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Analysis of β-Subgroup Proteobacterial Ammonia Oxidizer Populations in Soil by Denaturing Gradient Gel Electrophoresis Analysis and Hierarchical Phylogenetic Probing

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A combination of denaturing gradient gel electrophoresis (DGGE) and oligonucleotide probing was used to investigate the influence of soil pH on the compositions of natural populations of autotrophic β-subgroup proteobacterial ammonia oxidizers. PCR primers specific to this group were used to amplify 16S ribosomal DNA (rDNA) from soils maintained for 36 years at a range of pH values, and PCR products were analyzed by DGGE. Genus- and cluster-specific probes were designed to bind to sequences within the region amplified by these primers. A sequence specific to all β-subgroup ammonia oxidizers could not be identified, but probes specific for *Nitrosospira* clusters 1 to 4 and *Nitrosomonas* clusters 6 and 7 (J. R. Stephen, A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley, Appl. Environ. Microbiol. 62:4147–4154, 1996) were designed. Elution profiles of probes against target sequences and closely related nontarget sequences indicated a requirement for high-stringency hybridization conditions to distinguish between different clusters. DGGE banding patterns suggested the presence of *Nitrosomonas* cluster 6a and *Nitrosospira* clusters 2, 3, and 4 in all soil plots, but results were ambiguous because of overlapping banding patterns. Unambiguous band identification of the same clusters was achieved by combined DGGE and probing of blots with the cluster-specific radiolabelled probes. The relative intensities of hybridization signals provided information on the apparent selection of different *Nitrosospira* genotypes in samples of soil of different pHs. The signal from the *Nitrosospira* cluster 3 probe decreased significantly, relative to an internal control probe, with decreasing soil pH in the range of 6.6 to 3.9, while *Nitrosospira* cluster 2 hybridization signals increased with increasing soil acidity. Signals from *Nitrosospira* cluster 4 were greatest at pH 5.5, decreasing at lower and higher values, while *Nitrosomonas* cluster 6a signals did not vary significantly with pH. These findings are in agreement with a previous molecular study (J. R. Stephen, A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley, Appl. Environ. Microbiol 62:4147–4154, 1996) of the same sites, which demonstrated the presence of the same four clusters of ammonia oxidizers and indicated that selection might be occurring for clusters 2 and 3 at acid and neutral pHs, respectively. The two studies used different sets of PCR primers for amplification of 16S rDNA sequences from soil, and the similar findings suggest that PCR bias was unlikely to be a significant factor. The present study demonstrates the value of DGGE and probing for rapid analysis of natural soil communities of β-subgroup proteobacterial ammonia oxidizers, indicates significant pH-associated differences in *Nitrosospira* populations, and suggests that *Nitrosospira* cluster 2 may be of significance for ammonia-oxidizing activity in acid soils.

Chemolithotrophic oxidation of ammonia to nitrate via nitrate (autotrophic nitrification) is of major importance in the global cycling of nitrogen in terrestrial, aquatic, and marine ecosystems (22). The first, and rate-determining, step of nitrification, ammonia oxidation, is carried out by the autotrophic ammonia-oxidizing bacteria, whose growth in liquid batch culture rarely occurs at pH values below 6.5. Nevertheless, autotrophic nitrification has been reported in acid soils at pH values as low as 3.5 (5). Nitrification in acid soils may be explained to some extent by growth on surfaces (2) or in aggregates (6), by ureolytic activity (1, 4), and by heterotrophic nitrifiers (14). An additional explanation is the existence of strains adapted to low-pH environments (7, 26), but although an acidophilic nitrite oxidizer has been isolated (9), acidophilic ammonia oxidizers have proved difficult to isolate in pure culture. Low growth rates, low biomass yield, and the limited number of distinguishing phenotypic characters for ammonia oxidizers have prevented the analysis of natural communities, in particular those in acid soils. The application of 16S ribosomal DNA (rDNA)-based techniques, however, enables the study of community structure in environmental samples, without the requirement for laboratory cultivation (8). Such studies have been particularly productive when applied to ammonia-oxidizing bacteria. With the exception of a small number of cultured marine strains belonging to the γ-proteobacteria, the phylogenetic analysis of rDNA sequences places all ammonia oxidizers in a monophyletic group within the β-proteobacteria. This group consists of two distinct monophyletic genera, *Nitrosomonas* and *Nitrosospira* (10, 28, 30, 31). An analysis of natural ammonia oxidizer populations from a variety of envi-

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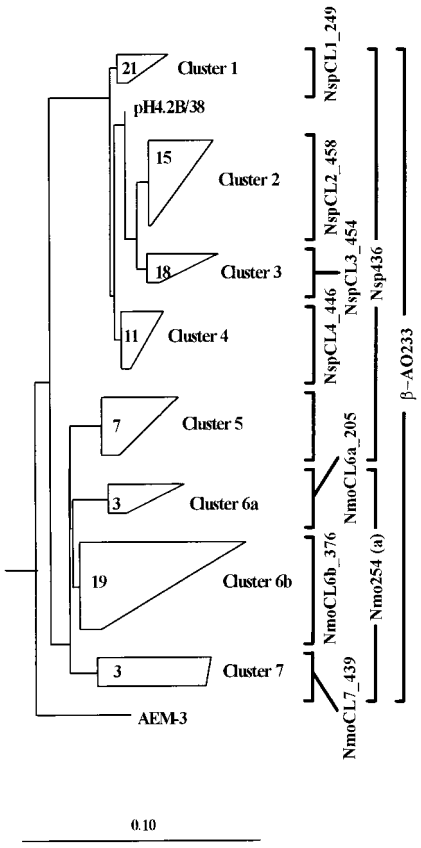

FIG. 1. Schematic tree of the β-subgroup ammonia-oxidizing bacteria based on 303 bp of 16S rDNA sequence spanning *E. coli* positions 198 to 500 (3) and target groups for probes described in Table 1. Ambiguous sites were removed by using the GDE 2.2 "mask" function (16) before transfer of the data to the ARB sequence analysis program (25). The tree was generated by neighbor joining (24) with the Jukes and Cantor (13) correction in ARB. Clusters of sequences are scaled vertically to represent the number of sequences and horizontally to represent the extent of variation within each cluster. Cluster designations are as described in Stephen et al. (26) with the exception of cluster 6a, which contains three soil clones (see text), and cluster 6b, which contains the remainder of cluster 6 sequences previously described (26). Soil clone pH4.2/28 and marine enrichment sequence AEM3 cannot be placed unequivocally in this scheme. The scale bar represents 0.1 estimated changes per nucleotide position. Sequence data were compiled from references 10, 15, 17, 21, 26, and 28).

ronments has revealed further subdivision (26), based on phylogenetic analysis of a 300-bp fragment of the 16S rDNA molecule spanning the V2 and V3 regions (20). An analysis of 1.1-kb regions (incorporating this 300-bp fragment) of representative environmental sequences has shown that *Nitrosospira* can be further subdivided into at least four clusters, designated 1 to 4 (26). The *Nitrosomonas* genus may also be subdivided, and Stephen et al. (26) recognized three clusters within this genus, designated 5 to 7 (Fig. 1). Of particular relevance to this study is a cluster containing three soil clones, here referred to as cluster 6a. These and other studies (11, 15) indicate that sequences from members of the genus *Nitrosospira* were more abundant than *Nitrosomonas* in a range of environments and suggest that the available pure cultures represent only a limited selection of the phylogenetic and, by inference, physiological diversity of natural ammonia oxidizer populations. These studies are based on the hypothesis that environmental sequences falling within the *Nitrosomonas* and *Nitrosospira* clades originate from autotrophic ammonia oxidizers (26). Evidence for this hypothesis is based on their phylogenetic positions relative to cultured taxa which uniformly possess this phenotype, including recently isolated cultures of *Nitrosospira* (28). In addition, no sequence from a nonautotrophic ammonia oxidizer falls within this clade.

The available 16S rDNA sequence data for β-subgroup ammonia oxidizers can be interpreted to suggest that particular phylogenetic clusters may be associated with specific habitats (26). For example, sequences from *Nitrosospira* cluster 2 and *Nitrosospira* cluster 3 were apparently more common in acid and neutral agricultural soils, respectively (26), while sequences from *Nitrosomonas* cluster 6 and *Nitrosospira* cluster 4 were detected at similar frequencies in both soils. Biogeographic or temporal studies of community structure by determination of sequence abundance in gene libraries are severely limited by the time required for sequence acquisition and analysis, and the approach is not necessarily quantitative. Denaturing gradient gel electrophoresis (DGGE) provides a complementary tool for the analysis of complex microbial communities (19, 25). It involves the separation of DNA fragments of identical length on the basis of differences in denaturant sensitivity in an acrylamide gel matrix, resulting from differences in the primary sequence. PCR products generated from different samples can thereby be compared directly, based on their mobility, without the need for cloning or DNA sequence analysis. Specific amplification and DGGE analysis of 16S rDNA for β-subgroup ammonia-oxidizing bacteria successfully demonstrated differences between communities in dune soil samples (15). However, differences in mobility between the various sequence clusters and overlapping banding patterns prevented precise identification of the community members, which necessitated band excision and sequence determination.

The recent expansion of the 16S rDNA sequence database for β-subgroup ammonia oxidizers provides the potential for the design of improved oligonucleotide probes for the analysis of natural populations (10, 15, 21, 26, 28). Published β-subgroup ammonia oxidizer probes have been based on sequence information from a limited number of cultured organisms (11, 12, 29) and would not detect much of the diversity determined by Stephen et al. (26), restricting their value for the analysis of natural communities.

The first aim of this study was to design and test oligonucleotide probes capable of distinguishing the subgroups within the different clusters of β-subgroup ammonia oxidizers from each other. The second aim was then to use these probes to identify bands separated by DGGE analysis of PCR products generated from soil with ammonia oxidizer-specific primers. In particular, we aimed to investigate further the hypotheses (26) that *Nitrosospira* may be of significance for ammonia oxidation in acid soils and that the relative abundances of different sequence clusters are related to soil pH.

MATERIALS AND METHODS

Design of oligonucleotide probes. Probes were designed with the assistance of the ARB "probe design" function (27) from a manually aligned data set of all 16S rDNA sequences related to the β-subgroup ammonia oxidizers derived from pure cultures, enrichment cultures, and environmental clones. Clustering of sequences (Fig. 1) was based on an analysis of 303 bases spanning the homologous *Escherichia coli* positions 198 to 500 (Fig. 1). Potential specific probe sequences showed at least one highly destabilizing base pairing (purine/purine) or two less-destabilizing mismatches (purine/pyrimidine or pyrimidine/pyrimidine) between the probe sequence and the most similar nontarget sequences towards the center of the target site. Table 1 shows the oligonucleotides synthesized for use in this study (Isogen Bioscience BV, Maarssen, The Netherlands). Potential specificities of probes were assessed by using the Ribosomal Database Project CHECK-PROBE facility (16) and FastA searches of the EMBL sequence databases to ensure that the target site did not occur in published sequences of non-ammonia-oxidizer organisms.

Probe hybridization analysis. Elution profiles were established for each oligonucleotide probe and its membrane-bound target sequence, and the temper-

TABLE 1. Oligonucleotide probes designed for this study[a]

Probe sequence 5′ - 3′	Target group	Probe name[b]	T_d (°C)[c]	Hybridization temp used (°C)[d]	No. of mismatches to nontarget organisms[e]
AGCTAATCAGRCATCGG	All β-subgroup ammonia oxidizers	β-AO233	44	44	0
TTTCGTTCCGGCTGAAAG	All *Nitrosospira*	Nsp436	45	45	≥1 (*T. thioparus*[f])
CTTTTACCTTACCAACAA	*Nitrosospira* cluster 1	NspCL1_249	42	42	0 (*Buchnera aphidi*[g])
TCACAGTTATTAACCGTG	*Nitrosospira* cluster 2	NspCL2_458	43	44	≥1 (*N. tenuis* St. 2)
RGGTATTAGCCGTGACCG	*Nitrosospira* cluster 3	NspCL3_454	44	47	≥2
ACCGTAACCTTTTCGTTC	*Nitrosospira* cluster 4	NspCL4_446	43	44	≥2
GTAGGCCSTTACCCYACC	All *Nitrosomonas*	Nmo254	43	43	0
GTARGCCWTTACCCYACC	All *Nitrosomonas*	Nmo254a	47		0
GCATAAGGTCTTTCGAT	*Nitrosomonas* cluster 6a	NmoCL6a_205	43	44	≥2
GGATCAGGCTTGCGCCC	*Nitrosomonas* cluster 6b	NmoCL6b_376	46	46	0
CTCTTTCTTTCCGACTAA	*Nitrosomonas* cluster 7	NmoCL7_439	44	44	≥2

[a] All probes were directed at the sense DNA strand.
[b] Probe nomenclature is as described by Mobarry et al. (18). NMO, *Nitrosomonas*; Nsp, *Nitrosospira*. The final number in each probe name is the number of the 5′ base complementary to the 3′ base of the probe (*E. coli* numbering [3]).
[c] T_d is as defined in Results.
[d] Hybridization temperatures are those necessary to prevent binding to the closest nontarget β-subgroup ammonia oxidizers.
[e] An entry of 0 mismatches indicates that a number of nontarget organisms carry identical sequences (the closest nontarget species or strain is in parentheses). Entries of ≥1 and ≥2 mismatches indicate that all nontarget organisms carry at least 1 and 2 mismatches, respectively.
[f] *T. thioparus* is the only species in the Ribosomal Database Project database with one mismatch.
[g] *Buchnera* spp. are obligate aphid endosymbionts.

ature at which 50% of the probe was eluted was determined. Profiles were also established for closely related heterologous sequences to optimize hybridization conditions for analysis of environmental samples. Plasmid or genomic DNA was amplified by using 20 pmol each of the ammonia oxidizer-specific primers CTO189f and CTO654r (15) and 2 U of Tbr polymerase (Dynazyme; Finnzymes, Espoo, Finland) under published amplification conditions. Each product (100 ng) was spotted in triplicate into a dot blot apparatus (Bio-Rad Laboratories, Surrey, United Kingdom) containing Hybond N+ hybridization support membrane (Amersham International plc, Bucks, United Kingdom).

Each probe (2 nmol) was end labelled by using T4 polynucleotide kinase (10 U) in the supplied buffer (New England Biolabs, Inc., Boston, Mass.) and 20 mCi of [γ-^{32}P]ATP (3,000 Ci mmol^{-1}; Amersham International plc) at 37°C for 30 min. Hybridization and prewashing steps were carried out at 37°C for all probes. Elution profiles were determined by a gradient temperature wash (23) and scintillation counting of the eluted material with a Packard 300 liquid scintillation counter (Canberra Packard, Berks, United Kingdom).

DGGE, membrane transfer, and hybridization. PCR, DGGE conditions, and the CTO primers were as described previously (15). Briefly, fragments of representative environmental library clones and genomic DNA from cultured representatives of each sequence cluster were amplified with PCR primer pair CTO189f-GC and CTO654r by using 1.25 U of Expand High Fidelity polymerase (Boehringer GmbH, Mannheim, Germany) in a 25-μl reaction volume according to the manufacturer's instructions. A fraction (10%) of each amplification reaction mixture was run on a 2% agarose–TAE (0.04 M Tris base, 0.02 M acetic acid, 1.0 mM EDTA, pH 7.5) gel, and DNA was visualized by fluorescence following staining with ethidium bromide. The concentrations of products were estimated by visual reference to molecular weight standards. Approximately 200 ng of each product (4 to 12 μl of crude PCR reaction mixture) was removed from the reaction tube for analysis by DGGE. DGGE gels (0.5× TAE, 8% acrylamide) were cast according to the protocol of Muyzer et al. (19) by using 38 to 50% denaturant (100% denaturant was 7 M urea with 40% [vol/vol] formamide [Boehringer GmbH]) and run at a constant temperature of 60°C. Gels were stained with ethidium bromide (0.5 mg liter^{-1}) in MilliQ water (Millipore B.V., Etten-Leur, The Netherlands) and destained in 0.5× TAE buffer. Images were captured by use of The Imager system (Ampligene, Illkirch, France). DNA was transferred to nylon hybridization membranes (Hybond N+; Amersham International plc) by use of a Semi-Dry electroblotter (model SD; Bio-Rad) according to the manufacturer's instructions, with transfer medium consisting of 0.5× TAE buffer, and run at 40 mA for 1 h. Following transfer, DNA was simultaneously denatured and covalently cross-linked to the hybridization membrane by incubation on a pad of 3MM paper (Whatman International Ltd., Kent, United Kingdom) soaked in 0.4 M NaOH for 30 min, followed by neutralization on two pads of 1 M Tris-HCl, pH 7.0, for 2 min each. Filters were then rinsed in distilled water and air-dried prior to prehybridization.

Prehybridization and hybridization were carried out in Quickhyb solution (Stratagene Inc., La Jolla, Calif.) at the temperatures given in Table 1 in a Mini Hybridisation oven (Hybaid Ltd, Teddington, United Kingdom). Membranes were washed free of unbound and nonspecifically bound probe by rinsing them in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate–0.1% sodium lauryl sulfate at room temperature, followed by 2 20-min washes in fresh buffer at the hybridization temperature. Autoradiography was for periods of between 1 h and overnight against Fuji-RX X-ray film (Genetic Research Instruments, Essex, United Kingdom) at −80°C. Quantification of the bound radioactive signal was achieved by use of a phosphorimager (Molecular Imager system equipped with a GS-363 loading dock; Bio-Rad) for 1 h, and the data were processed by using the profile analysis function of the Molecular Analyst software package (Bio-Rad). Relative abundances of different clusters were determined by imaging membranes probed with β–AO233, NspCL2_458, and NspCL3_454 (see below). Membranes were stripped prior to reprobing by multiple washes for 30 min each in 20 ml of 0.1 SSC–0.1% (wt/vol) sodium dodecyl sulfate at 65°C until radioactive counts returned to background, after which membranes were rinsed in distilled water and air-dried.

Analysis of environmental samples. The study site consisted of an agricultural field (Craibstone, Scotland) divided into seven pH-controlled plots held at approximately pH 6.6, 6.0, 5.5, 5.0, 4.5, and 4.2 since 1961 by the addition of lime. The unamended pH of this site is approximately 3.9. All plots were sampled in February 1997, having supported a crop of potatoes in the previous year. Soil samples (100 g) were collected from the surface 2 cm of each pH-controlled plot and homogenized by passing them through a 4-mm-mesh-size sieve. Soil (0.5 g) and glass beads (0.5 g, 0.1 mm in diameter; BioSpec Products, Techno Lab, Alkmaar, The Netherlands) were suspended in 0.5 ml of water-saturated phenol, pH 8.0, in a 2-ml screw-cap polypropylene tube. Cells were lysed mechanically by bead beating soil suspensions for three periods of 30 s in a minibeadbeater (BioSpec Products) set to 5,000 rpm. Suspensions were chilled on ice between shaking periods, and DNA was extracted in phenol followed by ethanol precipitation and passage through a two-stage agarose gel containing 1% polyvinylpolypyrillodone (Sigma, St. Louis, Mo.) (15). PCR amplification of 16S rDNA fragments related to the β-subgroup ammonia oxidizers was by 35 cycles of 92 (30 s), 57 (30 s), and 68°C (60 s) with the primer pair CTO189f-GC and CTO654r and 2.5 U of Expand High Fidelity polymerase (Boehringer). PCR products were subjected to DGGE analysis alongside products from each cluster group, transferred to positively charged hybridization membranes, and probed with genus- and cluster-specific ^{32}P-labelled oligonucleotides.

Quantification of relative abundances of sequence types. Gels were first probed with β–AO233 to quantify the total β-proteobacterial ammonia oxidizer signal in PCR products. Bands for clusters 4 and 6a are well separated, and the relative abundances of PCR products represented in these clusters in each environmental sample were calculated as the percentages of the total hybridization signal to β-AO233 within each lane represented by the cluster 4 or cluster 6a bands. The combined relative abundance of clusters 2 and 3 was calculated by subtraction from the total hybridization signal. Relative abundances of clusters 2 and 3 were determined by probing gels with NspCL2_458 and NspCL3_454, specific for clusters 2 and 3, respectively. Gels probed with β–AO233 showed that controls for clusters 2 and 3 were loaded at equal levels (within 0.5% of each other). Differences in binding efficiency and other factors between cluster 2 and 3 probes were determined by comparison of the hybridization signals for the respective controls, consisting of representative clones. Resultant correction factors were applied to hybridization signals for environmental samples to calculate relative proportions of cluster 2 and 3 signals. Finally, these values were expressed as proportions of the combined cluster 2 and 3 signal to give relative abundances of each cluster as a percentage of the total ammonia oxidizer signal. All analyses were carried out on two independent sets of soil samples, and results are expressed as means of duplicates.

RESULTS

Probe design. Probe specificities determined both empirically and by database searching are summarized in Table 1. Natural communities were analyzed by probing DGGE gels of PCR products obtained with primers specific to β-proteobacterial ammonia oxidizers (15). Probes were therefore chosen for their ability to distinguish between ammonia oxidizers belonging to different clusters. Some also have the potential to distinguish ammonia-oxidizing from non-ammonia-oxidizing bacteria, although finding such probes was not the main aim of the study. These carry at least one or two mismatches to nontarget sequences currently in the database (Table 1), although this is likely to represent a small percentage of natural diversity. No single probe target site which was specific to all β-subgroup ammonia oxidizers and which could differentiate these organisms from all other β-proteobacteria was found within the amplified region. The β–AO233 probe did not show complete specificity for β-subgroup ammonia oxidizers in an unconstrained search of the available data banks. However, all nontarget strains with similar sequences bore strongly destabilizing mismatches to the CTO PCR primer pair used to produce PCR products for DGGE. The β-AO233 probe was used to standardize radioactive probing of all products resulting from amplification with the CTO primer pair in quantifying the relative proportions of each band separated by DGGE.

Table 1 shows the T_d value for each probe, which is the temperature at which 50% of the probe was eluted from membrane-bound target DNA. Elution profiles were also obtained for each probe and target DNA consisting of one or more closely related heterologous DNAs from an ammonia oxidizer culture or environmental clone. For several probes, target and nontarget sequences could be distinguished under low-stringency conditions by the T_d value. For the remaining probes, and in particular those for target sequences detected in natural samples from this study, higher stringency was required. These situations are described in detail below, and hybridization temperatures required for the distinction of the target and the heterologous sequences are given in Table 1.

Genus *Nitrosospira*. Probe Nsp436 is specific to currently known sequences related to the *Nitrosospira* genus and showed at least two mismatches to all other proteobacterial sequences available except *Thiobacillus thioparus* (NCIMB 8370), to which it showed only a single weakly destabilizing mismatch, and *Comamonas testosteroni*, with which it is identical. The closest sequence which could be amplified with the CTO primer pair was that from *Nitrosomonas europaea*, from which target 95% of probe had eluted at the T_d value (45°C). *Nitrosospira* cluster 1 was specifically differentiated from related groups with probe NspCL1_249. Specific hybridization of probe NspCL2_458 to cluster 2 sequences was achieved at 44°C, retaining approximately 40% of maximum probe binding (Fig. 2a). Several sequences from *Nitrosospira* cluster 2 contained only weakly destabilizing mismatches to the probe NspCL3_454, which was designed to *Nitrosospira* cluster 3 (Fig. 2b). Elimination of cross-reaction to these heterologous sequences therefore required hybridization under very stringent conditions, at 47°C, where binding to cognate sequences was reduced to approximately 20% of the maximum. Elimination of binding of the probe NspCL4_446, designed to *Nitrosospira* cluster 4, against its closest nontarget sequence, EnvB1-7 (*Nitrosospira* cluster 1; derived from marine sediment), required high stringency with washing at 44°C, although dissociation from the closest target associated with the soil environment occurred at 42°C (Fig. 2c).

Genus *Nitrosomonas*. Probe Nmo254 was designed to recognize all *Nitrosomonas*-like sequences to the exclusion of all *Nitrosospira*-like sequences. Its specificity was improved, during the study, as new sequences entered the database, and probe Nmo254a provides fewer mismatches with the full range of target sequences. Nevertheless, two recently published *Nitrosomonas* sequences, those of *N. ureae* and *N. communis* (21), and that of an environmental clone (pH7C56) have a single mismatch, and further probe design is required for their detection. It was generally more difficult to design probes for the *Nitrosomonas* genus than for *Nitrosospira*, as might be predicted from the large degree of 16S rDNA sequence variation observed within *Nitrosomonas*. A single probe specific to *Nitrosomonas* cluster 6 could not be designed because of lack of sequence similarity between three library soil clones (cluster 6a) recovered in a previous study (26) and all other sequences in this cluster. Because of their potential importance in these soils, a highly specific probe, Nmo6a_205, was developed for the detection of these sequences; the probe provided clear differentiation between target and nontarget standards at 44°C Fig. 2d). Probe NmoCL6b_376 was designed to detect all remaining *Nitrosomonas* cluster 6 sequences to the exclusion of all other β-subgroup ammonia oxidizer sequences at a hybridization temperature of 46°C. *Nitrosomonas* cluster 7 (which is represented by *N. europaea*, *N. eutropha*, and *Nitrosomonas* sp. strain NM57) and a single marine enrichment sequence, B2aW2 (accession no., Z69138), were differentiated from all other *Nitrosomonas*-related sequences by the use of highly specific probe NmoCL7_439 at a hybridization temperature of 44°C. This probe carries at least four mismatches to other ammonia oxidizer sequences and three mismatches to the closest non-ammonia-oxidizer sequence, i.e., that from *Cytophaga uliginosa* NCIMB 1863. The only sequence cluster for which no specific probes could be designed within the amplified 16S rDNA region was *Nitrosomonas* cluster 5, previously detected only in highly polluted marine samples.

Analysis of environmental samples by DGGE and probing. Samples of soil from Craibstone sites maintained at a range of pH values were analyzed by DGGE followed by hybridization with probes designed against the different clusters of ammonia oxidizers. Very similar banding patterns were recovered for all samples, and amplified products from all Craibstone soil plots, irrespective of pH, could be separated into three groups of bands at 43.4 to 43.7, 44.0 to 45.3, and 45.5 to 46.4% denaturant (Fig. 3A). These bands comigrated with products from cloned standards representing sequence clusters *Nitrosomonas* cluster 6a, *Nitrosomonas* clusters 6b and 5 and *Nitrosospira* clusters 2 and 3, and *Nitrosospira* cluster 4, respectively (Fig. 3A). Bands comigrating with standards from clusters 2 to 6 were present in all samples, supporting studies of the community structure of ammonia oxidizers based on 16S rRNA gene libraries from the same soil (26). No bands comigrated with the *Nitrosospira* cluster 1 or *Nitrosomonas* cluster 7 standards used. None of the bands recovered could be ascribed to a sequence cluster on the basis of migration alone because of comigration of certain bands from different clusters.

Presence or absence of sequence clusters. All of the bands visible by ethidium bromide fluorescence were detected by hybridization with the general β-subgroup ammonia oxidizer probe β–AO233 following membrane transfer (Fig. 3B). Subsequent hybridization with cluster-specific probes identified the four sequence clusters previously shown by cloning and sequence analysis to be present in this soil: *Nitrosospira* clusters 2, 3, and 4 and *Nitrosomonas* cluster 6a (Fig. 3C to F). Hybridization with probe Nsp436, the general probe for the genus *Nitrosospira*, targeted all bands visible in Fig. 3A and B, except for the *Nitrosomonas* cluster 6a band (data not shown). This band hybridized strongly to general *Nitrosomonas* probe

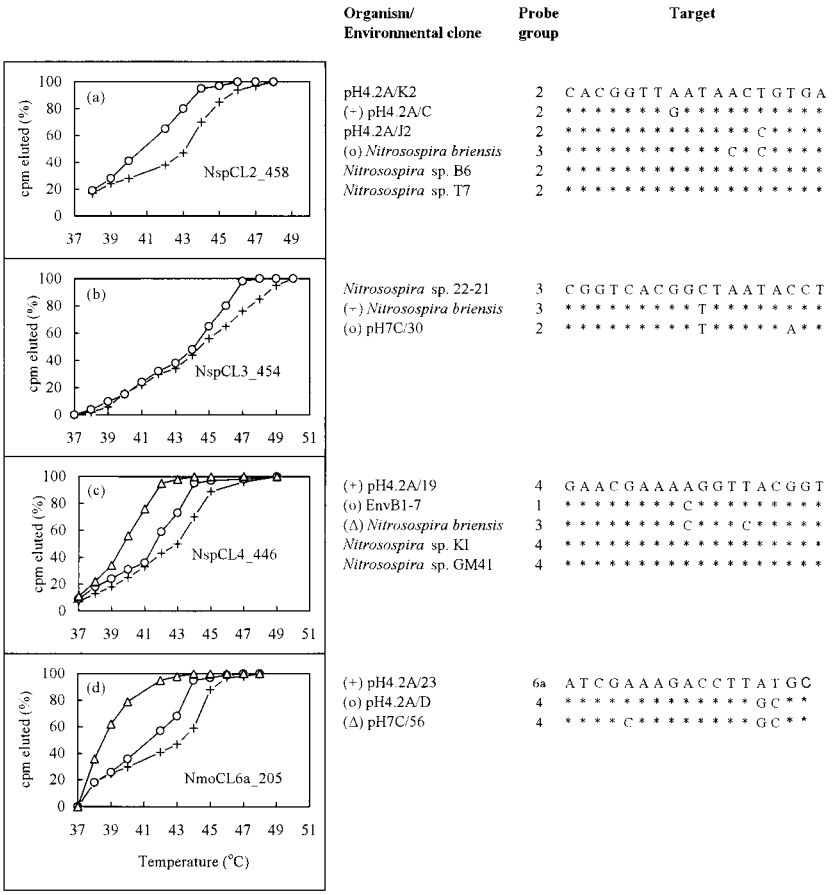

FIG. 2. Dissociation (DNA-DNA) kinetics for probes to clusters of *Nitrosospira* (a to c) and *Nitrosomonas* (d) detected on DGGE gels: NspCL2_458 (a), NspCL3_454 (b), NspCL4_446 (c), and NmoCL6a_205 (d). The rDNA targets used for *Nitrosospira* were derived from *Nitrosospira briensis* (accession no., M96396) and clones pH4.2A/K2 (accession no., Z69167), pH4.2A/C (accession no., Z69159), pH4.2A/J2 (accession no., Z69166), pH7C/30 (accession no., Z69191), pH4.2A/19 (accession no., Z69150), and EnvB1-7 (accession no., Z69102). *Nitrosospira* sp. strains B6, T7, KI, and GM41 are described by Utåaker et al. (28). The rDNA targets for *Nitrosomonas* were derived from *N. europaea* (accession no., M96399) and clones pH7C/54 (accession no., Z69196), pH4.2A/23 (accession no., Z69151), pH7C/56 (accession no., Z69197), DT1.9 (accession no., U62884), pH4.2A/3E (accession no., Z69155), and pH4.2/17 (accession no., Z69149). + denotes the cognate target sequence, and ○ and △ represent the closest available nontarget sequences. Asterisks denote that the sequence is identical to the top sequence in that position. Differences are denoted by the replacement nucleotides shown below the target sequence. Probes were named to agree as closely as possible with the scheme used by Mobarry et al. (18), except that the position of the 5′ nucleotide complementary to the 3′ probe nucleotide is given as a suffix (*E. coli* numbering [3]).

Nmo254. The central set of four bands comigrated with *Nitrosospira* cluster 2 and cluster 3 standards. These bands hybridized with probes designed for these groups, NspCL2_458 and NspCL3_454 (Fig. 3C and D). The lowest group of three bands comigrated with products from the *Nitrosospira* cluster 4 standard and hybridized strongly to NspCL4_446, the cognate probe for this cluster (Fig. 3E).

Hybridization with the probes designed to detect *Nitrosospira* cluster 1 and *Nitrosomonas* cluster 7, NspCL1_249 and NmoCL7_439, respectively, produced no signal from any of the soil samples under the conditions shown in Table 1. It was not possible, by using current data, to design a probe within the CTO-amplified region specific for *Nitrosomonas* cluster 5, but the absence of a hybridization signal in the denaturant range of 44.4 to 45.2% with general *Nitrosomonas* probe Nmo254 indicated that representatives of cluster 5 were absent from the amplified material. Additionally, no signal was generated with NmoCL6b_376, which was designed to detect *Nitrosomonas* cluster 6b. *Nitrosomonas* sequences were therefore represented only by cluster 6a, which was detected by the cluster 6a probe, NmoCL6a_205.

Distribution of clusters as a function of pH. Our earlier observations of clone libraries recovered from soil plots at pHs 4.2 and 7.0 suggested that the abundances in libraries of *Nitrosospira* cluster 2 and 3 sequences from soil were dependent on soil pH (26). Cluster 2 was more common in soil at pH 4.2, while *Nitrosospira* cluster 3 was more prevalent at pH 7. These data provided no evidence for pH-associated effects on the abundance of *Nitrosospira* cluster 4, while only three clones were obtained for *Nitrosomonas* cluster 6a. Hybridization of blotted DGGE gels loaded with DNA amplified from the full range of pH-controlled plots at the Craibstone site with probes NspCL2_458 and NspCL3_454 demonstrated that PCR products from neutral soil hybridized strongly with probe NspCL3_454 and poorly with probe NspCL2_458 (Fig. 3C and D). PCR products from acidic soil samples generated the opposite hybridization pattern, with a reduction of *Nitrosospira* cluster 3 PCR products and an increase in *Nitrosospira* cluster 2. *Nitrosospira* cluster 4 varied with pH (Fig. 3E) but no significant effect of soil pH on *Nitrosomonas* cluster 6a could be detected (Fig. 3F).

Quantification of hybridization signals was achieved follow-

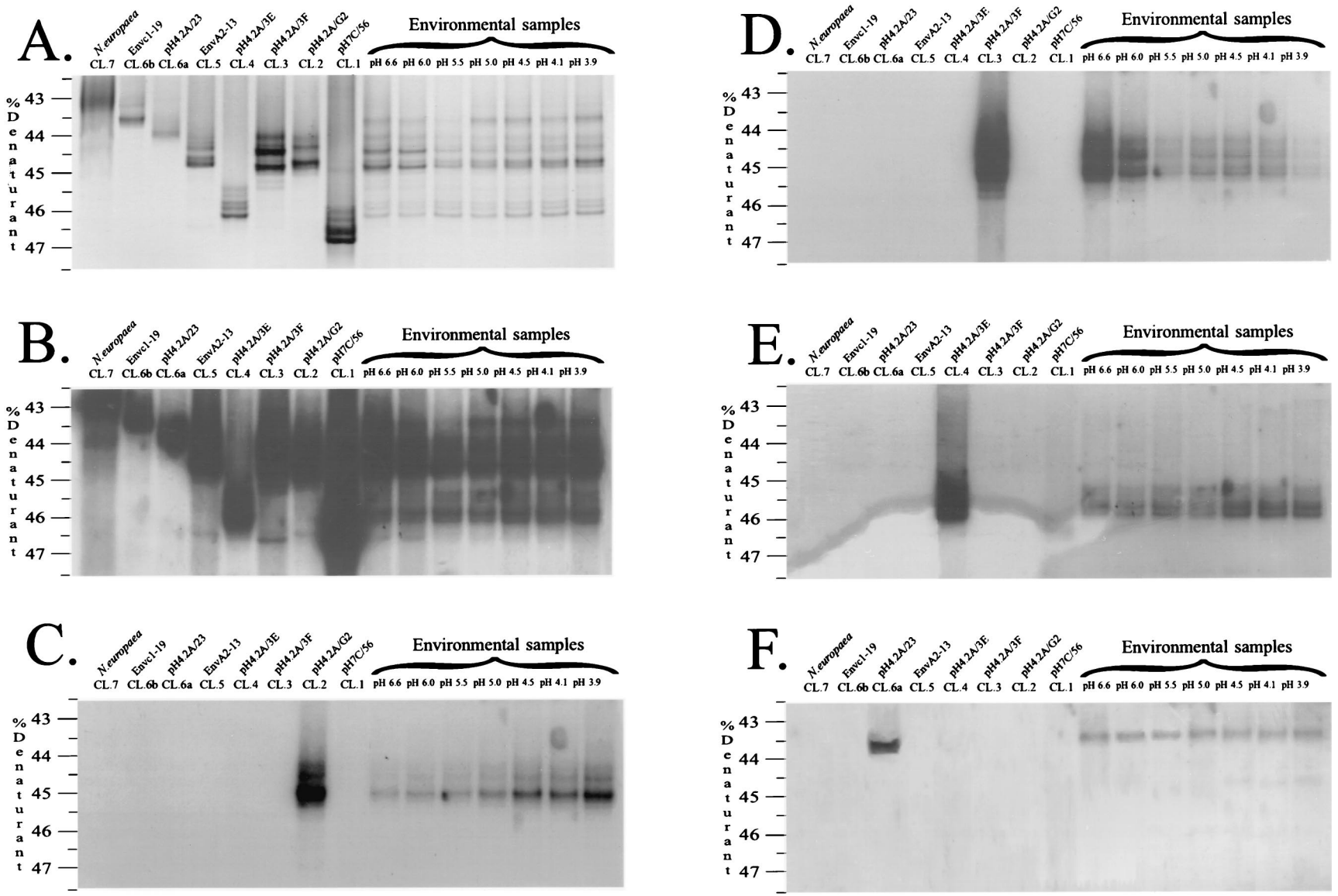

FIG. 3. Analysis of β-subgroup ammonia oxidizers by DGGE, membrane transfer, and hierarchical probing. Soil DNA was amplified by 35 cycles of PCR with the CTO primer pair, and the products were separated by electrophoresis on a 38 to 50% denaturant gradient gel. Phylogenetically defined amplification products from each sequence group were included as migration and hybridization standards (left eight lanes). The gel was stained with ethidium bromide (A) before transfer of the DNA to a nylon hybridization support membrane. The transfer membrane was subsequently hybridized with ^{32}P-labelled oligonucleotides β–AO233 (B), NspCL2_458 (C), NspCL3_454 (D), NspCL4_446 (E), and NmoCL6a_205 (F). The membrane was stripped of bound probe between experiments by being washed in 2× SSC–0.1% sodium dodecyl sulfate at 65°C. Hybridization conditions are given in Table 1.

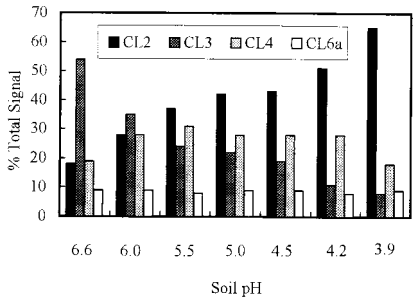

FIG. 4. Quantification of hybridization signals from DGGE gels of soil samples for Fig. 3C to F. Quantification was achieved by phosphorimaging and profile analysis of each lane of the membranes used. The percent total signal is the hybridization signal for each sample expressed as a percentage of the signal for the cluster standard, normalized as described in the text, and averaged over duplicate sets of lanes from independent DNA extractions from each soil. Least significant difference values for clusters 2, 3, 4, and 6a were 2.13, 2.36, 1.50, and 1.15, respectively.

ing probing with β–AO233 (Fig. 3A) and probes specific to clusters 2 and 3, enabling calculation of relative abundances of each cluster in samples of soil at each pH value. Values for each soil pH were averaged over duplicate experiments employing separate DNA extractions from the same samples. These data demonstrate a marked and gradual change in the balance of PCR products recovered from *Nitrosospira* clusters 2 and 3 with changing pH (Fig. 4). Cluster 2 signals decreased significantly with increasing pH (analysis of variance [ANOVA]; $P = 2.5 \times 10^{-5}$), while cluster 3 signals showed a significant increase ($P = 4.2 \times 10^{-5}$). The *Nitrosospira* cluster 4 signals increased with soil pH up to a maximum at pH 5.5, with a decrease at higher pH values (ANOVA; $P = 0.0033$). *Nitrosomonas* cluster 6 sequence signals showed no significant change with pH ($P = 0.978$). The data therefore indicate that strains belonging to clusters 2 and 3 show greatest relative abundances in low- and neutral-pH soils, respectively. The effect of pH on cluster 4 is less marked, but the relative abundance appears to be greatest at moderately acid pH values, while the relative abundance of cluster 6a is apparently unaffected by soil pH.

DISCUSSION

The recent expansion of 16S rDNA sequence information from the β-subgroup ammonia oxidizers has increased the potential value of molecular techniques in the study of the ecology of this environmentally and commercially important group of organisms. Phylogenetic analysis has demonstrated a division of β-subgroup ammonia oxidizers into a number of clusters or related sequences. Analysis of the relative abundances of sequences obtained by PCR amplification from environmental samples with ammonia oxidizer-specific primers further suggests that the distribution of different clusters is linked to environmental factors. An alternative approach to the study of distribution of groups within the environment is the use of ammonia oxidizer-specific oligonucleotide probes, but previous studies have been limited by the use of sequence information from pure cultures to design and test probes. The initial aim of this study was to use the expanded database to improve and extend phylogenetic probes for autotrophic ammonia oxidizers, in particular probes specific for the clusters defined by phylogenetic analysis of rDNA sequences.

On the basis of current rDNA sequence information, the CTO primer pair (15) is specific to all β-subgroup ammonia oxidizers. These primers, however, only provide specificity when used in combination, as in PCR amplification, and individual primers are not reliable as ammonia oxidizer-specific probes. Further analysis of the target site used in this study, 303 bases spanning the homologous *E. coli* positions 198 to 500, did not provide a unique sequence specific to all ammonia oxidizers. It was possible, however, to design a probe which appears to be specific for the *Nitrosospira* group. *Nitrosomonas* probe Nmo254 was specific for all *Nitrosomonas* sequences obtained from our study site but will not hybridize with *N. ureae* and *N. communis*, recently sequenced by Pommerening-Röser et al. (21). It is possible that the analysis of new environments will identify additional sequences which do not hybridize with Nmo254 or with the *Nitrosospira* probe. This stresses the need for the generation of clone libraries when initiating studies of new environments and the use of low-specificity PCR primers to detect related but uncharacterized groups (17).

The approach of using DGGE and probing adopted in this study involved initial amplification using the ammonia oxidizer-specific CTO primers prior to the use of probes. A hybridization analysis of amplification products from our agricultural soil samples demonstrated that all amplification products could be assigned to previously described cluster groups within the β-subgroup ammonia oxidizers. This confirms the specificity of the PCR primers and the described reaction conditions in diverse environments. Under these conditions, cluster-specific probes applied to the analysis of PCR products generated with the CTO primer pair need only be faithful within the β-subgroup ammonia oxidizer clade. None of the bacterial species which show closest sequence similarity to the PCR primer pair show close similarity to any of the probes presented. Additionally, some of these probes do show sufficient specificity in sequence similarity searches to hold potential for use in more complex analysis such as in situ probing (Table 1). It is also clear that further probes will need to be designed for the analysis of DGGE patterns described here, particularly for the genus *Nitrosomonas*, which cannot be exhaustively analyzed with the probe set presented. This probe set allows detection of, and discrimination between, each of the ammonia oxidizer clusters described by Stephen et al. (26), except for cluster 5, which has so far only been detected in polluted marine samples.

This study also provided information on the hybridization conditions required for use of each cluster probe. Hybridization at T_d values calculated from elution profiles of some probe and target sequences was capable of distinguishing target and known nontarget ammonia oxidizer sequences. This was not the case, however, for all probes to the clusters present in the soil samples used in this study, necessitating the use of more-stringent conditions for hybridization.

An earlier study (26) suggested the existence of two "specialist" clusters, *Nitrosospira* clusters 2 and 3, favored by acid (pH 4.2) and neutral (pH 7) conditions, respectively. DGGE analysis of PCR products followed by probing for the specialist clusters confirmed these findings. Signals from cluster 2 probes showed a continuous increase as soil pH values decreased from 6.6 to 3.9, while those from cluster 3 probes decreased over this range. Both studies also demonstrated similar levels of cluster 6 at all soil pH values investigated and indicated that *Nitrosospira* cluster 1 and *Nitrosomonas* clusters 5, 6b, and 7 were either absent from this environment or below the detection limits of this approach at the time of sampling. Sequences from the earlier study were obtained following PCR amplification with the βAMOf primers of McCaig et al. (17), while the present study involved initial amplification with a completely different primer set, CTO, targeting a different region of 16S rDNA. Soil samples were obtained from the same study sites.

Despite the use of different primers, both experimental approaches indicated similar effects of soil pH on the relative abundances of ammonia oxidizer clusters 2 and 3, although the earlier study was based on a relatively small number of sequences. The similarity in results from the two sets of primers indicates that primer bias is unlikely to be selecting for particular sequences, thereby greatly increasing confidence that the findings obtained represent in situ abundances. Other potential biases include lysis bias between clusters, although this is likely to be minimized by the use of bead beating. The suggestion that *Nitrosospira* cluster 4 is a "generalist" cluster was based on similar sequence abundances at only two pH values, 4.2 and 7, while the present study involved finer-scale analysis of soil pH. DGGE and probing analyses provided further evidence for similar levels of cluster 4 at low and neutral pH values but indicated that levels were greatest at pH 5.5, decreasing at lower and higher pH values. The effect of pH on the relative abundance of cluster 4 was less marked than on those of clusters 2 and 3 and would not have been detected in the earlier study, for which only two samples, one acid and one neutral, were analyzed.

Confirmation of the existence of generalist and specialist ammonia oxidizer strains highlights the need for physiological studies of these groups, in particular, representatives of *Nitrosospira* clusters 2, 3, and possibly 4, to identify factors leading to pH-related selection. Several cluster 3 strains are available in pure culture, and two pure cultures of *Nitrosospira* cluster 2 and *Nitrosospira* sp. strains B6 and T7 have recently been obtained (28). Currently, nothing is known of the physiological differences which may lead to pH-based selection, but our findings suggest that *Nitrosospira* cluster 2 may be an important genotype in acid soils and that physiological studies of representatives of this group are required.

The design of genus- and cluster-specific probes and their use in combination with DGGE analysis of PCR amplification products from directly extracted soil DNA provide a powerful, rapid, and quantitative technique for the analysis of the structure of ammonia oxidizer populations in natural environments. The technique has demonstrated clear links between genotype and ecotype in soils maintained at a range of pH values for 36 years, identifying candidate generalist and specialist strains. The findings provide the basis for further studies on the physiological characteristics of these strains leading to differences in their distribution and on their significance for nitrification rates and the persistence of ammonia oxidizers in natural environments.

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