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Phylogenetic Probes for Analyzing Abundance and Spatial Organization of Nitrifying Bacteria

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A hierarchical set of five 16S rRNA-targeted oligonucleotide DNA probes for phylogenetically defined groups of autotrophic ammonia- and nitrite-oxidizing bacteria was developed for environmental and determinative studies. Hybridization conditions were established for each probe by using temperature dissociation profiles of target and closely related nontarget organisms to document specificity. Environmental application was demonstrated by quantitative slot blot hybridization and whole-cell hybridization of nitrifying activated sludge and biofilm samples. Results obtained with both techniques suggested the occurrence of novel populations of ammonia oxidizers. In situ hybridization experiments revealed that *Nitrobacter* and *Nitrosomonas* species occurred in clusters and frequently were in contact with each other within sludge flocs.

The process of nitrification is carried out by two phylogenetically unrelated groups of autotrophic bacteria, the ammonia oxidizers and the nitrite oxidizers (5, 13). Lithoautotrophic ammonia-oxidizing bacteria transform ammonia to nitrite, which is subsequently converted to nitrate by nitrite-oxidizing bacteria. Although nitrifying bacteria are central to the global nitrogen cycle, they contribute to pollution problems through oxidation of ammonia fertilizers to nitrite and nitrate in water supplies, the production of greenhouse gases (NO) (6, 29), and biodeterioration of building materials (20). Yet, they can also be used to ameliorate anthropogenic damage to the environment, for example, by reducing the ammonia content of wastewater in sewage treatment before discharge into aquatic environments (23). This is particularly important, as ammonia is toxic to aquatic life (4) and creates a large oxygen demand.

Although increasingly strict regulations on nitrogen discharge are making nitrification a more important goal of biological treatment, nitrification is well recognized as being difficult to maintain in engineered systems. For example, nitrifying bacteria are sensitive to a variety of sewage compounds, to pH swings, and to temperature shifts, resulting in frequent process failures (9, 23). Improved process control will almost certainly depend upon a better understanding of their microbiology. However, the nitrifying bacteria have proved particularly difficult to study by cultivation techniques such as most probable number (18) and selective plating (10) because of their long generation times and poor counting efficiencies. The latter can be attributed in part to their tendency to form large, dense clusters within activated sludge flocs (32). Thus, a key question is whether the culture collection adequately represents environmental populations of nitrifying bacteria.

To address the question of diversity, we and others have used comparative 16S rRNA sequencing to characterize described species of ammonia- and nitrite-oxidizing bacteria (11, 22, 28, 34, 35). This has provided a molecular phylogenetic framework for evaluating the diversity and abundance of nitrifying environmental populations. In particular, the use of phylogenetically based hybridization probes targeting the 16S rRNAs of described groups has provided an important link between the culture collection and its environmental representation (7, 12, 19, 30). Since the presence of nitrifying bacteria can be correlated with their characteristic activities, the comparison of probe-based enumeration with nitrification rates should provide a basis to systematically evaluate the contributions of described species to nitrification in engineered systems and the environment. Initial studies using two probes for the genera *Nitrosomonas* and *Nitrobacter* demonstrated the utility of this approach for quantitative in situ studies (32, 33). In this study we describe the development and characterization of a more comprehensive set of probes for nitrifying bacteria. The initial application of these probes to the characterization of nitrifying reactors suggests undescribed diversity and a tendency for nitrite- and ammonia-oxidizing populations to grow in closely associated aggregates.

Probe design. Characterized ammonia-oxidizing bacteria are restricted to the γ and β subdivisions of the *Proteobacteria* (purple bacteria) (11, 28, 34, 35). Of these, all but one (*Nitrosococcus oceanus*) are affiliated with the β subdivision. Since *N. oceanus* is a marine isolate and no additional ammonia oxidizers within the γ subdivision have been described, we developed probes only for the β subdivision. Five new 16S rRNA-targeted oligonucleotide probes (Fig. 1) were characterized: probes Nso190 (S-Nso190Sb-19) and Nso1225 (S-Nso1225Sa-20) encompass all sequenced ammonia oxidizers of the β subclass of *Proteobacteria*, probe Nsm156 (S-Nsm156Sa-19) identifies members of the genus *Nitrosomonas* (together with *Nitrosococcus mobilis*), probe Nsv443 (S-Nsv443Sa-20) is specific for the *Nitrosovibrio-Nitrosolobus-Nitrosospira* group, and probe Nb1000 (S-G-Nit1000Sb-15) is specific for members of the genus *Nitrobacter*.

All probe sequences are listed in Table 1. Probe specificities with reference to available 16S rRNA sequences were checked with the BLAST program (1) at the National Center for Biotechnology Information (NCBI) (Washington, D.C.), the CHECK_PROBE program, supported by the Ribosome Database Project (Urbana, Ill.) (16), and the probe check program in the ARB software (27). Alignments of the 16S rRNAs encompassing the target region of each probe are presented in Fig. 2.

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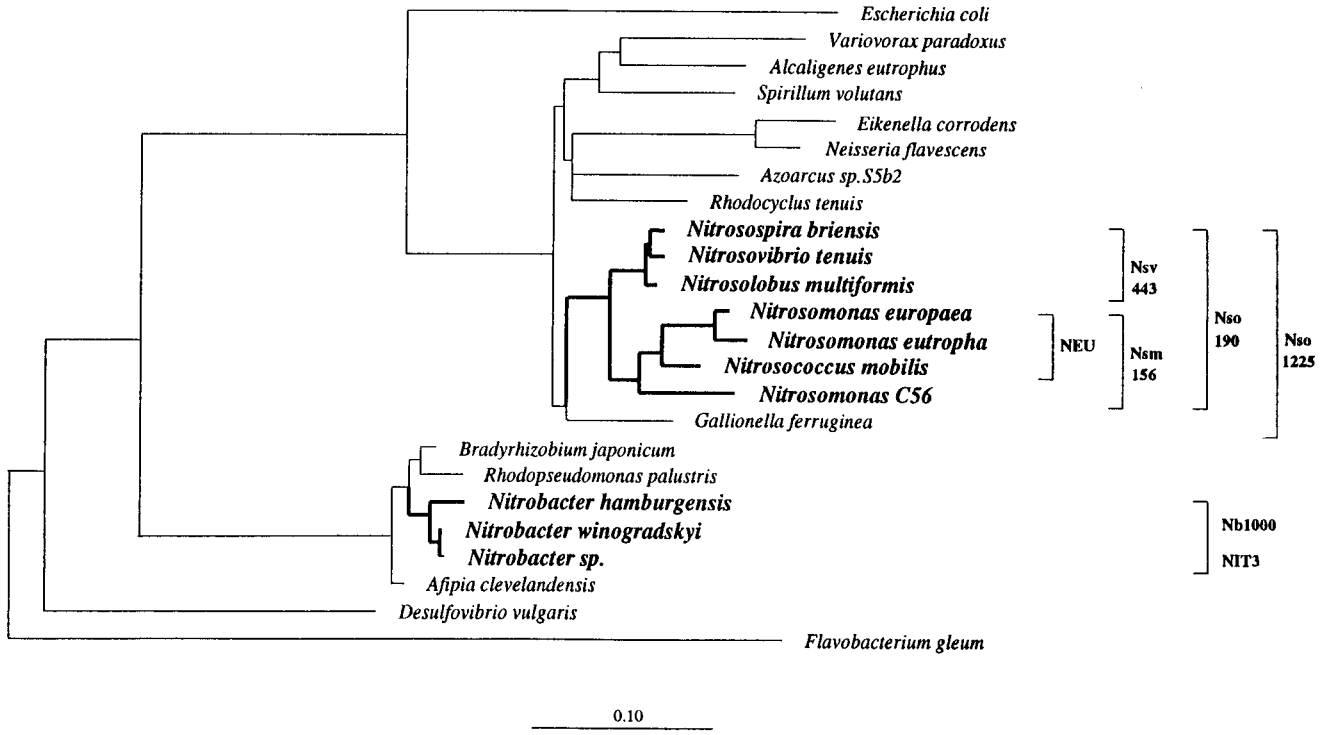


FIG. 1. Phylogenetic tree inferred from comparison of 16S rRNA sequences. Target organisms for probes Nso190, Nso1225, Nsm156, Nsv443, NEU, NIT3, and Nb1000 are indicated by brackets. The bar represents 0.1 estimated change per nucleotide.

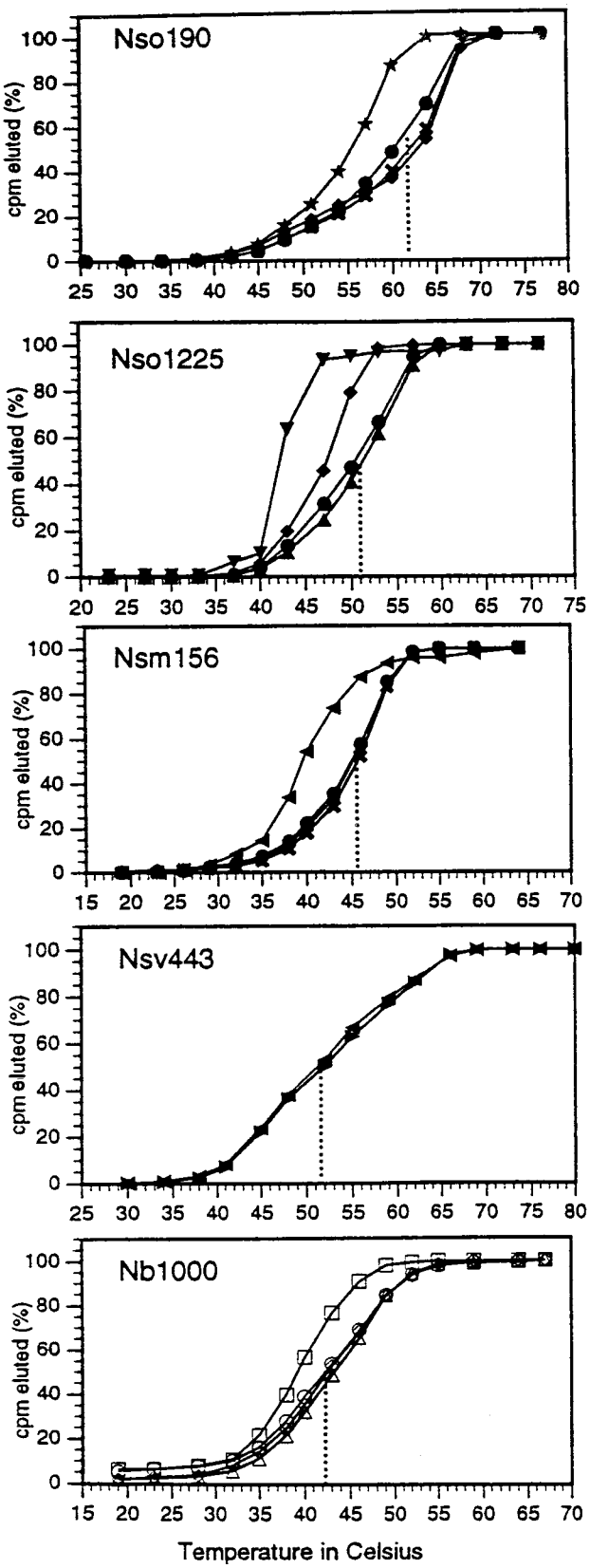
Although the ammonia oxidizers comprise a coherent group within the β subclass of the *Proteobacteria*, they are deeply branching. For this reason, target regions suitable for comprehensive probes are limited. We identified only two general target regions within the 16S rRNA (Nso190 and Nso1225), and they have some qualifications. Probe Nso190 has a T:G mismatch with the 16S rRNA target sequence of *Nitrosomonas eutropha* and a T:U mismatch with *Nitrosomonas* C-56. *Spirillum volutans* exhibits one mismatch with probe Nso190. However, all other characterized nontarget organisms have at least two mismatches with Nso190. The second general probe (Nso1225) has a single G:A mismatch with *Nitrosococcus mobilis*, but, with the exception of *Gallionella ferruginea*, all characterized nontarget organisms have at least two mismatches with this probe. Probes Nso1225 and Nso190 have target sites in different regions of the 16S rRNA, which means that they can be used simultaneously for whole-cell hybridization to confirm the intended target group specificity. Probe Nsm156 was designed to hybridize to *Nitrosomonas marina*, *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*. Unfortunately, the published sequences of *Nitrosomonas eutropha* and *Nitrosococcus mobilis* have some positional ambiguities in this target region. However, binding of probe to *Nitrosococcus mobilis* and *Nitrosomonas eutropha* was demonstrated by both in situ and slot blot hybridization. *Nitrosospira briensis* and related ammonia oxidizers have two mismatches within the Nsm156 target region. Probe Nsv443 was designed to hybridize with *Nitrosolobus multiformis*, *Nitrosospira briensis*, and *Nitrosovibrio tenuis*. All other available sequences have at least three mismatches with the target region of this probe.

In addition to the probes targeting ammonia-oxidizing bacteria, we developed a probe for nitrite-oxidizing species within the genus *Nitrobacter*, Nb1000. *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, *Afipia felis*, and *Afipia clevelandensis* each has one mismatch within the target site of the *Nitrobacter*-specific probe, reflecting their close phylogenetic relationship to the genus *Nitrobacter* (22).

Probe T_d determinations. Total RNA isolated from target

TABLE 1. Names, target positions, sequences, and specificities of the probes used in this study

Probe	*E. coli* 16S rRNA position	Probe sequence	Specificity
Nb1000	1000–1012	5′-TGCGACCGGTCATGG-3′	*Nitrobacter* spp.
NIT3	1035–1048	5′-CCTGTGCTCCATGCTCCG-3′	*Nitrobacter* spp.
NEU	653–670	5′-CCCCTCTGCTGCACTCTA-3′	Halophilic and halotolerant members of the genus *Nitrosomonas*
Nso190	190–208	5′-CGATCCCCTGCTTTTCTCC-3′	Ammonia-oxidizing β-*Proteobacteria*
Nso1225	1225–1244	5′-CGCGATTGTATTACGTGTGA-3′	Ammonia-oxidizing β-*Proteobacteria*
Nsm156	156–174	5′-TATTAGCACATCTTTCGAT-3′	*Nitrosomonas* C-56, *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*
Nsv443	444–462	5′-CCGTGACCGTTTCGTTCCG-3′	*Nitrosolobus multiformis*, *Nitrosospira briensis*, and *Nitrosovibrio tenuis*



Organism **Target**

Nso190

✖*Nitrosomonas europaea*	5'GGAGAAAAGCAGGGGAUCG3'
Nitrosococcus mobilis	5'.....3'
Nitrosolobus multiformis	5'.....3'
◆*Nitrosovibrio tenuis*	5'.....3'
Nitrosospira briensis	5'.....3'
Nitrosomonas eutropha	5'....G.....3'
●*Nitrosomonas* sp. (C-56)	5'.....U.....3'
★*Azoarcus* S5b2	5'....-.....U3'

Nso1225

Nitrosomonas europaea	5'UCACACGUAAUACAAUGGCG3'
Nitrosomonas eutropha	5'.....3'
●*Nitrosomonas* sp. (C-56)	5'.....3'
◆*Nitrosococcus mobilis*	5'.....A.3'
Nitrosolobus multiformis	5'.....3'
▲*Nitrosovibrio tenuis*	5'.....3'
Nitrosospira briensis	5'.....3'
Gallionella ferruginea	5'.....3'
▼*Alcaligenes eutrophus*	5'.....C.....U.3'
Desulfovibrio vulgaris	5'A.....C.....3'

Nsm156

✖*Nitrosomonas europaea*	5'AUCGAAAGAUGUGCUAAUA3'
●*Nitrosomonas* sp. (C-56)	5'.....3'
Nitrosomonas eutropha	5'.....NNNN....3'
◆*Nitrosococcus mobilis*	5'NNNN.....3'
Nitrosolobus multiformis	5'.C.....G.....3'
Nitrosovibrio tenuis	5'.C.....G.....3'
◀*Nitrosospira briensis*	5'.C.....G.....3'
Eikenella corrodens	5'.....UG.....3'
Neisseria flavescens	5'.....UA.....3'

Nsv443

Nitrosovibrio tenuis	5'CCGGAACGAAACGGUCACGG3'
▶*Nitrosolobus multiformis*	5'.....3'
◀*Nitrosospira briensis*	5'.....3'
Nitrosomonas eutropha	5'U.....A....GA....UA.3'
Nitrosomonas europaea	5'U.....A....GA..UG.AA3'
Nitrosococcus mobilis	5'UU..G.A.....A.UG.AA3'
Nitrosomonas sp. (C-56)	5'U..AG.A....A.A.UGU.A3'

Nb1000

△*Nitrobacter hamburgensis*	5'CCATGACCGGTCGCA3
◇*Nitrobacter winogradskyi*	5'.....3
⊙*Nitrobacter sp.* ATCC 25384	5'.....3
Bradyrhizobium japonicum	5'...G.....3
□*Rhodopseudomonas palustris*	5'...G.....3
Afipia clevelandensis	5'...G.....3

FIG. 2. Probe dissociation experiments for Nso1225, Nso190, Nsm156, Nsv443, and Nb1000. The rRNAs used were from the following organisms: *Alcaligenes eutrophus* (▼), *Azoarcus* S5b2 (★), *Nitrosomonas* C-56 (●), *Nitrosomonas europaea* (✖), *Nitrosococcus mobilis* (◆), *Nitrosolobus multiformis* (▶), *Nitrosospira briensis* (◀), *Nitrosovibrio tenuis* (▲), *Nitrobacter* ATCC 25381 (◇), *Nitrobacter* ATCC 25384 (○), *Nitrobacter hamburgensis* (△), and *Rhodopseudomonas palustris* (□). The T_ds (measured as cpm) are denoted by dotted lines. Next to the probe elution results, the 16S rRNA target sequences for each probe were aligned with the closest-matching 16S rRNA sequences from other bacteria. The top sequence is the target sequence. The periods in the sequences following signify that the nucleotides are identical. Differences are indicated by the replacement nucleotides shown below the target sequence nucleotides. The dash indicates a deletion.

TABLE 2. Quantitative slot blot hybridization in a nitrifying biofilm reactor

Sample date (mo/day/yr)	% of bacteria hybridizing with probe:				% of Nso190-labeled bacteria hybridizing with Nsm156
	Nsm156	Nsv443	Nso190	Nb1000	
6/10/94	59.5	0.11	77.8	0.57	76
7/4/94	64.9	0.09	87.2	0.49	74
7/26/94	30.7	0.2	46.0	0.76	67

and closely related nontarget reference organisms was immobilized on nylon membrane supports and hybridized with ^{32}P-labeled probes as previously described (15, 24, 26), except that 100 ng of reference RNA was applied per sample (slot blot). The temperature of dissociation (T_d), defined as the temperature at which one half of the bound probe is released from the hybrid, was determined by a graded-temperature wash series as previously described (24).

The specificities of the probes were evaluated by comparing the dissociation profiles of representative target bacteria and closely related nontarget organisms. All elution profiles, with the possible exception of that of Nso190, were symmetrical sigmoidal curves, but they differed significantly in the temperature range of dissociation. The measured T_d for Nso190 with *Nitrosomonas europaea* and *Nitrosovibrio tenuis* was 62°C (Fig. 2). A wash temperature of approximately 64°C would provide complete discrimination between target species and *Azoarcus* S5b2, the closest nontarget organism (two mismatches). The T_d for Nso1225 (51°C) was 4°C higher than that for *Nitrosococcus mobilis* (one mismatch) and 9°C higher than than for *Alcaligenes eutrophus* (two mismatches) (Fig. 2). The T_d for Nsm156 target rRNAs (46°C) was 7°C higher than that for *Nitrosospira briensis* (two mismatches) (Fig. 2). Thus, Nsm156 discriminates against the inclusion of *Nitrosospira briensis* and other organisms within its lineage. The T_d for Nsv443 was 52°C (Fig. 2), and no significant hybridization was observed for the closest nontarget bacteria (three mismatches).

Given the close relationship of several nontarget species, it was also essential to evaluate the *Nitrobacter* probe discrimination. Probe Nb1000 was hybridized to three *Nitrobacter* strains (*Nitrobacter hamburgensis* and *Nitrobacter winogradskyi* 25381 and 25384) and a nontarget organisms with one mismatch (*Rhodopseudomonas palustris*). The T_d for *R. palustris* was 3°C lower than the T_d for *Nitrobacter* spp. (42°C) (Fig. 2). Thus, total elimination of nonspecific hybridization would require a wash temperature as high as 45°C. However, this would reduce probe retention by target group RNA to approximately 20%, compared with a 50% probe retention at the T_d. Because of the low signal intensity of this probe at the 45°C wash temperature, we used 42°C for our slot blot hybridization experiments. Consequently, in slot blot experiments with environmental samples, a minor contribution by several nontarget species to the hybridization signal of Nb1000 cannot be excluded.

Slot blot hybridization. The probes characterized in this study and an additional probe previously characterized for the domain bacteria (2) were used to quantify rRNA isolated from a nitrifying biofilm reactor in France (Lyonnaise des Eaux, Paris). This reactor was loaded with 1.2 kg of NH_4^+ N per m^3 per day. Total RNA was recovered from these samples by mechanical disruption by bead beating in the presence of TRI reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's protocol. rRNA integrity was characterized by polyacrylamide gel electrophoresis prior to hybridization and visualized by ethidium bromide staining (25). Only those samples showing distinct 23S and 16S rRNA bands in a ratio of approximately 2 to 1 were used for hybridization. The RNA was immobilized on nylon membranes (Magnacharge; Micron Separation Inc., Westboro, Mass.) with a slot blotter (Minifold II; Schleicher & Schuell, Inc., Keene, N.H.) and hybridized to radiolabeled probe as previously described (15, 26). The wash temperature used for Nso190 was 53°C, in order to include *Nitrosomonas* C-56. Bound probe was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and expressed as a fraction of the total rRNA quantified with the bacterial-domain probe (Table 2). Very little hybridization was observed with the *Nitrobacter* and *Nitrosovibrio-Nitrosospira-Nitrosolobus* probes, indicating that their contribution to nitrification in this system is minor. The combined fraction hybridizing to the more specific Nsm156 and Nsv443 probes was always significantly less than the Nso190 signal for all samples, suggesting the presence of uncharacterized ammonia-oxidizing bacteria in these nitrifying environments.

In situ hybridization. Heterotrophic bacteria, ammonia-oxidizing bacteria, and nitrifying samples from a continuously stirred tank reactor (influent NH_4^+ N and chemical oxygen demand concentrations, 204 and 52 mg/liter, respectively; sludge retention time, 15 days), a sequencing batch reactor (200 and 53 mg/liter, respectively; 13 days), and an upflow aerated biofilter (120 and 60 mg/liter, respectively) were fixed for in situ hybridization (3, 32). These reactors oxidized more than 99% of the influent NH_4^+ N to NO_3^- N at the time of sampling, and they are more fully described elsewhere (8, 21). Fixed bacterial cells and fixed samples from the engineered systems were hybridized as previously described (3) with the following probes (Table 1): Nso190, Nso1225, Nsm156, Nsv443, NEU, and NIT3. The NIT3 and NEU probes were recently described (32, 33) and are specific for the genus *Nitrobacter* and a subgroup of the ammonia-oxidizing assemblage targeted by Nsm156, respectively (Fig. 1). Since only one target region in the 16S rRNA is inclusive for *Nitrobacter* species, probes Nb1000 and NIT3 have similar target sites and specificities. Neither of these two previously described probes has been characterized for use in membrane hybridization.

Oligonucleotides were synthesized with an amino linker at the 5′ end (Operon Technologies, Alameda, Calif.) and labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim, Indianapolis, Ind.) and the hydrophilic cyanine reagents Cy3 and Cy5 (Biological Detection Systems, Pittsburgh, Pa.) as previously described (3, 32). Although most rRNA-targeted probes can be used for slot blot hybridization, not all of them will also work for whole-cell hybridization. Therefore, probes Nso190, Nso1225, Nsm156, and Nsv443 were tested for their application in fluorescent in situ hybridization. Hybridization conditions for these four probes were optimized by using artificial mixtures of fixed bacterial target and nontarget cells. Specific hybridization with probes Nso190, Nso1225, Nsm156 (Fig. 3A), and Nsv443 (Fig. 3B) required the addition of different amounts of formamide (Table 3) to the hybridization buffer (17). The stringency of the washing step (at 48°C) was adjusted by lowering the sodium chloride concentration to achieve the appropriate specificity. Slides were viewed with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany) equipped with the Zeiss filter set 09 for FLUOS and high-quality (HiQ) filter sets for Cy3 and Cy5 (Chroma Technology, Brattleboro, Vt.). Mercury and xenon lamps were used for excitation of Cy3- and Cy5-labeled cells, respectively. Photomicrographs were taken with a Photometrics CE 250 camera incorporated with a cooled KAF 1400

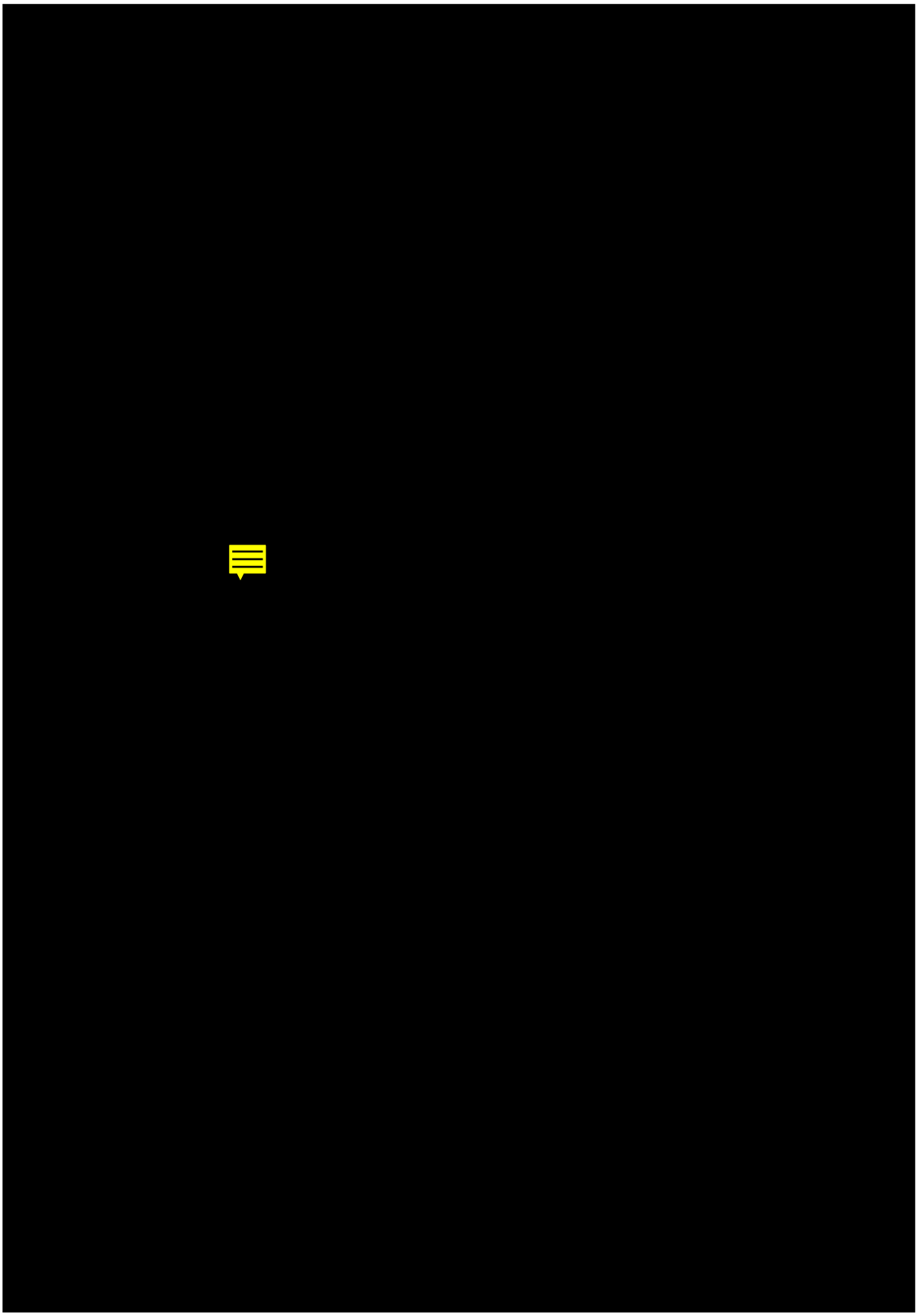


FIG. 3. In situ identification of nitrifying bacteria. Identical microscopic fields were viewed by phase-contrast microscopy (top) and epifluorescence microscopy (bottom). Bars, 10 μm. (A and B) Specific whole-cell hybridization with probes Nsm156 and Nsv443. An artificial cell mixture containing *Nitrosomonas europaea* and (a smaller number of) *Nitrosolobus multiformis* was hybridized with Cy3-labeled Nsm156 (A) and Nsv443 (B). While probe Nsm156 specifically stained *Nitrosomonas europaea*, probe Nsv443 hybridized exclusively with *Nitrosolobus multiformis* cells. (C) Simultaneous in situ identification of *Nitrosomonas* and *Nitrobacter* in a sample from the continuously stirred tank reactor after in situ double hybridization with Cy3-labeled probe NEU and Cy5-labeled probe NIT3. Cy5-labeled cells were colored green by image analysis. (D) Simultaneous double hybridization of the sample of the continuously stirred tank reactor with the probes Nso190-FLUOS and Nsm156-Cy3. Double-stained cells appear yellow because of the overlapping of labels. Cells exclusively labeled by probe Nso190 appear green.

charge-coupled device (Photometrics, Tucson, Ariz.) and a 63× apochromatic objective lens.

Different combinations of probes were used to characterize the nitrifying microbial communities of fixed samples obtained from the three reactors. Since optimum hybridization conditions for some of these probes are different, a successive hybridization procedure was used for dual or triple labeling (31). Initially, all samples were analyzed by combined triple hybridization with NEU, Nso190, and Nso1225 each labeled with a different fluorescent dyes. Together, probes Nso190 and Nso1225 encompass all characterized ammonia-oxidizing bacteria within the β subdivision of the *Proteobacteria*, while probe NEU targets a distinct group of halophilic and halotolerant members of the genus *Nitrosomonas*. In addition, the occurrence of members of the genus *Nitrosospira* (11) in the samples was examined by hybridization with probe Nsv443. Virtually all fluorescent cells were labeled simultaneously with probes Nso190, Nso1225, and NEU, while no target bacteria for probe Nsv443 could be detected, suggesting that most of the ammonia oxidizer diversity could be accounted for by organisms either identical or closely related to described species of the genus *Nitrosomonas*. Whole-cell hybridization also revealed that the nitrifying bacteria were not uniformly distributed within the biofilm-sludge floc matrix. The majority of ammonia oxidizers formed dense clusters similar to those previously observed in a variety of open and engineered nitrifying systems (32, 33). Standard DNA extraction techniques may not be sufficiently rigorous to extract fully representative DNA from samples containing these clustered *Nitrosomonas* cells. This could bias DNA-based detection techniques and may explain the failure to detect *Nitrosomonas* spp. in the environment by PCR (12).

A close physical association between *Nitrosomonas* spp. and *Nitrobacter* spp. was also demonstrated by dual probing with NEU and NIT3. *Nitrobacter* cells formed small clusters that were usually found in contact with *Nitrosomonas* cell clusters (Fig. 3C). This spatial organization may reflect a syntrophic association between *Nitrosomonas* and *Nitrobacter* spp. For example, the toxicity of nitrite for ammonia oxidizers and the poor energy yield of nitrite oxidation may promote associations of *Nitrosomonas* and *Nitrobacter* spp. by providing a competitive advantage for such associations over solitary occurrence. Most likely, ammonia-oxidizing bacteria grow first as microcolonies, which produce nitrite and then are colonized by nitrite-consuming *Nitrobacter* aggregates.

TABLE 3. Calculated and measured T_d values and formamide concentrations for specific fluorescent in situ hybridizations with probes presented in this study

Probe	T_d (°C)		% Formamide for FISH[b]
	Calculated[a]	Measured	
Nso190	50	62	55
Nso1225	47	51	35
Nsm156	40	46	5
Nsv443	55	52	30
Nb1000	42	42	ND[c]

[a] See reference 14.
[b] FISH, fluorescent in situ hybridization.
[c] ND, not yet demonstrated in situ.

The ammonia-oxidizing bacterial populations in the continuously stirred tank reactor sample were further characterized by double labeling with the probe pairs Nso190-Nsv443 and Nso190-Nsm156. None of the cells specifically stained with Nso190 were also detected with Nsv443 (results not shown), suggesting that members of the genus *Nitrosospira* are not quantitatively important in this bioreactor. Only a portion of the ammonia oxidizers stained with probe Nso190 were also detectable with probe Nsm156 (Fig. 3D). As probe Nsm156 specifically targets *Nitrosomonas europaea*, *Nitrosomonas* sp. C-56, and *Nitrosococcus mobilis*, which would all be simultaneously detectable with probe Nso190, cells exclusively stained with the general ammonia oxidizer probe do not correspond to ammonia oxidizers currently characterized by 16S rRNA sequencing.

Conclusions. The set of hierarchical probes for nitrifying bacteria characterized in this study was shown to be suitable for both quantitative slot blot and whole-cell hybridization. These formats are complementary and together provided a quantitative and structural characterization of nitrifier populations in complex communities. Initial studies revealed that *Nitrosomonas* populations predominated, often in close association with *Nitrobacter* spp. In addition, quantitative comparisons made by using this hierarchical collection of probes suggested a yet-undescribed diversity of ammonia oxidizers within the β subdivision of the *Proteobacteria*. These observations have considerable significance to our understanding of the microbiology of nitrification and the process control of engineered systems. For example, the close spatial contiguity of ammonia- and nitrite-oxidizing populations could be important. We anticipate that the availability of culture-independent tools for the characterization of nitrifying populations will provide an important research tool and a potentially powerful monitoring tool for the study of these bacteria in engineered and natural systems.

Ammonia-oxidizing bacterial strains used for the slot blot stringency tests were kindly provided by John Waterbury and Frederica Valois (Marine Biological Laboratory, Woods Hole, Mass.). Strains with ATCC numbers were received from the American Type Culture Collection and cultured by Jay Regan in our lab. Samples for in situ hybridization were received from a continuously stirred tank reactor and a sequencing batch reactor operated by D. G. V. de Silva and an upflow aerated biofilter reactor operated by Akiyoshi Ohashi and Doug Stilwell (both of Northwestern University).

B.K.M. and M.W. contributed equally to this publication.

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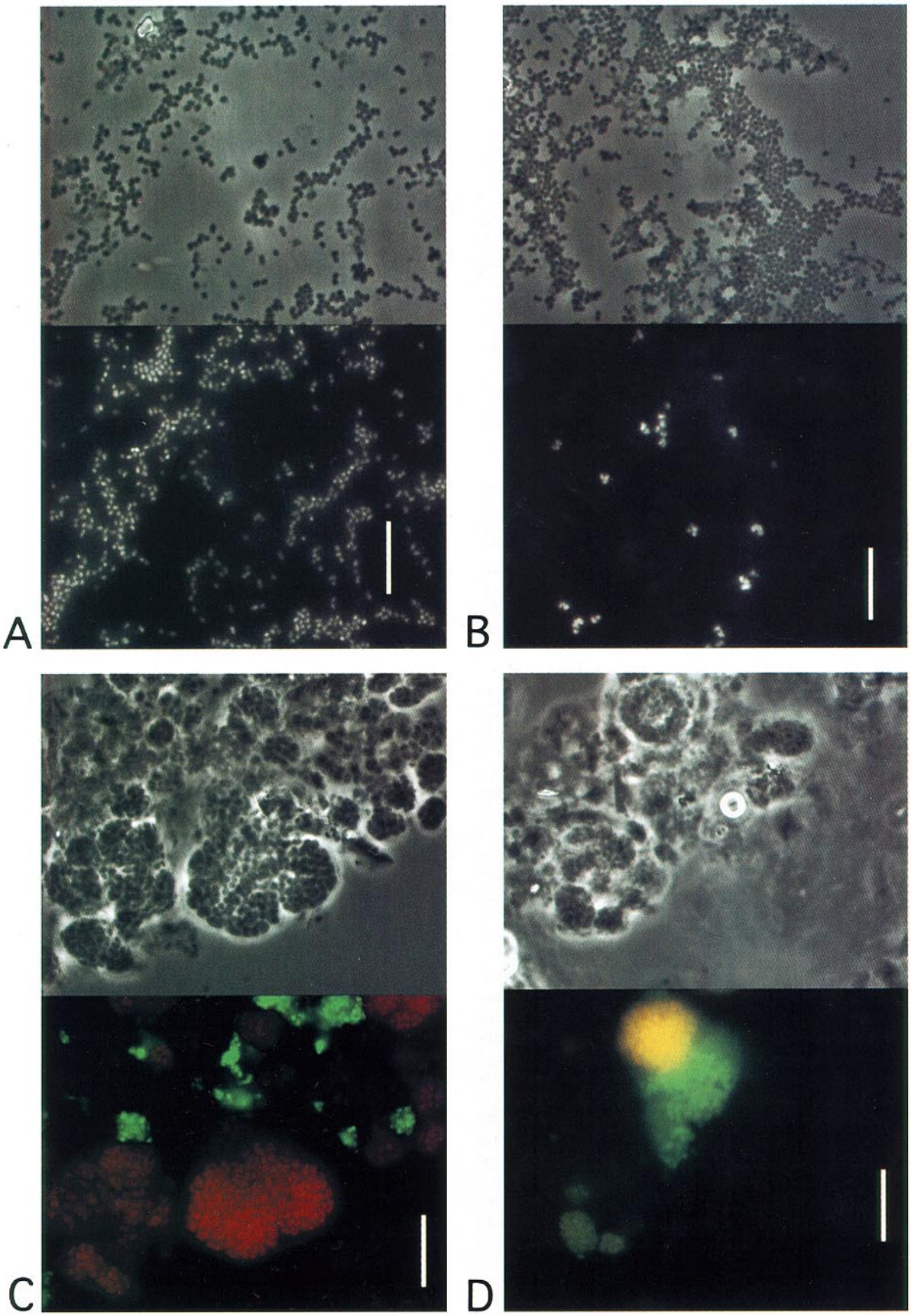
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