signals

FIG. 1. Phylogenetic relationship of *nirS* gene products (partial, 112 amino acids; accession numbers in brackets). The dendrogram was generated by phylogenetic distance analysis with a neighbor-joining algorithm with *Roseobacter denitrificans* [AJ224911] as the outgroup. Values indicate the percentage of 100 replicate trees supporting the branching order. Bootstrap values below 50 are omitted. Scale bar, 10 mutations per 100 sequence positions. Clones obtained from Puget Sound and Washington coast samples are labeled p and w, respectively. Phylogenetic positions of pure cultures based on 16S rDNA genes are indicated by  $\alpha$ ,  $\beta$ , and  $\gamma$  for the subgroups of the *Proteobacteria*. General taxonomic positions of marine isolates based on 16S rDNA genes in percent similarity determined by BLAST search is given in parenthesis after isolate designations. Roman numbers indicate the clusters of *nirS* sequences from isolates and environmental clones.



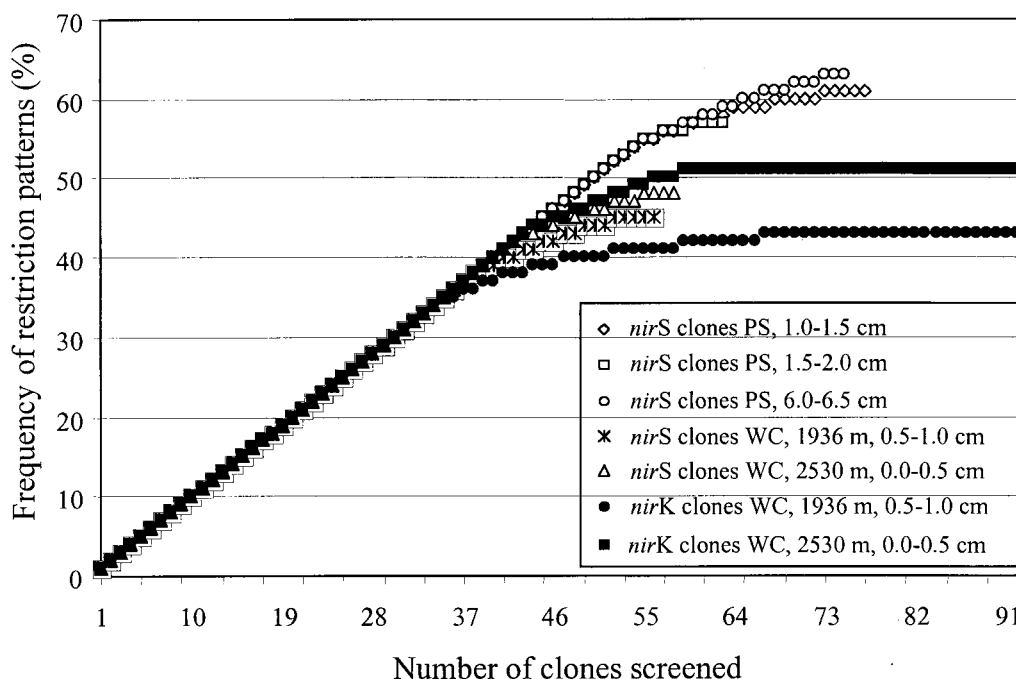


FIG. 2. Rarefaction curves indicating the diversity of denitrifying bacteria as detected by RFLP analysis of cloned nitrite reductase genes (*nirK* and *nirS*) from Puget Sound and Washington coast samples. *nirK* clones were digested with the tetrameric restriction enzymes *MspI* and *HaeIII*, and *nirS* clones were digested with *MspI* and *HhaI* in a single reaction. PS and WC stand for Puget Sound and Washington coast, respectively.

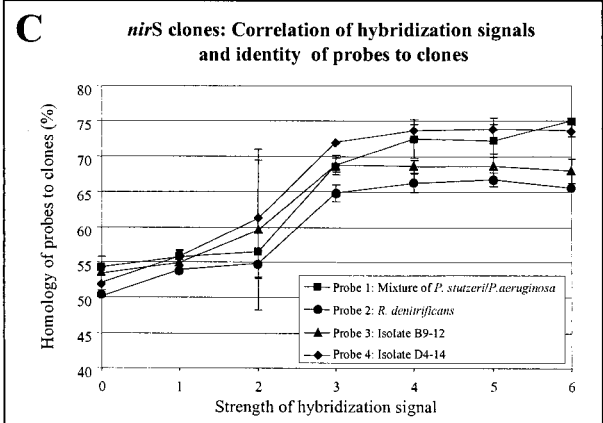
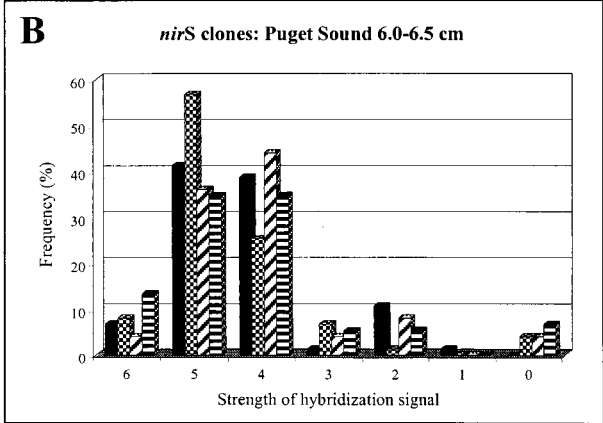
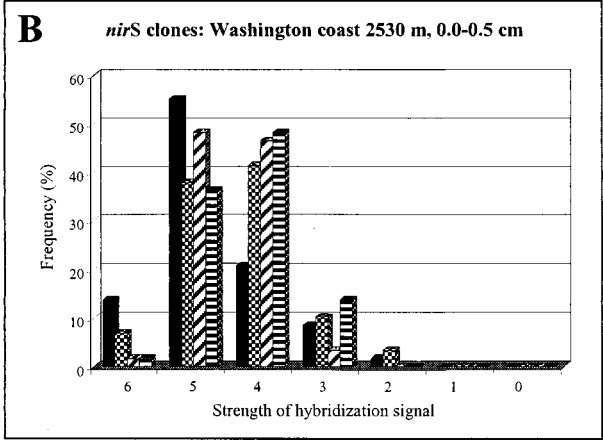
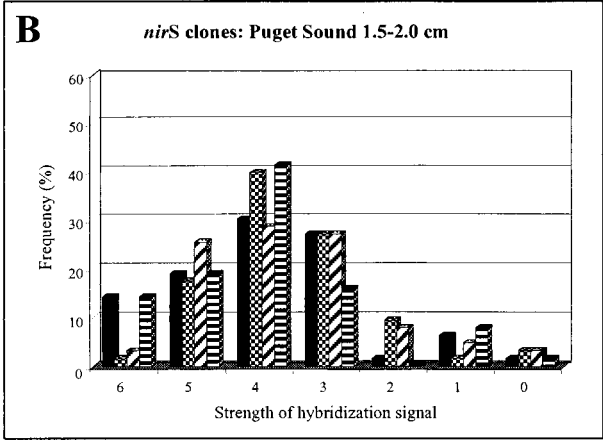
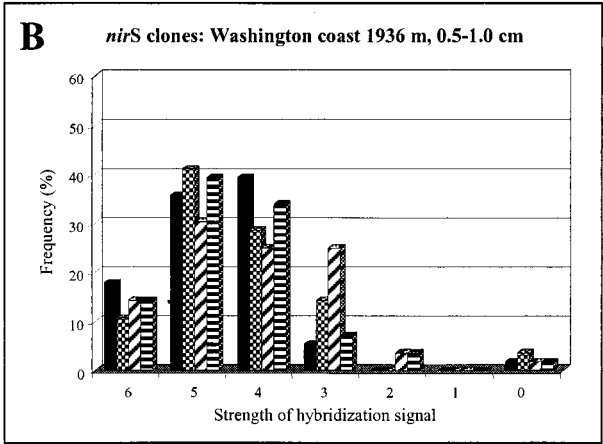
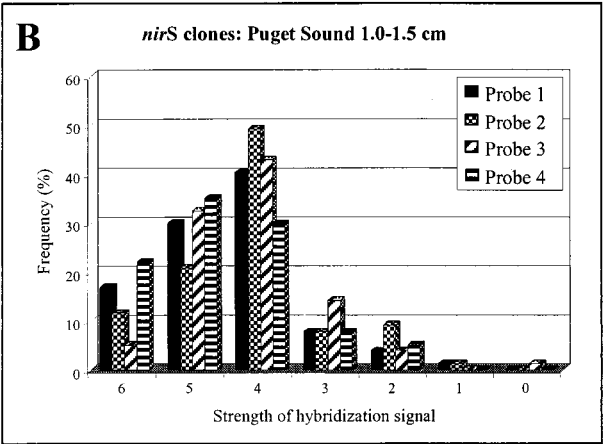
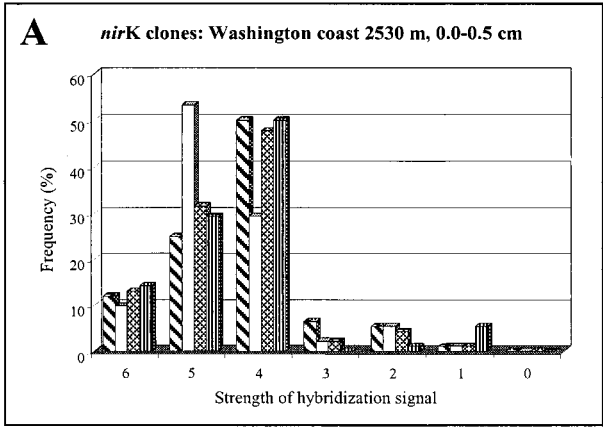
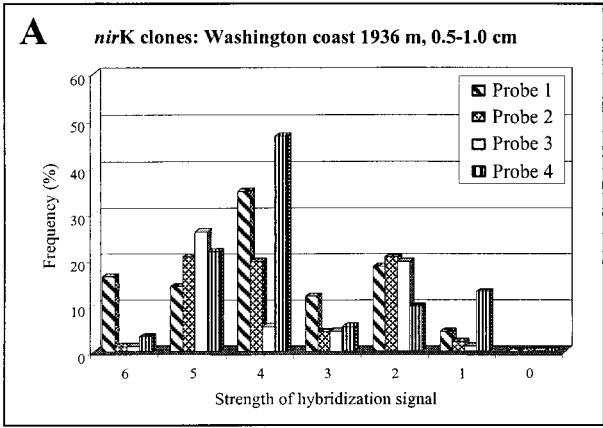
of 3 and stronger. This signal strength was correlated to a sequence homology of 65% and higher (Fig. 3C). None of the probes generally showed stronger or weaker signals, indicating no *nir* subfamily dominance in any samples.

**Sequence analysis of *nirK* and *nirS* clones.** Partial *nirK* sequences (336 bp) were obtained from eight clones representing the dominant RFLP groups and showing hybridization signals of 3 or stronger; they were confirmed as *nirK* genes. Sequences from *nirK* clones showing weaker hybridization signals could not be defined as *nirK* sequences by alignment or comparison to the EMBL database using FASTA or BLAST search. Levels of nucleotide identity of the *nirK* clones were from 78.6 to 100.0%, and levels of identity of the deduced amino acids were from 89.3 to 100% within the sequenced region (data not shown). A similar level of conservation over the gene (63.1 to 74.7% for the full sequence, in comparison to 65.6 to 79.9% for the sequenced 336 bp at the C terminus for the published sequences) supported the usefulness of this region for phylogenetic purposes. The *aniA* gene from *Neisseria gonorrhoeae* was not considered in this analysis but was used in the generation of the dendrogram. This gene encodes a putative gonococcal nitrite reductase with significant homologies to copper-containing nitrite reductases from denitrifiers (15). However, the level of conservation of this sequence in comparison to all *nirK* sequences is significantly lower (37.4 to 44.7% and 29.1 to 34.6% for nucleotides and amino acids, respectively, within the sequenced region). Dendrograms of nucleotide sequences and

deduced amino acid sequences generated by neighbor joining or parsimony were consistent for the major clustering except for minor differences within the clusters derived of sequences from known denitrifying strains. Regions of insertion or deletion were omitted from the analysis because of uncertain alignment. Trees (Fig. 4) showed two major clusters with the sequence from the enrichment clone branching with *nirK*-containing denitrifiers belonging to the  $\alpha$ -*Proteobacteria* except for *Alcaligenes faecalis* S-6 ( $\beta$ -*Proteobacteria*). Sequences from sediment clones clustered with none of these sequences. The second cluster consisted of known denitrifiers belonging to the  $\alpha$ -,  $\beta$ -, or  $\gamma$ -*Proteobacteria* and clones from marine sediment samples. The latter form a distinct subcluster of *nirK* sequences unknown in cultured organisms, suggesting that these genes are derived from novel denitrifiers.

As rarefaction analysis showed a higher level of diversity of *nirS* than *nirK* clones (Fig. 2) in marine sediment samples, 35 clones were chosen for partial sequencing (336 to 348 bp). Clones selected were from groups with distinct restriction patterns or were nonredundant clones without (clones wA20 and pB95) or with weak (clones pA17, pA63, pB6, and pB16) hybridization signals. Sequencing confirmed that all *nirS* clones from marine sediment samples and enrichments were *nirS* sequences except clone pB95, for which a matching sequence could not be found by FASTA and BLAST search. Nucleotide identity ranged from 45.3 to 100% and 40.0 to 100.0% for identity of deduced amino acids (data not shown). Similar

FIG. 3. Dot blot hybridization of *nirS* and *nirK* clones obtained from Puget Sound and Washington coast sediment samples. (A) Cloned *nirK* fragments were hybridized to four different probes: *nirK* PCR products from *Pseudomonas* sp. strain G-179 (1), from *B. denitrificans* (2), from *R. sphaeroides* f. sp. *denitrificans* (3), and from *Alcaligenes* sp. (4). Strength of hybridization signals was assigned as 0 (no hybridization signal) to 6 (strong hybridization signal). Frequency (%) expresses the total number of clones hybridizing with each probe. (B) Cloned *nirS* fragments were hybridized to four different probes: *nirS* PCR products from *P. stutzeri*/*P. aeruginosa* (1), from *P. denitrificans* (2), from marine isolate B9-12 (3), and from marine isolate D4-14 (4). Strength of hybridization signals was assigned as for panel A. (C) Strength of *nirS* hybridization signals was correlated to homology values of the probes to 37 sequenced *nirS* clones. Bars show standard deviation.



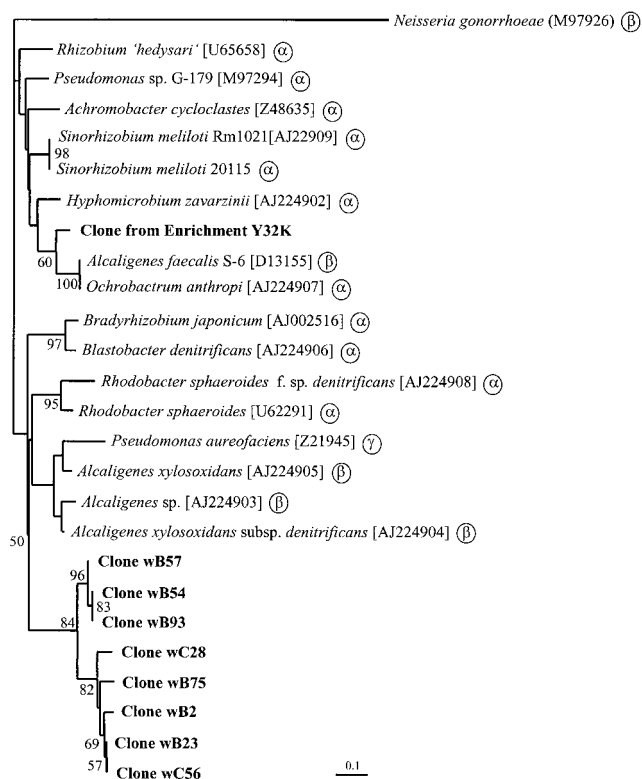


FIG. 4. Phylogenetic relationship of *nirK* gene products (partial, 111 amino acids; accession numbers in brackets). The dendrogram was generated by phylogenetic distance analysis with a neighbor-joining algorithm with *B. denitrificans* [AJ224906] as the outgroup. Values indicate the percentage of 100 replicate trees supporting the branching order. Bootstrap values below 50 are omitted. Scale bar, 10 mutations per 100 sequence positions. Phylogenetic positions of pure cultures based on 16S rDNA genes are indicated by  $\alpha$ ,  $\beta$ , and  $\gamma$  for the subgroups of the *Proteobacteria*.

levels of conservation of the entire gene (72.2 to 94.2%) in comparison to 69.5 to 95.0% for the sequenced region from published sequences suggests the usefulness of the sequenced region for phylogenetic purposes. Dendrograms were identical with both the distance and parsimony methods except for minor differences in the positions of clones wD4, pA5, and wB32. Regions of insertion or deletion were omitted from the analysis because of uncertain alignment. Trees showed three major clusters of *nirS* sequences with clones from marine sediment samples clustering in three distinct subclusters (Fig. 1). None of the clones was closely related to the *nir* gene of any isolate, whereas clone C32S from the enrichment supplemented with Casitone was related to the *nirS* gene of *Paracoccus denitrificans* within the first major cluster. Clone Y32S from the enrichment supplemented with yeast extract was located on a distinct branch. One major cluster consisted of a subcluster of closely related *nirS* sequences of all *P. stutzeri* strains and related isolates (Fig. 1, cluster I). A second subcluster within this branch consisted of very distantly related *nirS* sequences from both environments, Puget Sound and the Washington coast (cluster II). Within the other major cluster, the remaining *nirS* clones formed subclusters mostly according to the environment from which they were obtained. None of the clones from offshore Washington coast branched with a group of sequences from Puget Sound clones (cluster III) which clustered together with *nirS* sequences of *P. aeruginosa*. Clones from the Washington coast formed a separate subcluster (clus-

ter IV), although three clones from Puget Sound branched within this cluster. A third branch within this cluster was formed by three marine denitrifying isolates (cluster V). The distant relatedness and separate clustering of the *nirS* clones from marine sediments suggest that groups of novel denitrifying bacteria inhabit these sediments.

## DISCUSSION

PCR approaches amplifying 514- and 890-bp fragments from *nirK* and *nirS* genes from natural denitrifying populations, respectively, were applied to investigate the community structure of denitrifiers in marine sediment samples from two habitats in the Pacific Northwest. With the *nirS* primer pair, amplification was possible with all DNA extracts from all sediment samples tested. With the *nirK* primer pair, amplification was not possible with DNA extracts from Puget Sound. Even Southern blot hybridization with four different *nirK* probes did not detect any amplification from the Puget Sound sediment samples. The *nirK* primers seemed to amplify *nirK* genes closely related to those from which the primers were designed (3). Thus, they may not have detected whether more distantly related *nirK* genes were present in Puget Sound sediments. There was a small group of clones which could not be specified as *nirK* sequences although these hybridized weakly to the probes (Fig. 3A). Even though some of these sequences were partial open reading frames, BLAST search or alignments did not show close relationships to known *nirK* sequences and to each other. Additionally, analysis of the deduced amino acid sequences within the sequenced region showed that histidine B255 (*Achromobacter cycloclastes* numbering), which is not a copper ligand but close to the active site (2), was not conserved among these sequences but was conserved among all *nirK* sequences and the *aniA* sequence of *N. gonorrhoeae*. Comparison of deduced amino acids among *nirK* sequences revealed only two amino acids within this region which were specific for the marine clones, thus suggesting that the *nirK* primers indeed showed bias toward well-conserved *nirK* sequences. On the other hand, within the *nirK* gene tree the marine *nirK* sequences clustered together in a distinct subcluster of marine sequences, indicating that the marine *nirK* containing denitrifier genes were different from known *nirK* genes. Any suggestion about the phylogenetic affiliation of these denitrifiers was not possible. However, their grouping within *nirK* sequences from *Proteobacteria* suggests that they might be members of the *Proteobacteria* (Fig. 4).

For *nirS*, all except one clone could be identified as *nirS* by hybridization and sequencing. BLAST search and alignments did not show any homology to *nirS* sequences for this clone. A second clone (wA20) not hybridizing to any of the *nirS* probes was confirmed as a very distantly related *nirS* sequence. Levels of nucleotide identity were from 45.3 to 58.7% to other *nirS* sequences but analysis of deduced amino acids revealed conservation of key amino acids among all partial sequences. Histidine 352 (*P. aeruginosa* numbering) was conserved among all sequences whereas His 302 was not conserved among the distantly related clones (clone pA17, pA63, pB6, pB16, and wA20) and His 314 was not conserved among *Azospirillum brasiliense* Sp7 and clone pB16 but was conserved in all other sequences (data not shown). This suggests only His 352 as a heme  $d_1$  ligand (24). Despite weak hybridization signals, all *nirS* clones hybridizing to any of the probes were specific *nirS* sequences amplified by the *nirS* primer pair used in this study. Furthermore, despite a dominance of clones with hybridization signals of 4 and stronger, these *nirS* primers also amplified fragments from less conserved *nirS* genes, reflected by the

clones with hybridization signals of 3 and weaker (Fig. 3B). Strength of hybridization signals was correlated to the level of sequence conservation of *nirS* clones. For signal strength of 3 or weaker, there was a linear correlation of increasing signal strength to the increasing level of identity between probes and clones. For signal strength of 4 and stronger, it was assumed that an increase in strength was due to more conserved regions which strongly hybridized to the probes, although the overall level of identity for the sequenced clones to the probes did not exceed 65 to 75% (Fig. 3C).

RFLP analysis of clones and denitrifying strains revealed that clones from the marine sediment samples did not match any *nir* sequence from the database. Sequence analysis of selected clones confirmed these findings and grouped the *nirS* clones into three distinct subclusters of novel marine sequences within the *nirS* gene tree, suggesting that there were completely different *nir* genes if not groups of denitrifiers in this habitat. Interestingly, most of the clones grouped according to the environment from which they were obtained, suggesting that there are distinct populations of denitrifying bacteria within Puget Sound sediments and offshore Washington coast sediments.

The *nirS* trees show generally the same clustering as the 16S rDNA trees with the exception of the *nirS* genes of *Alcaligenes faecalis* A15 ( $\beta$ -*Proteobacteria*) and *A. brasiliense* Sp7 ( $\alpha$ -*Proteobacteria*) grouping with the *nirS* genes of *P. stutzeri* ( $\gamma$  subgroup). Therefore, it is likely that at least the more closely related clones from Puget Sound and the Washington coast belonged to *Proteobacteria*. More obscure remains the phylogenetic affiliation of the very unrelated cluster (cluster II) of *nirS* clones. Unfortunately, but not unexpectedly, clones from enrichments and *nirS* sequences from the marine denitrifying isolates did not group closely with the *nirS* environmental clones (Fig. 1). Further cultivation strategies are desirable for recovering the organisms with these more novel sequences so that any unique functional diversity can be identified.

With the caveat of primer preference for more conserved *nirS* genes and possible bias during PCR (27), it was assumed that these results reflect the community structure of denitrifying bacteria in these sediments, at least more so than isolation studies. Rarefaction analysis detected a high level of diversity within *nirS* genes in the sediment samples, and analysis of RFLP patterns showed that there were distinct populations present even in individual sections of the Puget Sound core although the first two sections were adjacent (1.0 to 1.5 cm and 1.5 to 2.0 cm). Clones were not redundant among Puget Sound sediment samples and those from the Washington coast. Only a few clones were redundant among the sections of the Puget Sound core. This suggested an independent development of cytochrome *cd*<sub>1</sub>-dependent nitrite reductase genes, especially in these two environments which are spatially distinct on a bacterial size scale and differ in quality and quantity of carbon input. Higher input of less degraded organic matter into Puget Sound sediments than into Washington coast sediments (13) together with long time and distance scales may have led to the development of distinct bacterial populations and hence *nirS* genes.

Comparison of the identity levels of nucleotides and of amino acids revealed that in many cases, the amino acids were less conserved than the nucleic acids which indicated that most mutations were not neutral and thus did not occur at the third base but within the first two bases of the codons. High rates of amino acid exchange indicating low selection pressure may be more likely under environmental conditions in which genes are not necessary for survival. For denitrification genes, this can readily occur where either aerobic growth or nitrate-free an-

aerobic conditions often predominate. Bioturbation in marine sediments disturbs chemical gradients periodically exposing denitrification genes to nonselective conditions. The enhanced bioturbation rates in shallow sediments than in deeper sediments (4) suggest lower selection pressure on Puget Sound versus Washington coast *nirS* gene populations, perhaps enhancing evolutionary rates of this functional gene.

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