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Molecular Diversity of Soil and Marine 16S rRNA Gene Sequences Related to β-Subgroup Ammonia-Oxidizing Bacteria

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We have conducted a preliminary phylogenetic survey of ammonia-oxidizing β-proteobacteria, using 16S rRNA gene libraries prepared by selective PCR and DNA from acid and neutral soils and polluted and nonpolluted marine sediments. Enrichment cultures were established from samples and analyzed by PCR. Analysis of 111 partial sequences of c. 300 bases revealed that the environmental sequences formed seven clusters, four of which are novel, within the phylogenetic radiation defined by cultured autotrophic ammonia oxidizers. Longer sequences from 13 cluster representatives support their phylogenetic positions relative to cultured taxa. These data suggest that known taxa may not be representative of the ammonia-oxidizing β-proteobacteria in our samples. Our data provide further evidence that molecular and culture-based enrichment methods can select for different community members. Most enrichments contained novel *Nitrosomonas*-like sequences whereas novel *Nitrosospira*-like sequences were more common from gene libraries of soils and marine sediments. This is the first evidence for the occurrence of *Nitrosospira*-like strains in marine samples. Clear differences between the sequences of soil and marine sediment libraries were detected. Comparison of 16S rRNA sequences from polluted and nonpolluted sediments provided no strong evidence that the community composition was determined by the degree of pollution. Soil clone sequences fell into four clusters, each containing sequences from acid and neutral soils in varying proportions. Our data suggest that some related strains may be present in both samples, but further work is needed to resolve whether there is selection due to pH for particular sequence types.

Autotrophic oxidation of ammonia to nitrite, the first stage in nitrification, is of major importance in the global cycling of nitrogen in terrestrial, aquatic, and marine ecosystems (29). Cultured ammonia oxidizers can be divided into two distinct monophyletic groups with 16S rRNA sequences, one containing strains of *Nitrosococcus oceanus* and *Nitrosococcus halophilus* (16) in the γ-proteobacteria and the other comprising species of *Nitrosomonas* and *Nitrosospira* (which includes strains formerly classified as *Nitrosolobus* and *Nitrosovibrio*) in the β-proteobacteria (13, 36, 45, 46). Most studies of the physiology and ecology of ammonia oxidation have focused on a single strain of *Nitrosomonas europaea*, which was originally isolated from soil and which can be grown most conveniently under laboratory conditions (29). However, there is culture-based evidence that *Nitrosospira* spp. may be more common in soils (3, 18, 19).

Investigation of autotrophic nitrification in natural systems has been limited by the difficulties in isolating and culturing ammonia oxidizers. The energy yield from ammonia oxidation is low, leading to small biomass yields and low maximum specific growth rates. Growth of visible colonies on solid media takes several months, and elimination of heterotrophic contaminants is difficult. Both have discouraged attempts to analyze species composition and diversity in natural habitats. Immunofluorescent antibody-based techniques have been used to study the species composition of marine (42, 43) and soil (4) environments, but these also require initial isolation of pure cultures. One possible solution is to use 16S rRNA sequences to investigate the phylogenetic affinities of members of natural communities without laboratory culture (12).

In the first of such studies, McCaig et al. (23) used PCR primers, designed to recover 16S rRNA sequences from β-proteobacterium ammonia oxidizers, to detect novel *Nitrosomonas*-like sequences in marine surface-water enrichment cultures. Subsequently, Voytek and Ward (38) used specific PCR and probing with a β-subgroup ammonia-oxidizer-specific probe to infer the presence of these bacteria in saltwater samples. In a more detailed study, Hiorns et al. (14) used a battery of specific probes and PCR primers to infer the occurrence of *Nitrosospira* spp. in activated sludge, soil, freshwater, and freshwater sediment samples. Recently, Wagner et al. (40) used a fluorescently labelled oligonucleotide probe to detect and enumerate some types of β-subgroup ammonia oxidizers in activated sludge and a trickling filter biofilm.

While these molecular investigations have undoubtedly yielded very interesting results, none has focused on the more precise identification of the in situ β-proteobacterium ammonia oxidizer population with 16S rRNA nucleotide sequencing (12). Only this approach gives data which, through tree diagrams of relationships, are directly comparable between different studies and habitats, allowing an increasingly comprehensive and more precise picture of sequence diversity and distributions to be revealed. The relationships between environmental sequences and cultured taxa can be investigated to provide a fresh perspective on the extent to which the latter are representative of the in situ population. Moreover, new sequence data, as they become available, can be used to devise or refine probes for use in determinative studies aimed at estimating the relative abundance of sequence types in natural samples.

In the present study we have made gene libraries of 16S

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genes coding for rRNA (rDNA) sequences related to β-subgroup (proteobacteria) ammonia-oxidizing bacteria from samples of DNA extracted from acid and neutral soils and from polluted and nonpolluted marine sediments. The environmental samples were chosen to represent a range of habitats in order to gain a perspective of the 16S rRNA sequence diversity of these bacteria under different environmental conditions. Polluted marine sediment samples were taken from beneath cages containing Atlantic salmon and were therefore subject to increased organic input. Comparison with samples distant from fish cages was intended to investigate whether fish feed pollution and the anaerobic conditions resulting from organic impact produced gross differences in the types of ammonia oxidizers detected. Soil samples were taken from two adjacent test plots which had been treated to maintain their pH at either 7.0 or 4.2. In sampling both sites we were interested to see if we could detect selection for different populations of ammonia oxidizers under acid or neutral conditions. Ammonia oxidation by pure cultures in liquid media is inhibited at acid pH values, but autotrophic soil nitrification is reported to occur at pH values as low as 3.5 (7, 11, 22, 29). One possible explanation is that ammonia oxidizers in acid soils differ from laboratory strains which have been selected by using enrichment and isolation media at neutral pH (29).

MATERIALS AND METHODS

Sample collection. Two surface samples (<2-cm depth) were taken randomly 0.5 m from the edges of each of two adjacent agricultural plots (3 by 4 m) at the Scottish Agricultural College, Craibstone, Aberdeen, Scotland (Ordinance Survey NJ867112) during February 1994. Details of the soil properties have been published previously (26). Plots had been maintained at pH 4.2 and pH 7.0 (pH determined after shaking in $CaCl_2$ for 1 h) by the addition of aluminum sulfate or calcium carbonate since 1961, before which they were identical. Both had been treated with ammonium sulfate each spring at a rate of 125 kg hectare^{-1} and had identical crop rotation, supporting a crop of potatoes the year prior to sampling.

Marine sediment samples were obtained at a depth of 16 m from Glenmore Bay, Loch Sunart, West Scotland (Ordinance Survey NM7162). Duplicate cores were collected from directly underneath a fish cage containing North Atlantic salmon fed on a high-nitrogen diet and at two sites located on a transect at distances of 20 and 40 m from the fish cage. The top 5 mm of each core was removed for DNA extraction and enrichment studies. Sediment from underneath the salmon cage (site A) was highly polluted, as indicated by both visual inspection and elevated (48.2%) organic content (27). In comparison, the 20-m (B) and 40-m (C) sites had organic levels of 5.1 and 3.9%, respectively. While gross visual examination indicated slight organic impact at the 20-m site, the 40-m site appeared to be pristine.

Preparation of marine enrichment cultures. Enrichment cultures were prepared from all marine sediment samples in duplicate in two different media. The first was an inorganic salt medium (M) containing 100 mg of N-NH_4^+ ml^{-1} and 0.01% (wt/vol) phenol red as a pH indicator (23). The pH of the medium was adjusted to approximately 7.5 by the addition of sterile 5% (wt/vol) Na_2CO_3. The second medium (W) consisted of artificial seawater ([ingredients are expressed in grams per liter of distilled water] NaCl, 25; $MgCl_2 \cdot 7H_2O$, 5; $CaCl_2$, 1; KCl, 1) supplemented with ammonium sulfate at a final concentration of 100 mg of N-NH_4^+ ml^{-1}. Enrichment was achieved by the addition of 1-g samples of sediment to 100 ml of each medium, followed by incubation at 23°C in the dark. Flasks were monitored weekly for the appearance of nitrite, by use of Quantofix nitrate-nitrite dipsticks (Camlab Ltd., Cambridge, United Kingdom) and by a change in the pH of the medium (determined by a change in the color of the pH indicator phenol red from pink to yellow). Growing first-generation cultures were neutralized once and subcultured by adding 1 ml of shaken culture into fresh medium (second-generation enrichments) after a second color change had indicated further growth. Subsequent subcultures were carried out in a similar fashion.

First-generation enrichments were centrifuged at 10,000 × *g* for 15 min to pellet both sediment and cells. DNA was extracted from the pellet and amplified as described for soil and sediment samples (see below). Second-generation enrichments were briefly spun to remove sediment, and then the resulting supernatant was centrifuged at 10,000 × *g* to pellet the cells. DNA was released from the pelleted cells by being boiled with Chelex 100 (23, 41).

Preparation of soil enrichment cultures. Soil enrichments were obtained in liquid medium inoculated with soil samples or from a continuous-flow soil column. For the first, 3 g of soil (pH 4.2) was used to inoculate 300 ml of modified Skinner and Walker (1, 28) medium (SW; pH 7.5 to 8.0) supplemented with 50 μg of NH_4^+-N ml^{-1} in a 500-ml Erlenmeyer flask. Growing cultures were neutralized as described above and subcultured three times by 1% inoculation into 100 ml of SW medium. Agar plates were prepared with Macdonald and Spokes (20) medium (pH 8.0) solidified with 1% (wt/vol) Noble agar (Difco Ltd., Surrey, United Kingdom) with phenol red replaced by 0.003% (wt/vol) neutral red and supplemented with 50 μg of NH_4^+-N ml^{-1}. Plates were spread with 100 μl of fully grown culture and incubated for 6 months in the dark. Red colonies (indicating acid production) were removed with a micromanipulator and inoculated into 100 ml of SW medium.

Continuous-flow soil (pH 7.0) columns consisted of a plastic cylinder (internal diameter, 1.5 cm; length, 20 cm) sealed at the base by a silicone rubber stopper on which was placed a 1.5-cm-diameter glass microfiber filter (Whatman International Ltd., Kent, United Kingdom). The column was closed at the top by a silicone rubber stopper. Supply of medium and air and collection of effluent were done with Pasteur pipettes inserted through the rubber stoppers. The column and connecting tubing were autoclaved for 30 min at 121°C and, after cooling, packed with 150 g of soil. A 4-cm-diameter glass microfiber filter was placed on the soil surface to allow even distribution of inflowing medium. The column was incubated at 30°C, and sterile SW medium and air were each supplied at a rate of 2 ml h^{-1} with an LKB 2132 Microperspex peristaltic pump. Effluent (pH 7.1) from the base of the column was used to inoculate fresh medium, and resultant enrichment cultures were subcultured and plated as described above.

Extraction and purification of DNA from soil and sediment samples. One-gram samples of soil or sediment were washed (twice) with 2 volumes of 120 mM sodium phosphate buffer (pH 8.0) in order to elute naked DNA (37). After centrifugation the pellets were resuspended in 500 μl of sodium phosphate buffer (pH 8.0) to which 0.5 g of glass beads (0.17- to 0.18-mm diameter; B. Braun Ltd., Melsingun, Germany) and 500 μl of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) were added. Lysis of cells in soil samples was done by bead beating for 1 min in a Mikrodismembrator U (Braun Ltd.) set to 2,000 rpm. Sediment samples were treated in a similar manner except that a vortex mixer was employed for two 1-min periods to lyse cells (lysis was checked by acridine orange staining). After centrifugation the aqueous supernatant was removed and the pellet was reextracted as described above. Pooled supernatants from soil samples were concentrated and repeatedly dialyzed with Tris-EDTA buffer (pH 8.2) in a Microcon 100 spin dialysis unit until no brown pigment appeared in the eluate. The retentate was further purified by gel electrophoresis through a 1% (wt/vol) low-melting-point agarose gel (Bio-Rad Laboratories, Surrey, United Kingdom). A band containing DNA of an estimated molecular mass greater than 8 kb was excised and trimmed of excess agarose, and the DNA was purified by use of Qiaex resin (Qiagen Ltd., Surrey, United Kingdom). Marine sediment samples were treated similarly, except that the repeated dialysis step was omitted and DNA extraction from agarose gels was performed with a Spinbind DNA Recovery System (Flowgen Instruments Ltd., Kent, United Kingdom).

PCR amplification of 16S rDNA, cloning, and sequencing. Approximately 20 ng of DNA was used in a 50-μl PCR reaction mixture. The primers βAMOf and βAMOr, designed to selectively amplify approximately 1.1 kb of 16S rDNA from β-subgroup ammonia oxidizers and their close relatives, have been described previously (23). Two units of *Taq* DNA polymerase (Promega Ltd., Southampton, United Kingdom) was used in PCR reaction mixtures with an annealing temperature of 55°C in a buffer supplied by the manufacturer. Other PCR conditions were as described previously (8). PCR products from five reactions were pooled to minimize possible bias due to random events within individual PCR reactions (39). The PCR band was purified for cloning by gel electrophoresis followed by extraction of the excised band with Qiaex resin. Ligations into the pGEM T vector (Promega Ltd.) followed the manufacturer's protocols. The ligations were transformed into XL1-Blue MRF Kan supercompetent *Escherichia coli* (Stratagene Ltd.) and plated on Luria-Bertani agar supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside [1 mM]), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [40 mg ml^{-1}]) and the antibiotics kanamycin, ampicillin, and methicillin according to manufacturer's instructions (Stratagene Ltd.). White colonies were grown overnight in Luria-Bertani broth (5 ml) containing ampicillin (50 μg ml^{-1}) at 37°C with shaking. Plasmids were purified by using the Promega Wizard Mini-Prep system. Approximately 200 plasmids were analyzed from each soil clone library by a single dideoxynucleotide (ddA; T-tracking)-sequencing method and primer 519r (31) in order to identify unique clones. These were then partially sequenced for about 300 bases with primer 519r. Approximately 40 clones were randomly chosen from the sediment libraries for partial sequencing.

Fifteen clones chosen to be representative of the different groups revealed by partial sequence analysis were fully sequenced for the entire 1.1-kb insert with the SP6 and T7 plasmid primers (Promega Ltd.) plus internal primers. Manual sequencing used Sequenase (United States Biochemical Corporation, Cleveland, Ohio) and 10% (vol/vol) dimethyl sulfoxide in all reaction mixtures. Double-stranded PCR products from enrichment cultures were precipitated and sequenced directly (8, 23).

Analysis of clone sequences. The clone sequences were aligned against representative prokaryote 16S rRNA sequences from the Ribosomal Data Base Project (21). All sequences were manipulated by using the Genetic Data Environment software version 2.2 distributed by the Ribosomal Data Base Project. Analyses of partial sequences used the Genetic Data Environment mask function to exclude missing data or positions which could not be unambiguously aligned. For distance matrix analyses the Jukes & Cantor (15) correction and neighbor-

joining method (30) implemented in PHYLIP 3.5 (10) operated through the Genetic Data Environment software were used. A more detailed analysis of complete clone sequences was also carried out. This alignment comprised 1,099 sites, which could be unambiguously aligned for all sequences including those from cultured ammonia oxidizers and reference β-proteobacteria. Distance matrix calculations were carried out as described above. Maximum parsimony analyses were done for 348 informative positions only and used the heuristic search option in PAUP 3.1.1 (35), with 10 random addition sequences and tree-bisection reconnection branch swapping. Bootstrapping (100 replicates) was used to investigate support for groups in maximum parsimony analyses, with one random addition sequence per replicate. For bootstrapping for distance matrix analyses, programs in PHYLIP 3.5 were used (10). Checks for chimeric sequences were conducted on the complete sequences with the program CHECK_CHIMERA developed by Niels Larsen (21) and by independently subjecting the first 5′ 300 base positions or the last 3′ 300 base positions of insert sequence to phylogenetic analysis.

Nucleotide sequence accession numbers. The clone sequences have been deposited in GenBank as Z69087 to Z69197.

RESULTS

The extraction method yielded an average of approximately 1 μg of DNA g of soil or sediment^{-1}. Samples gave single weak PCR products of the expected c. 1.1 kb in primary PCRs with primers βAMOf and βAMOr, and single weak PCR products of about 1.5 kb with positive-control universal bacterial primers (8).

Analysis of soil clone libraries by T-tracking revealed a number of unique patterns in the libraries from the different samples and some patterns which were shared between the different libraries. A large proportion of soil clone T-track sequences were very similar to each other. Partial sequencing of a representative selection of this subgroup revealed that they were related to a group of β-proteobacteria comprising *Comamonas testosteroni*, *Sphaerotilus natans*, and *Rubrivivax gelatinosus*. All of these clones shared the same T-track sequence close to the 519r priming site, and this was used as an ad hoc sorting criterion to exclude such clones from further analysis.

A total of 126 16S rDNA sequences sampled from the clone libraries and the PCR products sequenced from marine sediment and soil (only two enrichments were determined for 304 bases including variable regions V2 and V3 (25). Fifteen of the soil clones formed a cluster whose position at the base of the ammonia oxidizer clade was only weakly supported (tree not shown). Analyses of two complete clone sequences from this group suggested the possibility of their being chimeric sequences, so we excluded all members of this cluster pending further analysis.

The remaining 111 clone library and enrichment partial sequences formed a monophyletic group with partial sequences from cultured ammonia oxidizers (Fig. 1). Within this radiation the environmental sequences formed a number of separate sequence clusters (Fig. 1). None of the clone sequences was identical to any sequence from a reference ammonia oxidizer, although some were recovered in the same clusters (Fig. 1, clusters 3, 6, and 7). Bootstrap support for clusters based on partial sequences was less than 50%, probably because of the high overall similarity between the partial sequences and consequent small number of informative sites. For the same reason, support for specific relationships within the clusters was generally low as judged by bootstrapping.

To investigate further the relationships of the environmental clones to reference ammonia oxidizers, the entire 1.1-kb PCR product insert from 13 cluster representatives was sequenced (Fig. 2). The longer environmental sequences formed a strongly supported monophyletic group with reference ammonia oxidizers. The ammonia oxidizers separate into two groups, one based on the genus *Nitrosospira* (and including 10 of the new sequences) and the other based on the genus *Nitrosomonas* (plus 3 new sequences). For convenience, the partial sequence clusters are divided into two groups for the following discussion, *Nitrosospira*-like and *Nitrosomonas*-like, based on their inferred relationships (Fig. 1 and 2).

***Nitrosospira*-like sequences (clusters 1 to 4, Fig. 1 and 2).** Most of the soil clone partial sequences were recovered in three clusters (Fig. 1, clusters 2, 3, and 4) within the *Nitrosospira* group. Analyses of longer stretches of sequence (Fig. 2) from seven representatives of clusters 2, 3, and 4 support their relationship to the genus *Nitrosospira* and suggest that they are phylogenetically distinct from previously analyzed and cultured strains. Cluster 3 also contained most of the partial sequences from cultured *Nitrosospira* spp.

Within the resolution of our analysis, it did not appear that the *Nitrosospira*-like cluster composition was entirely determined by the pH of the soil from which the library was constructed. Each of the three soil clusters contained sequences from each pH, and in each cluster there are examples where the same partial sequence was recovered from both soils. However, cluster 2 contained mostly sequences from the pH 4.2 soil (16 of 20 sequences), and cluster 3 contained a higher proportion of sequences (10 of 12 sequences) from the pH 7.0 soil.

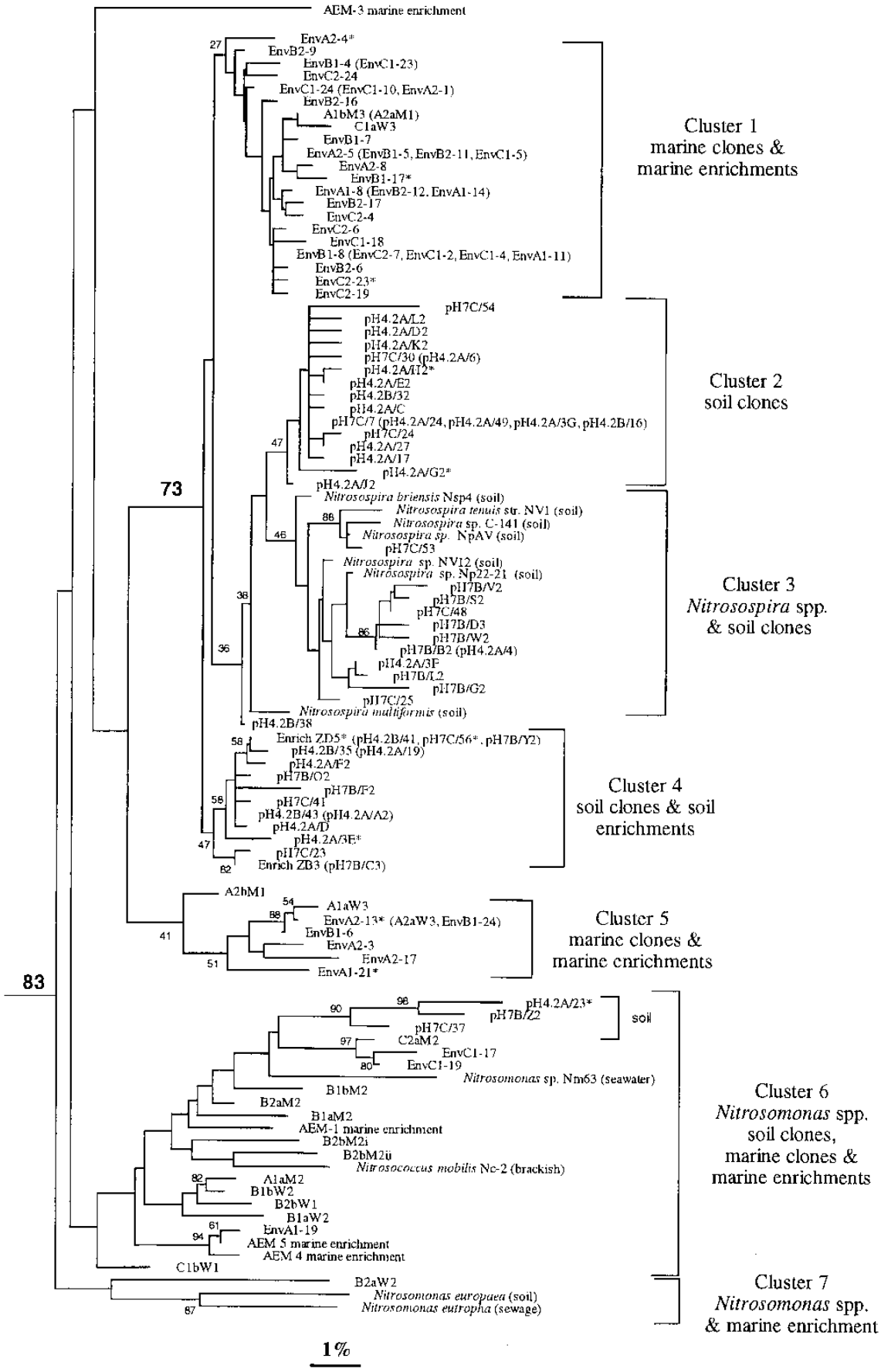
There is evidence from our analyses that marine *Nitrosospira*-like clones are distinct from soil *Nitrosospira*-like clone sequences. All of the marine clones formed a separate cluster (Fig. 1, cluster 1) whose relationship to the genus *Nitrosospira* was strongly supported by analysis of three longer sequences (Fig. 2). Within this marine clade there were sequences from all three sediment samples, and the same partial sequence was sometimes recovered from different samples.

***Nitrosomonas*-like sequences (clusters 5, 6, and 7).** Partial sequences clustering with cultured species of *Nitrosomonas* were detected in all samples. Two soil clones from neutral soil and one from acid soil formed a strongly supported monophyletic group (Fig. 1) within a radiation (Fig. 1, cluster 6) which otherwise comprised sequences from marine samples. Full-length sequencing of the acid soil clone (designated pH4.2/23; see the legend to Fig. 1 for an explanation of clone designations) supports its distinct status within the *Nitrosomonas* clade (Fig. 2).

The remaining nine *Nitrosomonas*-like sequences were from marine sediment. Six of these from the most-polluted site (A) and the site showing intermediate impact (B) formed a novel cluster (Fig. 1, cluster 5), which did not contain any sequences from the apparently nonpolluted site C. Analysis based on partial sequences gave a weakly supported relationship between clade 5 and the genus *Nitrosospira*. In contrast, analysis of longer sequences from two clones strongly supported membership of the *Nitrosomonas* clade, with moderate support for a relationship to *Nitrosomonas* sp. NM63 and soil clone pH4.2A/23.

Sequences from enrichments. Enrichment cultures were established with material from the sampled sites, to determine whether they revealed sequence types the same as or different from those recovered from the gene libraries and with the aim of isolating new cultures for physiological study. At present no enrichment, possibly because of the long incubation times necessary, has generated a pure culture, but all do contain, as judged by indicator reactions and nitrite production, cells which can oxidize ammonia autotrophically. PCR and sequencing of products from these cultures have revealed a number of sequences related to ammonia oxidizers (Fig. 1).

Two *Nitrosospira*-like partial sequences were recovered from soil enrichments. One of these (enrich ZB3) was recovered from mixed colonies from an enrichment at pH 7.5 to 8.0 from an original batch culture inoculated with pH 4.2 soil. The pH



of the enrichment medium was made alkali when it became apparent from preliminary experiments (data not shown) that no growth could be detected after extended incubation in media at pH 4.2 under the conditions that we applied. The other sequence (enrich ZD5) was isolated from mixed colonies from an enrichment inoculated with column effluent at pH 7.1. Interestingly, each of these gave the same partial sequence as one or more partial sequences in the soil libraries, and both occur in cluster 4, which does not contain any sequence from a cultured ammonia oxidizer. To investigate further the relationship between enrich ZD5 and a clone sequence pH7C/56, which appeared identical on partial sequence analysis (Fig. 1), we sequenced the entire 1,114 bases for each amplified gene. Sequence analysis revealed only 3 base changes between the two sequences, confirming that they are very closely related.

The marine enrichments gave two closely related sequences within cluster 1, which also contains the marine clone library *Nitrosospira*-like sequences, for which there are no cultured or sequenced representatives. The remaining 11 marine enrichment sequences clustered with the *Nitrosomonas*-like sequences (Fig. 1). Three partial sequence types, one of which was identical to a marine clone sequence, clustered with the novel *Nitrosomonas*-like cluster 5. The remaining enrichment sequences were recovered in cluster 6 or 7, which also contains the reference cultured *Nitrosomonas* sequences (Fig. 1).

DISCUSSION

The PCR primers (23) used in the generation of clone libraries produced clonable products from primary amplifications. Unlike previous studies (14, 38) it was not necessary to carry out nested secondary amplifications. It is not possible to identify why our reactions were apparently more successful, but possibilities include differences in samples and template preparation and the use of different PCR primers.

The PCR primers used in this study were not specific, under the conditions used, for sequences clustering within the radiation of β-subgroup ammonia oxidizers. A significant fraction of soil clones was related to a different β-proteobacterium clade containing *Comamonas testosteroni* and *Rubrivivax gelatinosus* (data not shown). The retrieval of these sequences appeared somewhat sample specific since only two such sequences were recovered among the 40 marine sediment clones. Similar sequences in varying proportions have been detected within libraries prepared from marine samples taken from the photic zone in the Mediterranean and from native soils in the United States (unpublished data). In the present investigation we were able to recognize *Comamonas* and *Rubrivivax*-like sequences using T-tracking (31), but it would be more desirable to prevent their amplification in the first place.

This work has revealed previously unknown sequence types, which we infer to be derived from novel, as-yet-uncultured (or if cultured then previously unsequenced) members of the β-subgroup proteobacterium ammonia oxidizer clade. At present, support for the hypothesis that they are themselves autotrophic ammonia oxidizers comes solely from their phylogenetic positions relative to cultured taxa which uniformly possess this phenotype. Parsimony suggests that the genera *Nitrosomonas* and *Nitrosospira* shared a common ancestor which possessed the ability to oxidize ammonia. To assume that the new environmental lineages (Fig. 1 and 2) originate from taxa which do not autotrophically oxidize ammonia thus requires additional ad hoc hypotheses postulating loss of this phenotype. At present we have no evidence that such losses have occurred, so in our further discussion we have adopted the working hypothesis that the novel environmental sequences, which form a strongly supported monophyletic group with the genera *Nitrosospira* and *Nitrosomonas*, do originate from ammonia oxidizers. This hypothesis should be tested by selective isolation experiments aimed at isolating pure cultures representative of the new environmental lineages.

The finding of similar sequences (Fig. 1) in active enrichments designed to select for autotrophic ammonia oxidizers is consistent with the above hypothesis. Most enrichments gave single unambiguous sequences from direct sequencing of PCR products, suggesting that some enrichment or selection for homogeneity has occurred. However, the extreme sensitivity of selective PCR could mean that the small amounts of carryover between flasks were sufficient to generate a signal. In this case only the most abundant cell types would be likely to persist on dilution, and this provides another plausible explanation of why only single sequences were recovered from enrichments (but see below).

The question of which ammonia oxidizers are most important in different habitats and under different conditions can only be addressed through detailed sampling, linked to abundance and process measurements. Data which are essentially qualitative from a limited number of sites and samples, as in this and previously published molecularly based studies (14), need to be interpreted with great caution. The relative proportions of sequences recovered by PCR from mixtures of homologous genes may be biased towards particular templates (34) and by the possibility that different prokaryotes may contain different numbers of rRNA genes (9). As such, our discussions below of quantitative cluster composition based on sampling of clone libraries should be viewed only as hypotheses, which need to be test by more quantitative methods such as probing (12, 33).

On the basis of partial sequence analysis the environmental partial sequences fell into seven clusters, four of which (Fig. 1, clusters 1, 2, 4, and 5) contained only novel sequences. Within-cluster differences between sequences, particularly among soil clones, sometimes involved only 1 or 2 base changes. It is entirely possible that some of this microheterogeneity might be artifactual since it is well-known that PCR may introduce point errors. Most changes did occur in regions known to be variable, but this in itself does not eliminate this potential source of error. The finding of the same partial sequences in different gene libraries suggests that at least some of the differences are real. Some microheterogeneity may also represent differences between different RNA gene copies from the same cell (5). However, the levels of 16S rRNA sequence dissimilarity displayed by some representatives of environmental sequence clusters were of a magnitude similar to or greater than that

FIG. 1. Relationships of environmental partial 16S rDNA sequences to partial sequences from reference ammonia oxidizers. The neighbor-joining (30) tree has been pruned from a larger one containing a selection of proteobacterium reference sequences. Bootstrap values are given at nodes when they exceed 50% of replicates or where values are of interest to the discussion. The scale equals 1% estimated substitutions calculated by using the Jukes and Cantor correction (15). Abbreviations are exemplified by the following examples: EnvA2-4, clone no. 4 from polluted sediment A in the 16S rDNA library, sediment sample replicate 2; C1aW3, marine enrichment from nonpolluted sample C, sample replicate no. 1, enrichment replicate a, medium W, third-generation enrichment; pH7C/54, clone 54 from 16S rDNA library from replicate soil sample C, pH 7.0 soil; enrich ZD5, pH 7.0 soil enrichment sequence; enrich ZB3, pH 4.2 soil enrichment; AEM-5, sequence from enriched surface water samples (23).

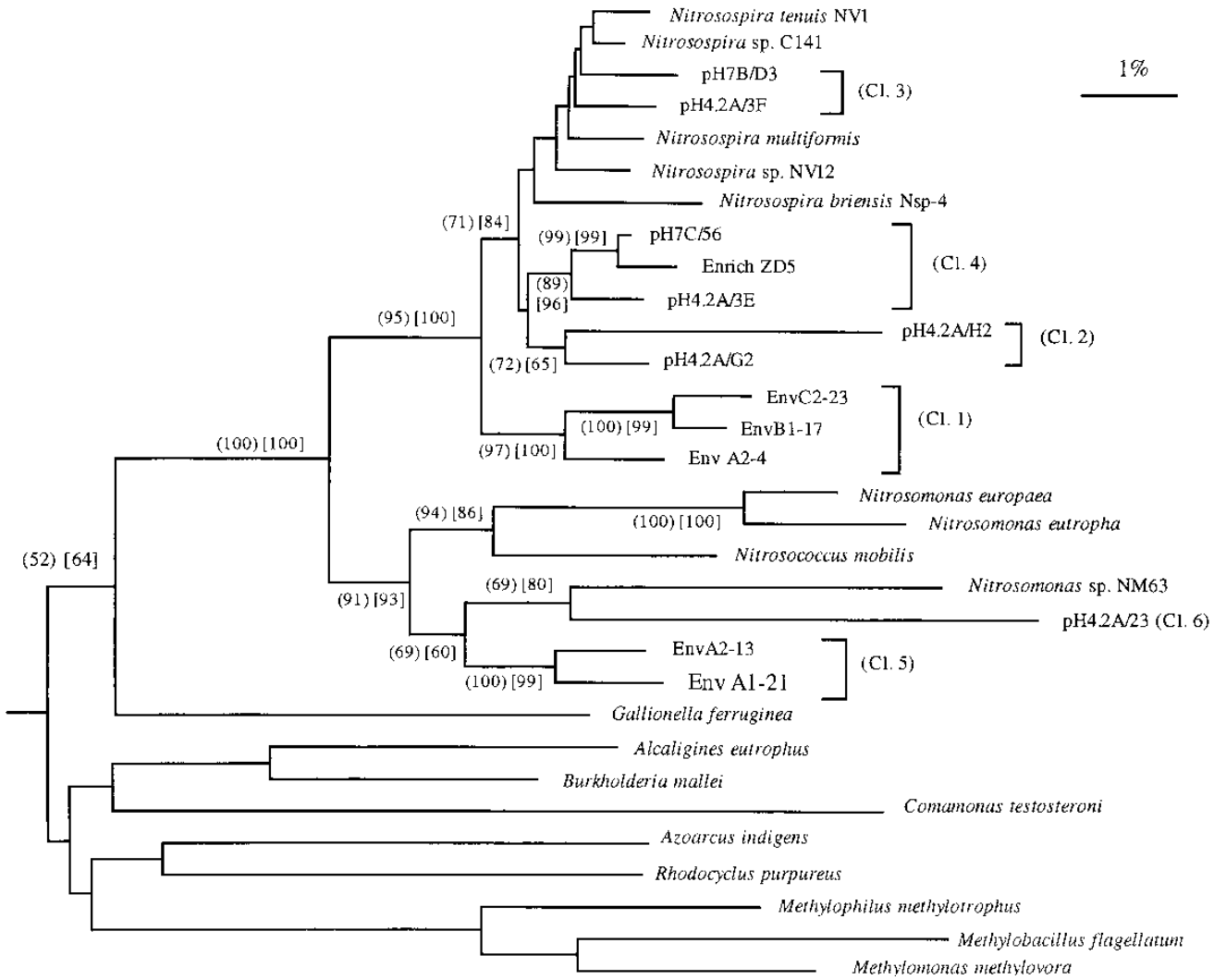


FIG. 2. Neighbor-joining tree showing relationships between environmental sequences and reference β-proteobacteria based on 1,099 bases of aligned 16S rDNA sequences. The percentages of bootstrap support above 50% are shown for parsimony (indicated in parentheses) and distance matrix (indicated in brackets) analyses at nodes concerning ammonia oxidizer relationships. For convenience the tree has been pruned from a larger one containing additional sequences from reference proteobacteria. The scale is 1% estimated change.

observed between named species of *Nitrosospira* and *Nitrosomonas* (Fig. 1 and 2). It would be naive to actually propose new species based on our limited sequence data: the prokaryote species is a concept which also requires phenotypic characterization from pure cultures (24, 44). However, it seems likely to us that from the level of sequence diversity observed, if cultures can be isolated, then each new sequence cluster may represent one or more new species.

The recovery of sequence clusters rather than a completely unresolved bush provides some evidence that natural discontinuities (6) might exist between ammonia oxidizer sequence types in natural samples. However, the bootstrap support for individual clusters based on partial sequences was low, and the apparent structure in the data needs to be investigated further by sequencing longer stretches from a comprehensive sample of clones. Encouragingly, analysis of longer stretches of sequence for 13 clones did improve bootstrap support for relationships between cluster representatives (Fig. 2).

Some identical or closely related partial sequences were obtained from different libraries and from enrichments. This finding infers that the same or closely related strains might be found in different samples. It cannot be certain that comparison of longer sequences will always confirm identity based on partial sequences, but our current limited data (Fig. 2) encourage us to suggest that overall similarity is likely to remain high.

None of the partial sequences was identical to any published sequence from a cultured ammonia oxidizer, and four of the seven recognized clusters contain only novel sequences. These findings suggest that cultured ammonia oxidizers for which sequences are available may not be entirely representative of the β-proteobacterium ammonia oxidizers in our particular samples. The significance of this observation may become more apparent when 16S rRNA sequences from more of the described (17) cultures of ammonia oxidizers become available. Previously published molecular and culture-based investigations (4, 14, 23) have also suggested that laboratory culture favors *Nitrosomonas* spp. over *Nitrosospira* spp. Our own data provide further evidence that molecular and culture-based (enrichment) methods may select for different community members. For example, most (15 of 18) partial sequences from

marine sediment enrichments were new lineages related to *Nitrosomonas* rather than to *Nitrosospira* (3 of 18) spp. This trend is in marked contrast to the sampled marine sediment clones, for which novel *Nitrosospira*-like sequences were three times more abundant than *Nitrosomonas* sequences.

From our library sampling it appears that *Nitrosospira*-like sequences are more common than those from *Nitrosomonas* in the soil and sediment samples that we processed. The situation is most apparent for the soil samples, only three clones of which were related to *Nitrosomonas* sequences. These observations are in agreement with some culture-based experiments suggesting that *Nitrosospira* spp. are common in soil (4, 18, 19). We also support the probing and PCR data of Hiorns et al. (14) which suggested that *Nitrosospira* spp. were present in soil and freshwater. Encouragingly, the probe AAO-258 designed (14) to bind to terrestrial ammonia-oxidizing β-proteobacteria performs well when challenged with our new sequence data from soil samples. The target site for AAO-258 is found in almost all of the new soil sequences from clusters 2, 3, and 4. The target site for this probe is not present in most marine sequences from clusters 5 and 6, nor is it present in marine *Nitrosospira*-like sequences from cluster 1 or the soil *Nitrosomonas*-like sequences in cluster 6. The other probes developed for more specific detection of different ammonia oxidizers generally fall outside the bounds of our new sequences and therefore cannot be similarly reevaluated.

Our data do not provide any support for the hypothesis (19) that *Nitrosospira* (formerly *Nitrosolobus*) *multiformis* is the most common ammonia oxidizer in soil, as the sequences of most of our clones were clearly distinct from this sequence. Neither ourselves nor Hiorns et al. (14) can provide any molecular evidence which supports the statement that *Nitrosomonas* spp. carry out most of the nitrification in soils (32). *Nitrosomonas europaea* has been the subject of most physiological studies (29), but our findings suggest caution in extrapolating information from this strain to all ammonia oxidizers and identify clusters whose sampling may form the basis of more informative studies of physiological diversity.

Most marine clone sequences (31 of 40 sampled) were related to *Nitrosospira* sequences. This is the first strong evidence, supported by an unambiguous phylogenetic placement (Fig. 2), for the presence of *Nitrosospira* spp. in marine samples. The marine *Nitrosospira*-like sequences were recovered from all three sediment samples, and they formed a novel sequence cluster (Fig. 1 and 2, cluster 1) separate from the soil *Nitrosospira*-like sequences. These observations are compatible with the hypothesis that they are indigenous to these samples, rather than the result of wash-in from land. We also detected a novel cluster (cluster 5) of marine *Nitrosomonas*-like sequences. This cluster and a small number of scattered clones within cluster 6, at least three of which suggest the presence of soil *Nitrosomonas* spp. which are likely to be distinct from cultured species, are the main evidence in our gene libraries for the environmental presence of *Nitrosomonas* spp.

The preliminary nature of our data on the different marine sediments necessitates caution in interpreting the results of these analyses, as regards any differences which might exist between the polluted and nonpolluted sites. Cluster 1, which is related to *Nitrosospira* sequences and which contained most marine clone sequences, contains sequences from all three sites. Interestingly, the smaller cluster 5, which is related to *Nitrosomonas* spp., does not contain any sequences from the nonpolluted site (C). Additional sampling and work are needed to further investigate the potential impact of pollution on the composition of clone libraries prepared from marine sediments.

Soil samples were taken from two adjacent test plots which had been treated to maintain their pH at either 7.0 or 4.2. In sampling both sites we were interested to see if we could detect selection for different populations of ammonia oxidizers under acid or neutral conditions. Sequences from each soil pH were found in all four clusters which contained soil clone sequences. These findings are compatible with the hypothesis that some closely related ammonia oxidizers exist in both neutral and acid soils. The increased ionization of ammonia to ammonium and the associated metabolic costs associated with ammonium transport have argued against the existence of ammonium oxidation at low pH in liquid culture. In acid soils, however, ammonia oxidizers can maintain activity in microsites with high pH, through simultaneous hydrolysis of urea (1, 7) and through attachment to surfaces (2). Detailed analysis of cluster composition does suggest that some sequence types might be more common in one soil than in the other. Cluster 2 contains mainly sequences from the pH 4.2 library (16 of 20 clones), so we cannot exclude the possibility that these originate from strains which are more common under acid conditions. Cluster 3 contains mainly sequences from the pH 7.0 soil library (10 of 12 clones) plus sequences from reference *Nitrosospira* spp. which were isolated under approximately neutral conditions. While considering any significance of these different cluster compositions, it is necessary to acknowledge that 16S rRNA sequences by themselves provide no information about potential physiological differences between closely related bacteria. Further work on pure cultures originating from the new clusters recognized by sequence analysis is now needed to investigate their phenotypic properties.

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