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Analysis of Ammonia-Oxidizing Bacteria of the β Subdivision of the Class *Proteobacteria* in Coastal Sand Dunes by Denaturing Gradient Gel Electrophoresis and Sequencing of PCR-Amplified 16S Ribosomal DNA Fragments

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Denaturing gradient gel electrophoresis (DGGE) is a powerful and convenient tool for analyzing the sequence diversity of complex natural microbial populations. DGGE was evaluated for the identification of ammonia oxidizers of the β subdivision of the *Proteobacteria* based on the mobility of PCR-amplified 16S rDNA fragments and for the analysis of mixtures of PCR products from this group generated by selective PCR of DNA extracted from coastal sand dunes. Degenerate PCR primers, CTO189f-GC and CTO654r, incorporating a 5′ GC clamp, were designed to amplify a 465-bp 16S rDNA region spanning the V-2 and V-3 variable domains. The primers were tested against a representative selection of clones and cultures encompassing the currently recognized β-subdivision ammonia oxidizer 16S rDNA sequence diversity. Analysis of these products by DGGE revealed that while many of the sequences could be separated, some which were known to be different migrated similarly in the denaturant system used. The CTO primer pair was used to amplify 16S rDNA sequences from DNA extracted from soil sampled from Dutch coastal dune locations differing in pH and distance from the beach. The derived DGGE patterns were reproducible across multiple DNA isolations and PCRs. Ammonia oxidizer-like sequences from different phylogenetic groupings isolated from gene libraries made from the same sand dune DNA samples but prepared with different primers gave DGGE bands which comigrated with most of the bands detected from the sand dune samples. Bands from the DGGE gels of environmental samples were excised, reamplified, and directly sequenced, revealing strong similarity or identity of the recovered products to the corresponding regions of library clones. Six of the seven recognized sequence clusters of β-subdivision ammonia oxidizers were detected in the dune systems, and differences in community structure between some sample sites were demonstrated. The most seaward dune site contained sequences showing affinity with sequence clusters previously isolated only from marine environments and was the only site where sequences related to the *Nitrosomonas* genus could be detected. *Nitrosospira*-like sequences were present in all sites, and there was some evidence of differences between *Nitrosospira* populations in acid and alkaline dune soils. Such differences in community structure may reflect physiological differences within β-subdivision ammonia oxidizers, with consequent effects on nitrification rates in response to key environmental factors.

Chemolithoautotrophic ammonia-oxidizing bacteria, which convert ammonia to nitrite, play an important role in the global cycling of nitrogen (28, 32). This group of organisms has been refractory to conventional techniques for isolation of pure cultures, hampering physiological and ecological studies. As a result, most studies have focused upon a single isolate of *Nitrosomonas europaea*, which is perhaps the most easily isolated and cultured ammonia-oxidizing species (28). However, several culture-based studies have indicated that *Nitrosospira* species are common in terrestrial habitats (1, 2, 17).

Known ammonia oxidizers comprise two monophyletic groups based on comparison of 16S rDNA sequences from cultured strains. One of these comprises strains of *Nitrosococcus oceanus* within the γ subdivision of the *Proteobacteria*, and the other contains the genera *Nitrosomonas* and *Nitrosospira* (the latter now encompasses the genera *Nitrosolobus* and *Nitrosovibrio*) within the β subdivision of the *Proteobacteria* (12, 36, 39, 44, 45). Several recent studies have used 16S rDNA sequence information to assess the diversity and distribution of the β-subdivision ammonia oxidizers in natural environments (13, 14, 20, 21, 33, 40, 41).

Previous investigations have been of three types. Specific or semispecific PCR of 16S rDNA fragments, followed by direct or cloning-assisted sequence analysis, has discovered new sequences and allowed their precise identification through phylogenetic analysis, but this approach is time-consuming and not well suited to the analysis of multiple samples in ecological studies (20, 33). Hybridization with oligonucleotide probes of directly extracted rRNA or 16S rDNA fragments recovered by PCR (13, 14, 40) is quicker but suffers from the inability of probes to identify novel sequences or to detect sequence variation which may occur outside of the probe target region. Whole-cell hybridization with fluorescent oligonucleotide probes provides precise spatial information and has perhaps the best

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long-term potential for accurate quantification of cell numbers (21, 41).

In the present investigation, a fourth molecular approach, denaturing gradient gel electrophoresis (DGGE) (24) of specifically amplified 16S rRNA gene fragments, has been used to analyze the β-subdivision ammonia-oxidizing populations from coastal sand dune samples. Samples taken from a lime-poor dune region differed from each other in both proximity to the beach (from the seaward dune face through the older, landward dunes) and pH (7.9 to 4.5, respectively). Autotrophic ammonia oxidation has been detected in all of these samples (5), although most known ammonia-oxidizing isolates fail to nitrify below pH 6 in liquid culture (28). Lime-rich dune sites (pH 7.7) with similar vegetation were also sampled to allow the comparison of acidic and neutral landward dune sites. As this study employed PCR primers which specifically targeted the β-subdivision ammonia oxidizers, this approach did not detect ammonia-oxidizing bacteria of the γ subdivision of the *Proteobacteria* or as-yet undescribed nitrifiers which might also contribute to the autotrophic ammonia oxidation detected in the samples under investigation. This investigation also failed to address heterotrophic organisms which might contribute to nitrification, although it has been shown elsewhere that autotrophic nitrification is the chief route to nitrate production in this dune system (5).

DGGE provides information about sequence variation in a mixture of PCR fragments of identical length based upon differential mobility in an acrylamide gel matrix of increasing denaturant concentration; it can be used conveniently to infer differences in the composition of microbial communities (9, 22, 35, 43). In addition, clone libraries (33) of PCR-amplified 16S rDNA fragments from each site were made to identify, by phylogenetic analysis, some of the potential β-subdivision ammonia oxidizers present in the dune samples. DGGE patterns from environmental samples were compared with the migration of reference clones of known sequence, and the major environmental bands were excised, reamplified, and sequenced to investigate their identities further. DGGE, coupled with sequence analysis, allowed the detection of specific groups of β-subdivision ammonia oxidizers and permitted their presence to be correlated with key environmental factors.

MATERIALS AND METHODS

Terschelling and Meijendel sand dune soil samples. Soil samples were collected in September 1994 from two sand dune areas in the Netherlands, the island of Terschelling (53°23′N, 5°16′E) and the coastal nature reserve Meijendel (52°08′N, 4°19′E). Characteristics for these sites are shown in Table 1. At the Terschelling site, samples were taken along a transect from the seaward dune face through the older landward dunes. The lime-poor nature of this region has led to decalcification in the top layers of landward dune sites, which is reflected in an acidic soil pH (10). A succession of the vegetation along this transect has been described (39) and includes marram grass (*Ammophila arenaria*) in the seaward sites, with a transition to mainly grasses and a mixture of lichens inland (*Calamogrostis epigejos* and *Elymus* sp.; *Cladina patentosa* and *Campylopus ithoglexus*), and grasses and sedges at the locations furthest inland (*Corynephorus canescens* and *Carex arenaria*). Meijendel samples were relatively lime rich (alkaline) and were taken from one protected seaward dune location (M-6), where *Ammophila arenaria* was the dominant vegetation, and one landward dune location (M-7), whose vegetation was dominated mainly by grasses and herbs (*Phleum arenatium* and *Sedum acre*). Each soil sample was prepared by homogenizing (sieving through a <4-mm mesh sieve) at least 30 100-mm cores taken from beneath tussocks of marram grass. Analyses of pH, moisture, total nitrogen, total $CaCO_3$, NO_3^-, N mineralization after 100 days, and relative nitrification rates were as described by Troelstra et al. (37) and De Boer et al. (5). Samples were placed in an insulated container during transport and stored at 4°C until use. All values represent the mean of two measurements, except for N mineralization after 100 days, which was performed in triplicate. The results of these analyses are summarized in Table 1.

TABLE 1. Characteristics of Terschelling and Meijendel sand dune samples at the time of sampling

Site[a]	pH	Moisture content (%)	Total N (mg kg [dry wt]$^{-1}$)	Total $CaCO_3$ (g kg [dry wt]$^{-1}$)	Min-N*	N mineralization/100 days (mg kg [dry wt]$^{-}$)[b]	Relative nitrification rate (%)[c]
T-1	7.9	3.0	50	0.71	0.84	2.3 ± 0.1	100
T-2	7.9	2.7	176	0.72	2.80	12.5 ± 4.2	100
T-3	6.1	4.3	405	0.00	3.01	23.4 ± 6.0	100
T-4	4.9	2.8	240	0.04	2.80	16.0 ± 3.9	38
T-5	4.8	4.7	301	0.02	1.40	16.4 ± 0.8	61
M-6	7.8	3.1	125	2.74	1.96	11.6 ± 0.4	100
M-7	7.6	2.9	292	2.54	4.97	13.6 ± 1.0	100

[a] T and M represent samples from Terschelling and Meijendel, respectively. Terschelling sites are numbered from the most seaward site (T-1) to the most landward site (T-5). M-6 represents a seaward dune location that is protected from sprayed seawater and mist and exposure to wind-blown sand. M-7 represents a landward, older dune site.

[b] ± indicates 95% confidence limits.

[c] Relative nitrification is defined as (total nitrate production/total mineralized N) × 100%.

DNA extraction. Direct DNA extractions were performed by cell disruption by a modification of the method described by Stephen et al. (33). The soil sample (0.5 g [wet weight]), 0.5 ml of 120 mM K_2HPO_4 buffer (pH 8.0), 0.5 g of glass beads (diameter, 0.1 mm; BioSpec Products, Techno Lab, Alkmaar, The Netherlands), and 0.5 ml of water-saturated phenol (pH 8.0) (Aquaphenol; Ampligene, Illkirch, France) were mixed in a 2-ml screw-cap polypropylene tube (Anthos Labtec, Heerhugowaard, The Netherlands). The samples were shaken at 5,000 rpm three times for 30 s in a mini-beadbeater (BioSpec Products) and chilled on ice between shaking periods. After centrifugation (5 min at 5,000 × *g*), 0.4 ml of the aqueous layer was removed and extracted with 0.5 ml of water-saturated phenol and then with a 1:1 mixture of water-saturated phenol and chloroform-isoamyl alcohol (24:1). DNA was precipitated for 2 h at −20°C with 2 volumes of ice-cold 96% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). After centrifugation for 15 min (14,000 × *g* at 4°C), the pellet was washed once with ice-cold 70% ethanol and allowed to air dry. The dried pellet was resuspended in 40 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and run on a composite 0.5× TAE (1× TAE is 0.04 M Tris base, 0.02 M acetic acid, and 1.0 mM EDTA [pH 7.5]) agarose gel, prepared by pouring a 2% agarose–1% polyvinylpolypyrrolidone (PVPP; Sigma, St. Louis, Mo.) gel in 0.5× TAE and cutting away all except the top 20 mm containing the slots after solidification. The remainder of the gel was then prepared with 1% agarose in 0.5× TAE. During electrophoresis, most brownish-yellow humic substances remained within the PVPP portion of the gel while DNA passed into the 1% agarose portion of the gel. All DNA longer than 10 kb was excised for purification with the QIAquick gel extraction kit (Qiagen, Inc., Chatsworth, Calif.). DNA was eluted in a final volume of 60 μl of sterile deionized water.

Preparation of DNA templates from standards. Liquid cultures (50 ml) of ammonia oxidizers were grown in 250-ml flasks at 20°C in the dark for 6 weeks without shaking in a medium containing 330 mg of $(NH_4)_2SO_4$, 100 mg of KH_2PO_4, 40 mg of $MgSO_4 \cdot 7H_2O$, 15 mg of $CaCl_2$, and 1 ml of a trace element solution (4) per liter of deionized water. DNA extractions from *Nitrosomonas europaea* ATCC 25978, *Nitrosospira briensis* C-128 (12) and *Nitrosospira* sp. strain AHB1 (6) were performed by a standard mini-chromosomal DNA extraction method (30), except that cells were first pelleted by centrifugation for 30 min at 14,000 × *g* prior to extraction. Cultures of *Comamonas testosteroni* NCIMB 8955, *Variovorax paradoxus* NCIMB 9899, *Methylobacillus glycogenese* NCIMB 11375, and *Methylomonas methylotrophus* NCIMB 10515 and 11376 were grown in 50 ml of liquid media as directed by the supplier (25), and templates for PCR were prepared by boiling cells in the presence of Chelex 100 (Bio-Rad Laboratories, Hercules, Calif.) (20, 42). Plasmid extractions from recombinants from clone libraries used either the Wizard mini-plasmid preparation kit (Promega, Madison, Wis.) or the Qiaquick mini-plasmid preparation kit (Qiagen, Inc.).

PCR primer design and PCR conditions. All PCRs for DGGE were conducted with an equimolar mixture of three forward primers (CTO189fA-GC, CTO189fB-GC, and CTO189fC-GC), each with a GC clamp (31), and a reverse primer containing a single ambiguous base. The forward primers CTO189fA and CTO189fB (CCGCCGCGCGGCGGGGCGGGGGCACGGGGGGAGRAAAGCAGGGGATCG [GC clamp underlined]) and CTO189fC-GC (CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAG**G**AAAG**T**AGGGGATCG)were synthesized separately and collectively referred to as CTO189f-GC. The reverse primer sequence was CTO654r (CTAGCYTTGTAGTTTCAAAC GC) (*Escherichia coli* numbering [3]). The primers were designed to amplify partial rDNA sequences from β-subdivision ammonia-oxidizing bacteria while excluding other taxa for which sequences are available. The ability of this primer set to amplify a 465-bp β-subdivision ammonia oxidizer 16S rDNA fragment was tested experimentally against 100 ammonia oxidizer-like 16S rDNA clones representing the currently recognized sequence clusters (33). The CTO189f-GC

TABLE 2. Summary of the PCR conditions used in this study

Template and reaction vol (μl)	Amt of template used (ng)	Amt and type of enzyme used	Thermocycling program
Environmental samples for DGGE, 50	10–100	2.5 U of Expand (Boehringer)	60 s at 93°C, followed by 35 cycles of 30 s at 92°C, 60 s at 57°C, and 45 s at 68°C (+1 s cycle^{-1}), followed by 5-min final extension at 68°C
Cloned sequences for DGGE, 25	10	1.25 U of Expand (Boehringer)	60 s at 93°C, followed by 30 cycles of 30 s at 92°C, 60 s at 57°C, and 45 s at 68°C (+1 s cycle^{-1}), followed by 5-min final extension at 68°C
Cloned sequences for PCR assays, 25	10	1.25 U of Dynazyme (Finnzymes)	120 s at 94°C, followed by 30 cycles of 30 s at 94°C, 60 s at 57°C, and 45 s at 72°C (+1 s cycle^{-1})

primer showed a 1-bp mismatch with the following published β-subdivision ammonia oxidizer 16S rDNA sequences (*E. coli* numbering [3]): *Nitrosomonas* strain NM.83 (accession number M96400 [12]) (position 196-T), environmental clones pH7B/S2 (191-T) and pH4.2B/35 (199-G), and marine enrichment sequence B2bM2ii (193-T) (32). The only mismatch detected for the reverse primer was for *Nitrosomonas ureae* (position 649-A; accession number Z46994 [27]). Under the described PCR conditions, cloned sequences containing single mismatches with the CTO primer pair produced amplification products. *Nitrosomonas* strain NM.83 and *N. ureae* were not tested.

The possibility of amplifying rDNA from other bacterial groups was evaluated by FastA searches of the EMBL sequence database and by use of the CHECK-PROBE facility of the Ribosomal Database Project (18, 26). No other sequence with less than a total of three differences from the primer sequences was detected.

All PCRs were conducted with the buffers supplied by the manufacturers and under the conditions presented in Table 2. All reaction mixtures were overlaid with an equal volume of mineral oil (Sigma, molecular biology grade) and run in an OmniGene Thermal Cycler (Hybaid, Teddington, United Kingdom). The PCR products were examined by standard agarose gel electrophoresis (1.5% agarose, 0.5× TBE [1× TBE is 90 mM Tris-borate [pH 8.3] and 2 mM EDTA) with visualization of DNA by ethidium bromide fluorescence.

DGGE. DGGE of PCR products generated by the CTO primer pair was performed by the method described by Muyzer et al. (23) with the use of a D-Gene system (Bio-Rad Laboratories). Polyacrylamide gradient gels (8% polyacrylamide; 1.5 mm thick; 0.5× TAE; 37:1 acrylamide-bisacrylamide; 38 to 50% denaturant; 200 by 200 mm) were poured with the aid of a gradient maker (CBS, Del Mar, Calif.) and an Econo-pump (Bio-Rad Laboratories) at a speed of 5 ml min^{-1} (40-ml gradient volume). Denaturing acrylamide (100%) was defined as 7 M urea with 40% formamide (24). Gels were poured from bottom to top by pricking a hole through the sealing rubber gasket. A 10-ml stacking gel containing no denaturants was subsequently added on top, before polymerization had occurred. Gels were run either for 6.5 h at 200 V or for 16 h at 85 V in 0.5× TAE buffer at a constant temperature of 60°C. The gels were stained in MilliQ (Millipore B. V., Etten-Leur, The Netherlands) water containing 0.5 mg of ethidium bromide liter^{-1} and destained twice in 0.5× TAE buffer prior to UV transillumination. Gel images were stored by using The Imager system (Ampligene). Quantification of ethidium bromide-stained bands for competitive PCR experiments was performed with the ImageQuant program, version 3.3 (Molecular Dynamics, Inc., Sunnyvale, Calif.).

16S rDNA gene libraries from dune soil DNA samples. Clone libraries were generated as described by Stephen et al. (33). PCR products generated with the βAMOf and βAMOr primer pair (20) were recovered from dune soil DNA extractions and ligated into a plasmid vector with the pGEM-T system (Promega Corp.) before being used for transformation of *E. coli* (Epicurian Coli XL1-Blue MRF supercompetent cells; Stratagene, La Jolla, Calif.) as specified in the manufacturer's standard protocol. The presence of inserts of the expected size was confirmed by PCR with the flanking vector primers SP6 and T7 (Promega) and a small portion of untreated white colony as the template. Amplification was done by 30 rounds of 94°C for 30 s, 50°C for 30 s, and 72°C for 70 s with 2 U of *Tbr* polymerase (Dynazyme; Finnzymes, Espoo, Finland) and the buffer supplied by the manufacturer. Colonies containing reannealed vector generated a product of approximately 160 bp as predicted from the manufacturer's map. A total of 34 recombinant colonies were grown overnight at 37°C with shaking at 200 rpm in 3 ml of Luria-Bertani medium (Difco Laboratories, Detroit, Mich.) supplemented with 50 mg of ampicillin ml^{-1}. Plasmid isolation used the Wizard miniprep kit (Promega). DNA sequence was generated with the vector-priming sites SP6 and T7 and the internal 16S rDNA primer sites described by Edwards et al. (7). Sequencing reactions were performed with an ABI PRISM Dye Terminator Cycle Sequence Ready Reaction kit as specified by the manufacturer (Perkin-Elmer, Foster City, Calif.). The products were analyzed with an Applied Biosystems (San Jose, Calif.) automatic sequencer (model 373 with "Stretch" adapter; Department of Biotechnology, Wageningen, The Netherlands), and sequences were assembled with the Sequence Navigator program (version 1.0, release 3.0.1; Applied Biosystems).

Analysis of clone sequences. All sequences were aligned against a representative selection of prokaryote sequences extracted from the Ribosomal Database 16S rRNA sequences (18). Sequences were manipulated with SeqApp, version 1.9a169 (11). The alignment used for sequence analysis comprised 1,114 sites which could be unambiguously aligned for all recovered clones and published sequence data. All sequence analyses were implemented in PHYLIP 5.57 (8). Distance matrix analyses were done with the Jukes and Cantor (15) correction, tree construction was done by the neighbor-joining method (29), and bootstrapping was conducted with 100 replicates with the program SeqBoot (8). Affiliations of recovered ammonia oxidizer-like sequences are shown in Fig. 1. Bootstrap support for the sequence clusters was similar to that found previously (33).

Recovery and sequence analysis of bands from DGGE gels. The middle portion of bands derived from environmental samples was excised for DNA extraction. Approximately 60 mg of acrylamide gel material per band was put into a 2-ml screw-cap polypropylene tube (Anthos Labtec) with 0.1 g of glass beads (diameter, 0.1 mm; BioSpec Products) and 0.1 ml of TE buffer and shaken, as described above, in a mini-beadbeater (BioSpec Products). After centrifugation at 14,000 × *g* for 2 min, the gel fragments and buffer were removed with a pipette, transferred to a 1.5-ml microcentrifuge tube, and incubated overnight at 37°C. Buffer containing extracted DNA (1 μl) was subsequently used as the template for a 50-μl PCR mixture under the same conditions as described for environmental samples. A sample of each PCR product (5 μl) was subjected to agarose gel electrophoresis, and the other 45 μl was purified with Wizard PCR Preps (Promega) and used as template in three double-stranded sequencing reactions with both flanking CTO primers (forward without GC clamp) and the internal primer 536r (16). Reactions were carried out by the same method as used for plasmid templates.

Nucleotide sequence accession numbers. The near-full-length clone sequences (1.1 kb) which showed affinity with the β-subdivision ammonia oxidizers have been deposited in GenBank with accession numbers U62867 to U62886. The sequences of bands 2 and 5 from the DGGE gels have been deposited in the EMBL database under accession numbers U70444 and U70445, respectively.

RESULTS AND DISCUSSION

Testing of the CTO primer pair for β-subdivision ammonia-oxidizing bacteria. DGGE provides the potential for analysis of sequence diversity of complex natural microbial populations (22). In this investigation, DGGE was evaluated for the identification of β-subdivision ammonia oxidizers based upon the mobility of PCR-amplified 16S rDNA fragments and for analysis of mixtures of PCR products from this group generated by selective PCR from DNA recovered from dune samples varying in location and pH (Table 1).

McCaig et al. (20) previously designed PCR primers to recover selectively 16S rRNA gene fragments from β-subdivision ammonia oxidizers. Application of these primers to natural samples revealed that while they did indeed recover sequences related to ammonia oxidizers, they also amplified a variety of other β-subdivision sequences (33). In the present study, recently published sequence data (20, 33, 38) were used to design new primers for β-subdivision ammonia oxidizers. The new primers, CTO189f-GC and CTO654r, produced PCR products of the expected size from 100 recombinant plasmids chosen to sample the currently recognized 16S rDNA sequence diversity of β-subdivision ammonia oxidizers (Fig. 1) and from DNA of pure cultures of *Nitrosospira* sp. strain AHB1 (6), *Nitrosospira briensis* C-128, and *Nitrosomonas europaea* ATCC 25978. Un-

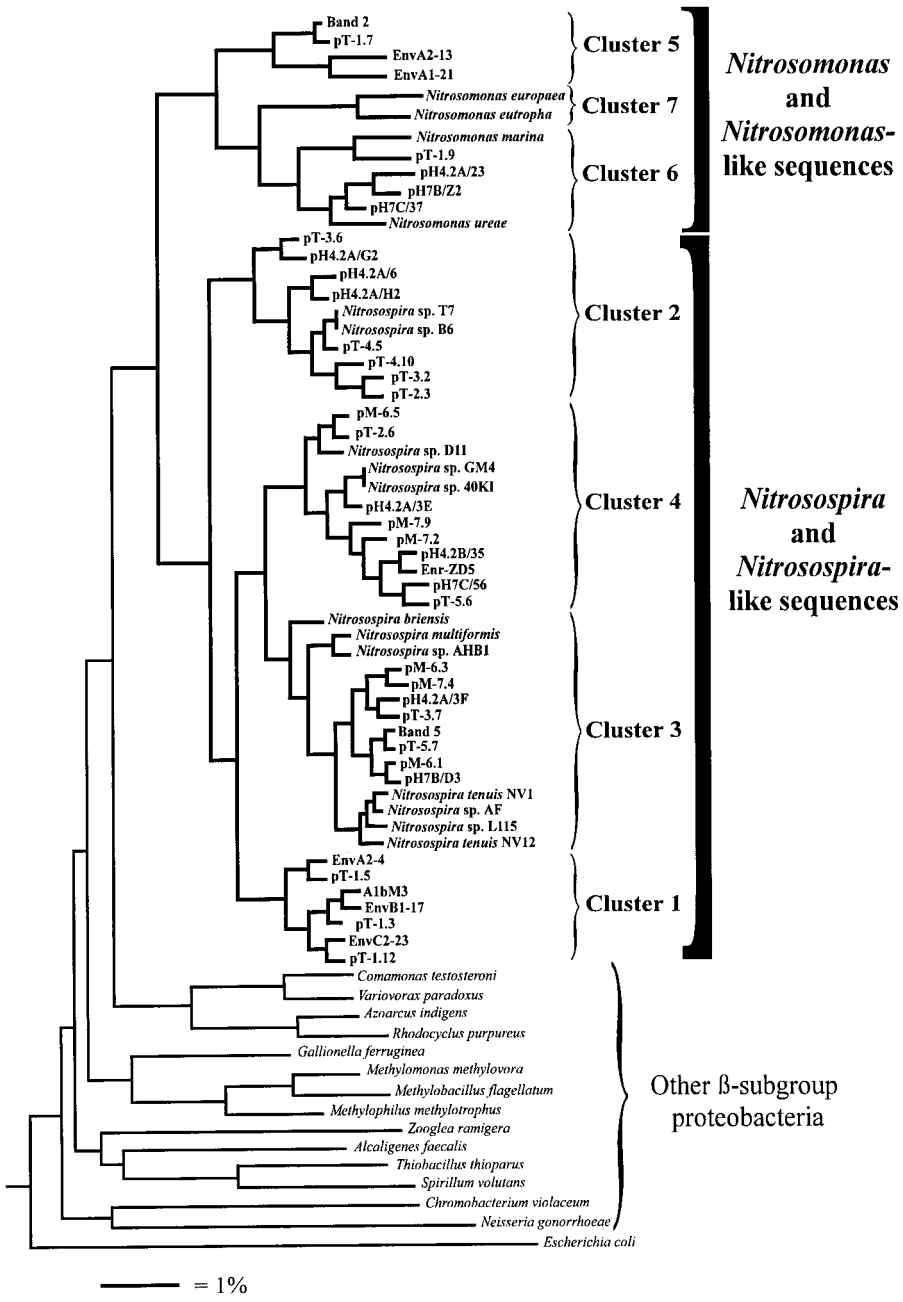

FIG. 1. Neighbor-joining tree based upon partial 16S rDNA sequences from β-subdivision ammonia oxidizers. Sequence analysis was done as described in the text, except that to include all sequences used in the text, only 303 base positions were included in the calculations. Cluster designations are those used by Stephen et al. (33). Environmental clones from this study are referred to with reference to the dune sites from which they were recovered, with p indicating plasmid, T indicating Terschelling, and M indicating Meijendel, as in Table 1. The first numerical value indicates the sample site, and the second numeral is the clone designation; e.g., 1.9 is clone 9 from sample site 1. Sequence names beginning with "pH" or "Env" refer to clones derived from environmental samples from soil (of the pH indicated) and from marine sediment, respectively. Sequence A1bM3 is derived from a marine sediment enrichment culture (33). *Nitrosospira* isolates 40KI, AF, B6, D11, GM4, L115, and T7 were purified from soil (39). For band 2 and band 5, see the description of data in Fig. 5.

der the PCR conditions used, no products were obtained with DNA from the type strains of the related β-subdivision species *Comamonas testosteroni*, *Variovorax paradoxus*, *Methylobacillus glycogenese*, and *Methylomonas methylotrophus*. The new primers did not amplify products from all tested representatives (n = 50) of the non-ammonia oxidizer sequences recovered from soil samples with the primers of McCaig et al. (20).

To evaluate further the behavior of the new primers, CTO189f-GC and CTO654r, a series of PCRs was performed with different ratios of template DNA from clones pH4.2A/6 (*Nitrosospira*, cluster 2), and pH4.2A/23 (*Nitrosomonas*, cluster 6) (Fig. 2). Our aim was to investigate the extent to which the relative initial template concentration in a simple mixture of two templates might influence the ratio of products formed. The two DNA templates produced bands of similar intensity in separate reactions and when mixed at a ratio of 1:1. The expected and

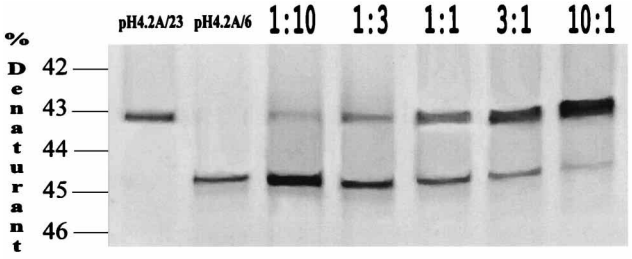

FIG. 2. DGGE of PCR products from different mixtures of two clones, pH4.2A/6 and pH4.2A/23. A DGGE gel displays the products of PCRs with different ratios of initial templates. The two cloned sequences, pH4.2A/6 (*Nitrosospira*, cluster 2) and pH4.2A/23 (*Nitrosomonas*, cluster 6) were either amplified separately (lanes 1 and 2) or mixed in the given ratios. Samples of 5 μl were loaded for each single template PCR, and 10 μl was loaded for each mixed reaction.

observed intensities of DGGE bands across a range of different template ratios showed some deviation from the expected values, but the observed signal was always within 25% of that expected. Thus, at least for these particular templates, the ratio of products produced by the new primers was in good agreement with the concentration of starting template. While these results are encouraging, it probably should not be assumed that such equivalence will hold for all primer-template combinations in more complex mixtures (19, 34).

DGGE reproducibility. As a test of reproducibility, replicate PCRs with a mixture of equal amounts of 11 clones as the template gave DGGE patterns which were indistinguishable from each other (Fig. 3, lanes C1, C2, and C3). Bands a and d (Fig. 3) represent single sequences, while bands b and c/c′ each represent two comigrating sequences. These bands were quantified from lanes C1, C2, and C3 in relation to band a from lane C1, which was given a value of 1. Values for C1 were 1.00 (a), 1.81 (b), 2.03 (c/c), and 0.87 (d), which correspond to the expected values of 1, 2, 2, and 1, respectively. Equivalent values for C2 were 1.11, 2.52, 2.41, and 0.95, and those for C3 were 1.17, 2.54, 1.87, and 0.99. Thus, the observed values were within 25% of the expected values. Furthermore, DGGE patterns of PCR products from replicated environmental DNA extracts from the T-4 and T-5 dune samples showed no variation in the number of fragments and only a slight variation in the relative intensities of the same fragments in different reactions (results not shown).

Multiple banding from single templates. DGGE examination of PCR products amplified with the CTO189f-GC and CTO654r primers often revealed more than one band from a single unique starting template (as shown in Fig. 3). The generation of multiple bands was not entirely consistent, as can be seen if the different results for clone pH7C/56 are compared in Fig. 3 and 4. The simplest explanation of the multiple bands is that PCR with degenerate primers produced a mixture of PCR products which could be separated by DGGE. Degenerate primers are conveniently used in environmental PCR to recover all members of a target group, without compromising specificity by having to relax the annealing temperature to accommodate potential mismatches. In this study, the forward primer, CTO189f-GC, actually comprises three different sequences. However, the two variable positions are located close to the GC clamp, and analysis of the simulated melting behavior of products obtained with these primers (MacMelt version 1.0; Bio-Rad) predicted that these substitutions should not influence DGGE migration. The reverse primer contains one ambiguous site (either C or T at position 647; *E. coli* numbering [3]), and a base substitution at this position was predicted to influence the melting behavior of the PCR product molecule. Furthermore, the formation of heteroduplexes between the different sequence variants, which are probably sufficiently similar for this to occur, might introduce further complexity into the DGGE pattern. The possibility that primer ambiguities are the source of additional bands was tested by synthesizing each of the reverse primer variants separately and using them in a PCR followed by DGGE. Each single reverse primer did indeed yield a single DGGE band from cloned templates, and the respective products differed in mobility according to prediction (data not shown).

Predictive and informational limitations of DGGE. Analysis of the PCR products from different β-subdivision ammonia oxidizers and from related environmental clone sequences demonstrated that the limitations inherent in any chromatographic/electrophoresis technique apply to DGGE. Thus, while nonidentity is easy to infer when different fragment mobilities are observed, identity cannot be reliably inferred when two or more fragments have the same mobility. Figures 3, 4, and 5a show the results of DGGE analysis of PCR products from ammonia oxidizer sequences sampled from the different clusters in Fig. 1 and from the cultured strains *Nitrosospira briensis*, *Nitrosospira* sp. strain AHB1, and *Nitrosomonas europaea*. Denaturation occurred in the range of 43.0 to 46.5%

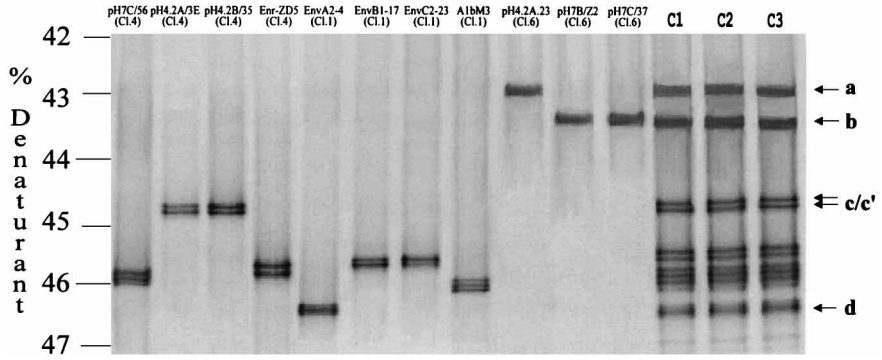

FIG. 3. DGGE analysis of variations within and between clusters. DGGE analysis was performed separately on 11 cloned 16S rDNA sequences, of which 4 show affinity with *Nitrosospira*, cluster 4 (lanes 1 to 4); 3 show affinity with *Nitrosospira*, cluster 1 (lanes 5 to 8); and 4 show affinity with *Nitrosomonas*, cluster 6 (lanes 8 to 11). C1, C2, and C3 represent three separate PCR samples, using an equimolar combination of all 11 clones as the template. Samples of 5 μl were added for each single-sequence reaction, and 30 μl per sample was added for mixed reactions. Clearly separated bands were quantified by using "a" from C1 as a reference (see the text). The c/c′ double band was treated as a single band for the purposes of quantification.

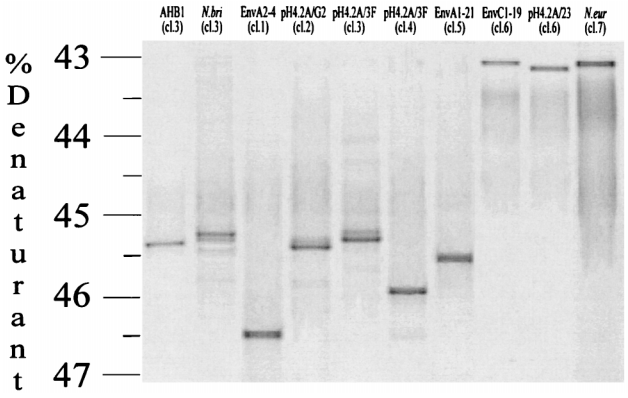

FIG. 4. DGGE of 16S rDNA fragments from different sequence clusters. Seven cloned sequences and three pure cultures, representing the seven sequence clusters of the β-subdivision ammonia oxidizers (33), were amplified with the CTO primer pair and run on a 38 to 50% DGGE gel. Samples were 5 μl of an amplification reaction plus 3 μl of loading dye per lane. The culture strains used were as follows: AHB1, *Nitrosospira* sp. AHB1; *N.bri*, *Nitrosospira briensis* C-128 (both *Nitrosospira*, cluster 3); *N.eur*, *Nitrosomonas europaea* ATCC 25978 (*Nitrosomonas*, cluster 7).

denaturant for all sequences at a constant temperature of 60°C. Many of the fragments could be distinguished from each other on the basis of their mobility, but fragments from some sequences which were known to be different exhibited very similar mobilities. For example, in Fig. 4, compare fragments from pH4.2A/G2 (*Nitrosospira*, cluster 2) with *Nitrosospira* sp. strain AHB1, *Nitrosospira briensis*, and clone pH4.2A/3F (*Nitrosospira*, cluster 3). It follows that the number of bands generated by DGGE may not accurately reflect the number of different sequences which are present in a given mixture. The observed overlap in band mobility also means that it is not generally possible to assign a band to a recognized sequence cluster without the use of further data (Table 3).

Analysis of cloned 16S rDNA sequences recovered by PCR. As part of an ongoing survey of the distributions and natural diversity of sequences related to β-subdivision ammonia oxidizers, the primers of McCaig et al. (20) were used to make gene libraries of environmental 16S rDNA PCR products from the two sand dune areas of Terschelling and Meijendel. Our aim was to compare the sequences of clones recovered from these samples with sequences recovered previously from marine samples and from acidic and neutral agricultural soil (20, 33). Before discussing these data, it must be stressed that the hypothesis that novel environmental sequences actually originate from autotrophic ammonia oxidizers is based solely upon their phylogenetic positions relative to cultured *Nitrosospira* and *Nitrosomonas* species, which uniformly possess this phenotype. It can be argued that the new sequences which fall within the known range of bona fide ammonia oxidizers are most parsimoniously interpreted as being from ammonia oxidizers. Although pure cultures are needed to confirm this inference, all bacteria isolated to date whose 16S rDNA sequences show affinity with this clade of the β subdivision of the *Proteobacteria* have proven to be ammonia oxidizers. The recent discovery of new ammonia oxidizers (38), whose 16S rDNA sequences are closely related to novel environmental sequences isolated previously (33), is also very encouraging. Only sequence clusters 1 and 5 now lack representatives in pure culture, and to reflect this, they are referred to as "*Nitrosospira*-like" and "*Nitrosomonas*-like," respectively (Fig. 1).

At the Terschelling site, samples were taken along a transect from the seaward dune face through to the older landward dunes. The lime-poor nature of this region has led to decalcification in the top layers of landward dune sites, which is reflected in an acidic soil pH and gives a gradient in pH across the transect (Table 1) (10). The Meijendel samples were relatively lime rich (pH 7.6 to 7.8) and were taken from one protected seaward dune location (M-6) and one landward dune location (M-7). Autotrophic ammonia oxidation has been detected previously in all of these samples (5), and utilizes all mineralized ammonia except in the most acidic sites (T-4 and T-5) (Table 1). Thirty-four recombinant clones were randomly sampled from the different gene libraries and sequenced. Phylogenetic analysis revealed that 20 of the clones formed a strongly supported group with sequences from the β-subdivision ammonia oxidizers (33) (Fig. 1). All 20 of these clones also produced the expected size of PCR product when amplified with the CTO primer pair (results not shown). Fourteen clones contained sequences which were related to other members of the β subdivision of the *Proteobacteria*. Interestingly, these clones did not amplify when tested with the CTO primer pair.

All of the new ammonia oxidizer-like sequences could be placed within previously defined sequence clusters, constituting sequences from cultured strains and/or enrichments and environmental clones (Fig. 1). Interestingly, sequences from all but one of the previously recognized *Nitrosospira*-like or *Nitrosomonas*-like sequence clusters were detected (Fig. 1). No sequences which grouped within *Nitrosomonas* cluster 7, which includes the most extensively studied chemolithotrophic ammonia oxidizer, *Nitrosomonas europaea*, were recovered. The small number of clones compared limits the potential for quantitative inference, but 18 of the new sequences were related to the genus *Nitrosospira* rather than to *Nitrosomonas*. These data are compatible with previous molecular studies which have suggested that *Nitrosospira* may be more common than *Nitrosomonas* in some natural samples (13, 33).

DGGE analysis of environmental dune samples. The CTO primers and DGGE were also used to analyze (Fig. 5) the same DNA samples used to make the gene libraries discussed above. It appeared from these analyses that the two approaches were in good agreement, since library clones were identified which produced bands that comigrated with most of the bands detected by "community analysis" by DGGE (Fig. 5). Since we have previously shown that comigration does not ensure sequence identity for these products, the DGGE bands were excised from the gel, reamplified, and directly sequenced. All of the excised bands comprised sequences which were closely related to those of β-subdivision ammonia oxidizers, supporting the specificity of the CTO primer pair for this phylogenetic group. The results of the sequencing were in excellent agreement with the results based upon sequencing fragments amplified with the McCaig et al. primers (20), as sequences recovered from several of the DGGE bands were identical to the sequence of a reference clone showing the same mobility.

Sequencing of bands revealed that where doublets occurred, the upper band differed from the lower band only by a single base corresponding to the ambiguous position in the reverse primer (C or T at position 647). As expected from the predicted melting behaviors, the lower band contained the C · G pair at this position.

The T-1 site (pH 7.9) produced the most distinctive DGGE pattern and was the only site for which *Nitrosomonas*-like sequences were detected. The CTO primer-generated DGGE bands contained sequences which were identical, or closely related, to *Nitrosomonas*-like library clones pT-1.7 (sequence cluster 5) and pT-1.9 (sequence cluster 6) isolated from this site. The remaining sequenced band in the T-1 sample contained a sequence which was identical to a library clone pT-1.3,

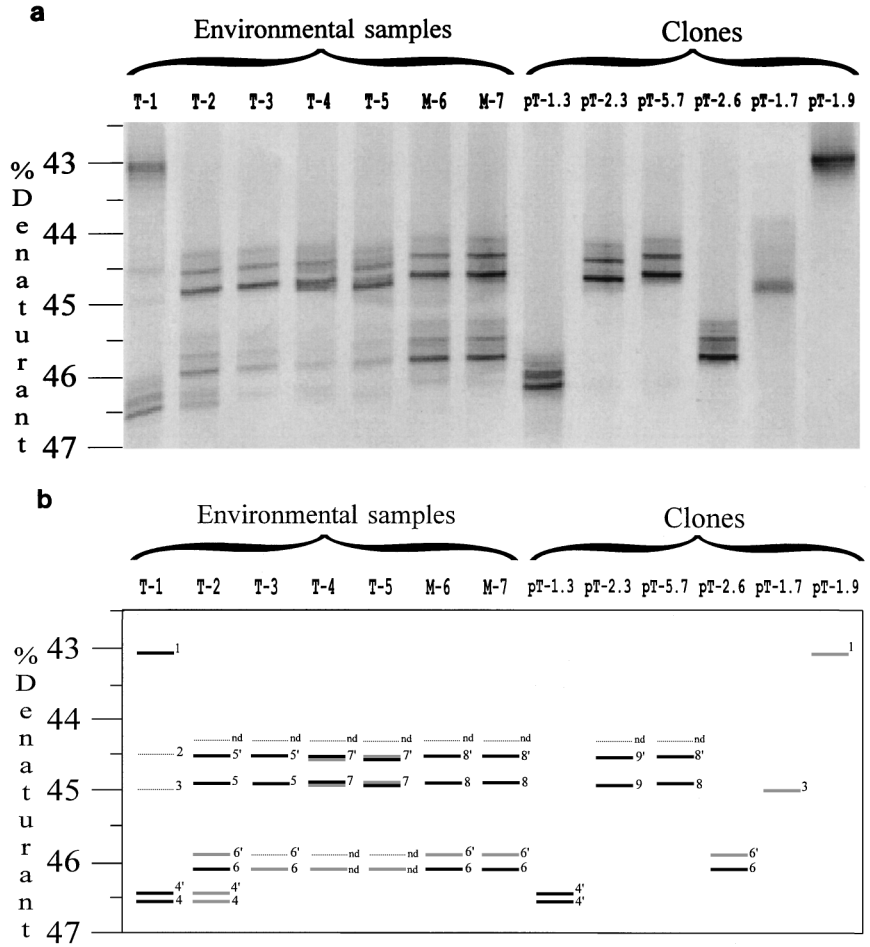

FIG. 5. DGGE of the Terschelling and Meijendel samples with representative clones. (a) DGGE analysis of DNA samples extracted from sand dune samples following PCR with the CTO primer set (lanes 1 to 7). Cloned sequences recovered from environmental sites are shown as references (lanes 8 to 13). Site descriptions can be found in Table 1. Clone designations and cluster associations are as follows: pT-1.3, *Nitrosospira*-like, cluster 1; pT-2.3, *Nitrosospira*, cluster 2; pT-5.7, *Nitrosospira*, cluster 3; pT-2.6, *Nitrosospira*, cluster 4; pT-1.7, *Nitrosomonas*-like, cluster 5; pT-1.9, *Nitrosomonas*, cluster 6. (b) Schematic drawing of the DGGE gel from panel a, showing bands which were excised for sequence analysis. Bands marked n.d. were not determined. The sequences of the numbered bands are as follows: 1, identical to the corresponding region of clone pT-1.9 (*Nitrosomonas*, cluster 6); 2, two differences (C→T at position 225 and G→A at position 461) from clone pT-1.7 (*Nitrosomonas*-like, cluster 5); 3, identical to clone pT-1.7 (*Nitrosomonas*-like, cluster 5); 4, identical to clone pT-1.3 (*Nitrosospira*-like, cluster 1); 5, two differences (T→C at position 475 and C→T at position 476) from clone pT-5.7 (*Nitrosospira*, cluster 3); 6, identical to clone pT-2.6 (*Nitrosospira*, cluster 4); 7, the sequence was ambiguous at 14 positions, not allowing phylogenetic placement; 8, identical to clone pT-5.7 (*Nitrosospira*, cluster 3); 9, clone pT-2.3 (*Nitrosospira*, cluster 2). Sequences which are labeled with a prime have a 1-bp difference (T for C) from the corresponding clone sequence at position 647 introduced by the ambiguity in the reverse primer (*E. coli* numbering [3]). Sequences from clones were derived from plasmids, not determined from excised bands.

which clustered with *Nitrosospira*-like sequences (sequence cluster 1) isolated from marine samples (33). This sequence was also detected as a minor band (band 4, Fig. 5) in the sample from site T-2. The T-1 site is low in nitrogen, has the lowest measured nitrogen mineralization rate, and is frequently inundated by sea spray. T-2 is also exposed to some sea spray, and this cannot be excluded as a potential source of some of the sequences detected.

The DGGE patterns obtained for sites T-2 to T-5, which represent a descending pH profile (pH 7.9 to pH 4.8 [Table 1]), were very similar. All of the sequences were *Nitrosospira*-like, mainly representing clusters 2, 3, and 4, which contain sequences previously obtained from soil clones and isolates (12, 33, 36). These data support the inferences made from the library clones that *Nitrosospira* might be more common than *Nitrosomonas* in these samples. Sequencing confirmed that some comigrating bands were identical between the samples (Fig. 5b), demonstrating that some sequences are common to multiple sites. However, one of the comigrating bands (band 7)

TABLE 3. Mobility ranges for cloned sequences from the different β-subdivision ammonia oxidizer sequence clusters[a]

Sequence cluster	Denaturant range (%)	Mean denaturant (%)	No. of sequences tested
Nitrosospira-like, cluster 1	45.6–46.6	46.3	21
Nitrosospira, cluster 2	44.4–45.3	44.8	19
Nitrosospira, cluster 3	44.2–45.2	44.7	19
Nitrosospira, cluster 4	44.7–46.0	45.6	16
Nitrosomonas-like, cluster 5	44.4–45.2	44.8	8
Nitrosomonas, cluster 6	43.0–43.5	43.2	14
Nitrosomonas, cluster 7	43.1–43.6	43.3	3

[a] A total of 100 clones representing the known diversity of the β-subdivision ammonia oxidizers (33) were amplified with the CTO primers and subjected to DGGE. The range of denaturant within which bands derived from the different sequence clusters fell is given, along with the average denaturant percentage and number of clones tested per sequence cluster.

comprised multiple sequences in the two most acidic samples (T-4 and T-5) but only a single identical sequence (band 5) in T-2 and T-3. The sequence of band 7 failed to resolve at a total of 14 positions, demonstrating that these bands probably comprise a mixture of sequences. Cloning is necessary to resolve further the identities of individual sequences and to determine if some resemble *Nitrosospira*-like sequences previously inferred to be more common in acidic soils (33). The use of oligonucleotide probes is also currently being evaluated for the sequence cluster determination of DGGE bands. A FastA search of the EMBL database, using the positions which could be resolved, was consistent with the sequences in band 7 being related to β-subdivision ammonia oxidizers. The DGGE patterns from the lime-rich M-6 and M-7 samples (pH 7.8 and 7.6) were identical as confirmed by sequencing. Some bands and sequences found in these samples were also detected in the Terschelling samples (band 6 and clone pT-5.7).

In the present study, we have shown that DGGE of specifically amplified 16S rDNA fragments is a viable technique for the analysis of β-subdivision ammonia-oxidizing populations in natural samples. With the help of sequence data from cloned 16S rDNA fragments and excised DGGE bands, differences could be detected between some dune soil samples differing in important environmental factors such as pH and proximity to the sea. Such data not only provide insight into the diversity and distribution of this ecologically important group of organisms but also help generate testable hypotheses regarding potential physiological differences between 16S rDNA sequence clusters and their response to changing environmental conditions.

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