MICROBIAL ECOLOGY

Microb Ecol (2001) 42:228–237
DOI: 10.1007/s00248-001-0016-x


# Species Diversity of Uncultured and Cultured Populations of Soil and Marine Ammonia Oxidizing Bacteria

Z. Smith,[1] A.E. McCaig,[1,2] J.R. Stephen,[1,2] T. M. Embley,[2] J.I. Prosser[1]

[1] Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Aberdeen, Scotland, AB25 2ZD, UK
[2] Department of Zoology, Natural History Museum, Cromwell Rd., London, SW7 5BD, UK

Received: 11 September 2000; Accepted: 16 February 2001; Online Publication: 17 August 2001

## ABSTRACT

Although molecular techniques are considered to provide a more comprehensive view of species diversity of natural microbial populations, few studies have compared diversity assessed by molecular and cultivation-based approaches using the same samples. To achieve this, the diversity of natural populations of ammonia oxidising bacteria in arable soil and marine sediments was determined by analysis of 16S rDNA sequences from enrichment cultures, prepared using standard methods for this group, and from 16S rDNA cloned from DNA extracted directly from the same environmental samples. Soil and marine samples yielded 31 and 18 enrichment cultures, respectively, which were compared with 50 and 40 environmental clones. There was no evidence for selection for particular ammonia oxidizer clusters by different procedures employed for enrichment from soil samples, although no culture was obtained in medium at acid pH. In soil enrichment cultures, *Nitrosospira* cluster 3 sequences were most abundant, whereas clones were distributed more evenly between *Nitrosospira* clusters 2, 3, and 4. In marine samples, the majority of enrichment cultures contained *Nitrosomonas,* whereas *Nitrosospira* sequences were most abundant among environmental clones. Soil enrichments contained a higher proportion of identical sequences than clones, suggesting laboratory selection for particular strains, but the converse was found in marine samples. In addition, 16% of soil enrichment culture sequences were identical to those in environmental clones, but only 1 of 40 marine enrichments was found among clones, indicating poorer culturability of marine strains represented in the clone library, under the conditions employed. The study demonstrates significant differences in species composition assessed by molecular and culture-based approaches but indicates also that, employing only a limited range of cultivation conditions, 7% of the observed sequence diversity in clones of ammonia oxidizers from these environments could be obtained in laboratory enrichment culture. Further studies and experimental

*Correspondence to:* J.I. Prosser; Fax: +44 1224 273144; E-mail: j.prosser@abdn.ac.uk

approaches are required to determine which approach provides better representation of the natural community.

## Introduction

The use of molecular techniques to characterize bacterial communities in natural environments has greatly altered perceptions of bacterial diversity. Broad-scale measures, such as those involving DNA:DNA reassociation kinetics, suggest the existence of several thousand distinct genomes per gram of soil [34]. Fine-scale techniques include the analysis of sequences in clone libraries of 16S rRNA and functional genes amplified from DNA extracted directly from environmental samples. Application of fine scale techniques to broad microbial groups, e.g., Bacteria [3, 12, 13], and more specific phylogenetic and functional groups, such as methanotrophs [9] and ammonia oxidizers [31], has demonstrated the dominance of such libraries by sequences markedly different from those of cultivated organisms in databases. Molecular techniques do not require laboratory cultivation and, although themselves subject to potential biases [22, 33], can reveal aspects of bacterial community structure that are lost during laboratory growth. The information recovered by such studies has been interpreted as supporting the view that traditional, cultivation-based techniques underestimate the diversity of natural bacterial populations [29]. Quantitative studies indicate that 0.1–10% of the bacterial biomass of an environmental sample can be induced to grow under laboratory cultivation [2, 29, 35, 37, 38]. Similar values are indicated by comparisons of bacterial cell concentrations estimated using competitive PCR with traditional viable cell counts [11, 20].

Molecular studies are considered to assess the species diversity of total (culturable and uncultured) populations. However, no molecular data are available on the many organisms that have been isolated and cultivated in ecological studies using traditional approaches. It is therefore dangerous to conclude that diversity studies based on laboratory cultivation and isolation provide a biased or even different view of environmental diversity without analysis using both approaches on the same samples. The enormous diversity within broad groupings, e.g., Bacteria, makes such comparisons difficult, arguing for analysis of smaller taxonomic groups with defined physiology facilitating specific cultivation and enrichment. Generalizations based on particular microbial groups must, of course, consider potential differences in culturability between groups.

Analysis of natural populations of autotrophic ammonia-oxidizing bacteria using cultivation-based techniques presents particular problems, due to their slow growth, difficulties in separating ammonia oxidizers from heterotrophic contaminants, and the limited number of characteristics for identification and classification. This has hampered meaningful study of the population ecology of ammonia-oxidizing bacteria, limiting our understanding of their important role in carrying out oxidation of ammonia to nitrite, the first step in nitrification. Increased understanding of the relationship between taxonomic and physiological diversity is important for our understanding of nitrogen cycling in natural environments.

With the exception of a small number of cultured marine strains belonging to the γ-Proteobacteria, analysis of 16S rRNA gene sequences places autotrophic ammonia oxidizers in a monophyletic group within the β-Proteobacteria, containing two genera, *Nitrosomonas* and *Nitrosospira* [8]. Analysis of 16S rRNA gene sequences recovered from natural populations of ammonia oxidizers indicates further subdivisions, with the *Nitrosospira* and *Nitrosomonas* genera containing at least four clusters and three clusters, respectively, of related sequences [31]. Such studies also suggest that the distribution of different clusters is linked to environmental factors, including tillage, marine organic pollution, soil pH, marine aggregate formation, and manure application [4, 7, 18, 19, 21, 30, 31].

Although it is difficult to obtain pure cultures of ammonia-oxidizing bacteria from environmental samples, preparation of enrichment cultures is relatively easy. The aim of this study was, therefore, to compare the species composition of natural populations of ammonia oxidizers from arable soil and marine sediments generated by analysis of 16S rDNA sequences from enrichment cultures and from 16S rDNA cloned from DNA extracted directly from the same environmental samples.

## Materials and Methods

### *Soil and Marine Samples*

Soil was sampled in February 1994 from two adjacent 3 × 4 m agricultural plots at the Scottish Agricultural College, Craibstone, Aberdeen, Scotland (OS NJ867112 (see [31] for details). The plots had been maintained at pH 4.2 and pH 7.0 since 1961, prior to

which soil treatments and properties were identical. Duplicate samples of bulk soil from the surface 20 mm were taken randomly 0.5 m from the edges of each plot. The pH of the soil prior to amendment was approximately 3.9. Marine sediment samples were obtained from a depth of 16 m in a seawater loch, Loch Sunart, with fish cages containing North Atlantic salmon. Samples were taken from directly underneath a fish cage and at distances of 20 m and 40 m on a transect from the fish cage and the top 5 mm removed for analysis (see [31] for details of sampling methods).

### *Enrichment Cultures*

Soil enrichments were prepared by incubation of soil samples using 1% (w/v) inocula into either 100 or 300 ml liquid inorganic medium [24] contained in a 500 ml Erlenmeyer flask and with an initial pH value of 5 or 7.5 and either 5 or 50 μg $NH_4^+$–N $ml^{-1}$. Separate batch enrichments were also set up consisting of 10 g soil and 2 ml medium in Universal bottles. Cultures were incubated at 23°C, in the dark, and growth was assessed by the appearance of nitrite, detected by use of Quantofix nitrate/nitrite dipsticks (Camlab Ltd., Cambridge, UK), and by a change in pH of the medium (determined by change in color of the pH indicator phenol red from pink to yellow). First-generation cultures were neutralized, until ammonium was completely oxidized, by addition of 5% (w/v) $Na_2CO_3$, and subcultured by 1% (v/v) inoculation of fresh medium. Subsequent subcultures were carried out in a similar fashion. First generation enrichments were also plated onto Macdonald and Spokes [15] medium (pH 8.0) solidified with 1% (w/v) Noble Agar (Difco Ltd., Surrey, UK) with phenol red replaced by 0.003% (w/v) neutral red and supplemented with 50 μg $NH_4^+$–N $ml^{-1}$. Plates were incubated for 6 months in the dark and red colonies (indicating acid production) were removed using a micromanipulator and inoculated into 100-ml liquid inorganic medium. Enrichments were also prepared in continuous flow columns packed with soil from pH 7.0 plots, supplied continuously with air and inorganic medium containing 50 μg $NH_4^+$–N $ml^{-1}$ and incubated at 30°C for several months. Effluent from the column was used to inoculate fresh liquid medium and resultant enrichment cultures were subcultured and plated as described above.

Duplicate enrichments from marine sediment samples were prepared by inoculation of 100 ml of both inorganic salt medium or artificial seawater containing 100 μg N–$NH_4^+$ $ml^{-1}$ as ammonium sulfate with 1 g sediment [17]. The former medium contained 0.01% (w/v) phenol red as a pH indicator, and pH was adjusted to approximately 7.5 by addition of sterile 5% (w/v) $Na_2CO_3$. Cultures were incubated and growth assessed as described above. First-generation cultures were neutralized twice, by addition of 5% (w/v) $Na_2CO_3$, and subcultured by 1% (v/v) inoculation of fresh medium. First-generation marine 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 for soil and sediment samples (see below). Second-generation enrichments were briefly spun to remove sediment, followed by centrifugation of the resulting supernatant at 10,000 *g* to pellet cells. DNA was released from cell pellets by boiling with Chelex 100 [17, 36].

### *Extraction and Purification of DNA from Soil and Sediment Samples*

DNA was extracted from soil and sediment samples, as described by Stephen et al. [31], by bead beating and vortexing, respectively. Extracted DNA was purified by dialysis and gel electrophoresis (soil) or by passing through a SpinBind column (Flowgen Instruments Ltd., Kent, UK (marine).

### *PCR Amplification of 16S rDNA, Cloning and Sequencing*

PCR amplification of 16S rDNA was carried out on 20 ng of DNA in 50 μl PCR reactions using the primers βAMOf and βAMOr, designed to amplify selectively approximately 1.1 kb of 16S rDNA from β-subgroup ammonia oxidizers and their close relatives [17]. PCR conditions were those described previously [17]. PCR products pooled from five reactions, to minimize possible PCR bias, were purified and ligations then carried out into the pGEM-T vector (Promega Ltd., Southampton, UK) following the manufacturer's protocols. The ligations were transformed into supercompetent *Escherichia coli* XL1-Blue MRF′ Kan (Stratagene Ltd., Cambridge, UK) and plated on Luria–Bertani agar supplemented with IPTG (1 mM), X-Gal (40 mg $ml^{-1}$), and the antibiotics kanamycin, ampicillin, and methicillin according to manufacturer's instructions. 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 using the Promega Wizard Mini-Prep system. Randomly selected recombinant colonies were analyzed from each soil clone library using a single dideoxynucleotide (ddA—"T-tracking") sequencing method and primer 537r [28], in order to identify non-ammonia-oxidizer clones and to remove them from subsequent analysis.

### *Sequence Analysis*

Enrichment and clone sequences were aligned against representative prokaryote 16S rRNA gene sequences from the Ribosomal Database Project (RDP) [16] and ammonia oxidizer sequences [8, 17, 23, 25] using the Genetic Data Environment running in ARB [32]. Phylogenetic trees based on 307 bases (soil sequences) and 303 bases (marine sequences) of aligned sequence data were constructed by using the Jukes and Cantor model [10] and Neighbor joining [27] with PHYLIP, version 3.5 [6]. Data sets were bootstrapped using SEQBOOT (PHYLIP, version 3.5).

### *Nucleotide Sequence Accession Numbers*

Partial enrichment and clone sequences determined in this study have been deposited in the GenBank database under accession numbers Z69087 to Z69197 and AF178087 to AF178112.

## Results

### *Arable Soil*

Craibstone soil plots, maintained at either pH 4.2 or pH 7.2, yielded a total of 31 enrichment cultures. The effects of

**Table 1.** Numbers and (in parentheses) percentages of ammonia oxidizer enrichment cultures derived from soil samples of different initial pH values, using different enrichment and subculturing procedures, and related distributions between β-proteobacterial ammonia oxidizer clusters defined by Stephen et al. [31][a]

| | | Nitrosospira | | Nitrosomonas | |
|---|---|---|---|---|---|
| | Total | Cluster 2 | Cluster 3 | Cluster 4 | Cluster 7 |
| PH of inoculated soil | | | | | |
| pH 4.2 | 20 (69%) | 4 (20%) | 12 (60%) | 3 (15%) | 1 (5%) |
| pH 7 | 9 (31%) | 0 (0%) | 7 (78%) | 2 (22%) | 0 (0%) |
| Conc. of ammonium in medium | | | | | |
| 5 μg $NH_4^+$–N $ml^{-1}$ | 4 (14%) | 1 (33%) | 1 (33%) | 1 (0%) | 1 (33%) |
| 50 μg $NH_4^+$–N $ml^{-1}$ | 25 (86%) | 3 (12%) | 18 (69%) | 4 (19%) | 0 (0%) |
| Subculture medium | | | | | |
| All subcultures in liquid medium | 23 (79%) | 3 (13%) | 16 (70%) | 4 (17%) | 0 (0%) |
| One subculture on solid medium | 6 (21%) | 1 (17%) | 3 (50%) | 1 (17%) | 1 (17%) |
| Enrichment culture system | | | | | |
| Flask culture | 19 (66%) | 1 (5%) | 14 (74%) | 4 (21%) | 0 (0%) |
| Soil microcosm | 10 (34%) | 3 (30%) | 5 (50%) | 1 (10%) | 1 (10%) |

[a] Two *Nitrosospira* sequences were omitted from analysis (see legend to Table 2).

enrichment conditions were assessed by determining the number and percentage of cultures with sequences representative of different clusters resulting from the different enrichment procedures adopted (Table 1). (Two enrichments were omitted from analysis for reasons given below.) All enrichment cultures were obtained in medium with an initial pH >7 and none was obtained from medium adjusted to low pH, containing either 50 or 5 μg $NH_4^+$–N $ml^{-1}$, during the period of incubation. The majority (90%) of cultures were obtained from initial enrichment in medium containing the higher ammonium concentration, although equal numbers of enrichments were set up at each concentration. The majority (66%) of cultures were obtained from initial enrichment in liquid medium, rather than microcosms or soil columns, and only 21% involved one subculture on solid medium. The low proportions of cultures obtained on solid medium and at low ammonia concentration reflect, in part, the respective difficulties in detecting microscopic colonies on solid medium and low growth rates and yield at low ammonium concentrations. Only one enrichment culture, Enrich_ZD5, was obtained from a soil column, which was supplied with medium containing the higher ammonium concentration at an initial pH of 7. Interestingly, a greater number of enrichment cultures were obtained from acid soil plots (69%) despite all enrichment cultures being obtained in medium at neutral pH.

All cultures were subcultured at least twice following the initial enrichment step, after which DNA was extracted and partial 16S rRNA gene sequences were determined using primers selective for β-proteobacterial subgroup ammonia-oxidizing bacteria. The 31 enrichment culture sequences contained representatives of *Nitrosospira* clusters 2, 3, and 4 and *Nitrosomonas* cluster 7 (Fig. 1, Table 1; employing cluster designations of Stephen et al. [31]). In addition, two enrichment cultures (E18_pH4/5/FL and E25_pH7/50/FL) were obtained whose sequences fell within the *Nitrosospira* clade (98% bootstrapping support) but that were not clearly affiliated to any of the previously defined clusters. Possible selection for particular clusters was assessed by calculating the proportion of cultures obtained using a particular enrichment procedure. The total number of enrichments available for analysis was not high, making firm conclusions difficult, but there was no evidence of selection for particular ammonia-oxidizer clusters by different enrichment procedures.

Ammonia-oxidizer populations in soils of different pH were characterized by analysis of 16S rDNA sequences in enrichment cultures and in environmental clones amplified from DNA extracted from soil, and relative abundances of sequences falling within different clusters are presented in Table 2. The majority of enrichments (66%) fell within *Nitrosospira* cluster 3. This includes the enrichment E26_pH7/50/FL, which is identical to *Nitrosospira briensis* and which branches closer to cluster 2 in Fig. 1. In analysis of longer stretches of sequence data (348 and 1099 bases), however, *N. briensis* tends to branch within cluster 3 [31]. The majority (80%) of the enrichment culture sequences belonging to *Nitrosospira* cluster 3 fell within the same grouping, which also contained sequences from pure cultures of *Nitrosospira* spp. NpAV and C-141. This group con-

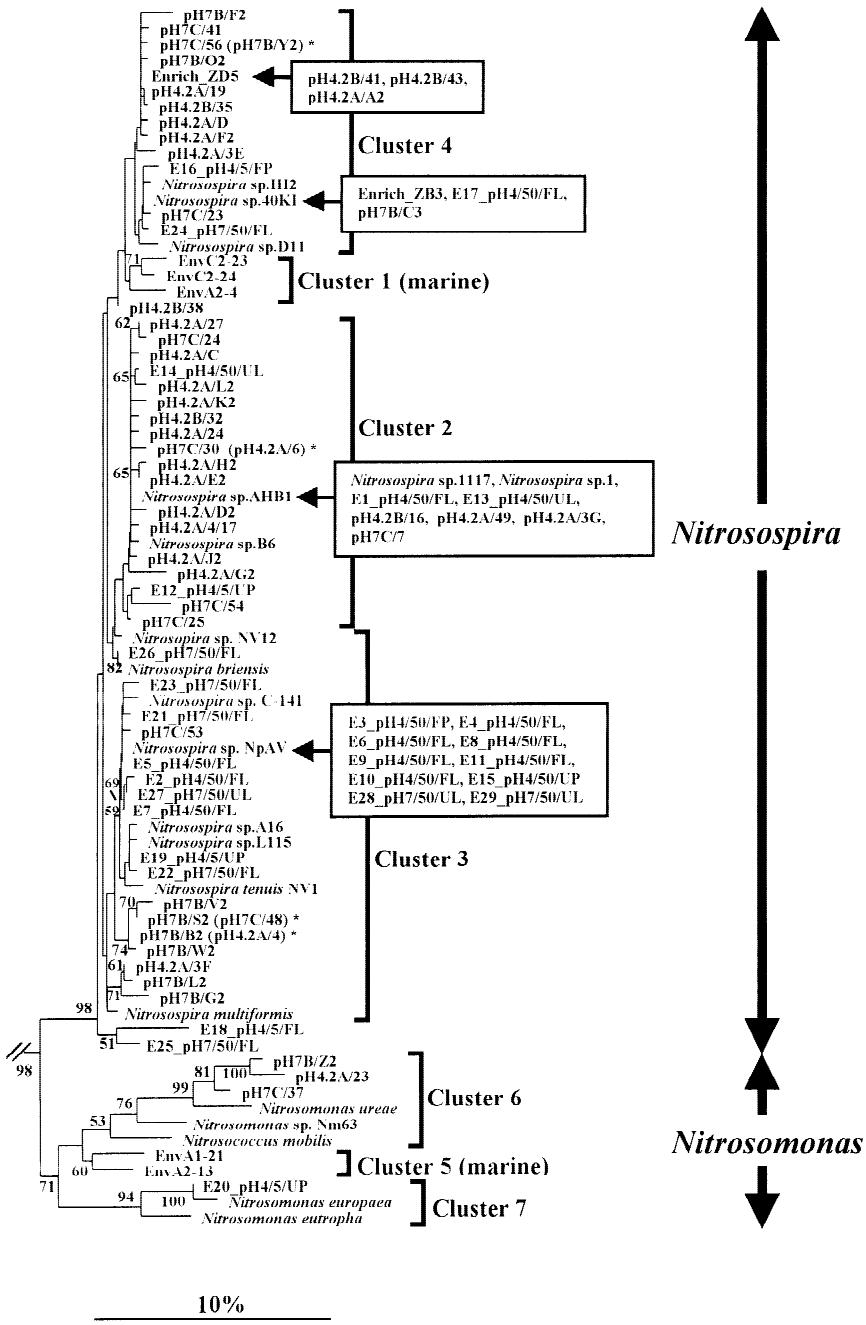


Fig. 1. Neighbor-joining tree showing the relationships between pure cultures, enrichments, and environmental clones of β-subgroup ammonia-oxidizing bacteria from samples of soil maintained at pH 4.2 or pH 7 based on analysis of 307 bases of aligned 16S rRNA gene sequences. The scale bar represents 10% estimated nucleotide substitutions. Soil clones from low and neutral pH soil are prefixed "pH4.2" or "pH7", respectively. Soil enrichments are prefixed "Enrich" or "E1", "E2", "E3" etc., followed by soil pH value (pH 4 for pH 4.2 soil), ammonium concentration (5 or 50 μg $N–NH_4^+$ $ml^{-1}$), and enrichment method (F and U refer to liquid enrichment in flask or enrichment in soil microcosm, respectively; L and P refer to subculturing in liquid medium only or use of plating step, respectively). Clones prefixed "Env" are from marine sediment samples. Clusters 1 to 7 are indicated on the tree and are based on the designations of Stephen et al. [31]. Pairs of identical sequences are indicated with an asterisk, and identical sequences belonging to groups with more than two sequences are shown in boxes.

tained 10 sequences obtained from independent enrichment cultures that were identical to *Nitrosospira* sp. NpAV, with a further three sequences differing by only one or two ambiguous bases. Sequence identity was also observed in other groups. Two of the four enrichment culture sequences falling within *Nitrosospira* cluster 2 were identical to each other, to four cloned sequences, and to three pure cultures. Two of the five sequences within *Nitrosospira* cluster 4 were also identical. Thus, from a total of 31 enrichment culture sequences, only 20 (65%) differed over the region of 16S rDNA investigated.

16S rRNA gene sequences of environmental clones, derived from DNA extracted directly from soil, were distributed more evenly than those of enrichment cultures, with 21, 10, and 15 sequences in *Nitrosospira* clusters 2, 3, and 4, respectively, a single *Nitrosospira* clone (pH4.2B/38) that cannot be assigned with certainty to the recognized clusters, but only 3 sequences in *Nitrosomonas* cluster 6. Identical sequences were rare, but one, two, and one pairs of clones with identical sequences were found in clusters 2, 3, and 4, respectively (Fig. 1). *Nitrosospira* clusters 2 and 4 also contained four clones and three clones, respectively, with iden-

**Table 2.** Numbers and (in parentheses) percentages of partial 16S rDNA sequences from terrestrial and marine environmental clones and enrichment cultures between β-proteobacterial ammonia oxidiser clusters defined by Stephen et al. [31]

| | Terrestrial samples | | Marine samples | |
|---|---|---|---|---|
| | Enrichment cultures | Environmental clones | Enrichment cultures | Environmental clones |
| *Nitrosospira* cluster 1 | | | 3 (17) | 31 (78) |
| *Nitrosospira* cluster 2 | 4 (14) | 21 (43) | | |
| *Nitrosospira* cluster 3 | 19 (66) | 10 (20) | | |
| *Nitrosospira* cluster 4 | 5 (17) | 15 (31) | | |
| *Nitrosomonas* cluster 5 | | | 2 (11) | 6 (15) |
| *Nitrosomonas* cluster 6 | | 3 (6) | 11 (61) | 3 (8) |
| *Nitrosomonas* cluster 7 | 1 (3) | | 1 (6) | |
| Other[a] | 2 (6) | 1 (2) | 1 (6) | |
| Total | 31 | 50 | 18 | 40 |

[a] The soil clone pH4.2B/38 and the branch comprising soil enrichments E18_pH4/5/FL and E25_pH7/50/FL belong to the *Nitrosospira* clade but cannot be placed within any clusters previously defined in Stephen et al. [31] using the available data. The marine sediment enrichment A2bM1, although belonging to the ammonia oxidizer clade, is not associated strongly with either genus. These sequences were not used in calculating percentages.

tical sequence. Thus, from a total of 50 clones examined, 41 (82%) had different sequences over the region examined. Several sequences were identical to enrichment culture sequences. In *Nitrosospira* cluster 4, identity was found between three clone sequences and one enrichment culture and between a pair of identical enrichment culture sequences and a single clone sequence. In *Nitrosospira* cluster 2, a pair of identical enrichment sequences shared sequence identity with the group of four identical clones and the pure culture (see above). Thus, 5 of the 31 enrichment cultures (16%) had sequences that were identical to members of the 50 clones that were sequenced, providing a measure of the "culturability" of the natural community. A single enrichment culture (E20_pH/4/5/UP) generated a sequence highly similar to that of the *Nitrosomonas* type strain, *Nitrosomonas europaea,* a member of cluster 7, whereas no enrichment culture was recovered with similarity to the *Nitrosomonas* cluster 6 sequences recovered from the clone libraries.

### *Marine Sediments*

Marine sediments yielded 18 enrichment cultures, whose partial 16S rRNA gene sequences were compared with those of 40 clones from the same samples. Both sets of sequences contained representatives of *Nitrosospira* cluster 1 and *Nitrosomonas* clusters 5 and 6, with one enrichment sequence representative of *Nitrosomonas* cluster 7 (Table 2, Figure 2). An additional enrichment sequence A2bM1 was obtained that, although branching with 63% bootstrapping support within the *Nitrosospira* clade (Fig. 2), is unstable when the length of sequence analyzed is increased [31]. Clone sequences were dominated by representatives of *Nitrosospira* cluster 1, with 31 from a total of 40 (78%). No clone had a partial sequence identical to that of a pure culture in the database. In contrast, enrichment sequences were dominated by *Nitrosomonas* cluster 6, which contained 61% of the enrichment sequences. Few representatives were found of *Nitrosospira* cluster 1 and *Nitrosomonas* cluster 5, and a single representative only was found of *Nitrosomonas* cluster 7. Within *Nitrosospira* cluster 1, clone and enrichment culture sequences appear to cluster separately. One pair, two sets of triplets, one set of four, and one set of five identical clone sequences and one pair of identical enrichment sequences were found in *Nitrosospira* cluster 1. In addition, three clone sequences were found with identity to that derived from an enrichment culture in *Nitrosomonas* cluster 5. Thus, of 18 and 40 enrichment and clone sequences analysed, 17 (94%) and 26 (65%), respectively, had different sequences over the region examined. This contrasts with terrestrial samples, where the proportion of identical sequences was greater in clones than in enrichment cultures. Only one enrichment sequence was represented in the 40 clones examined (equivalent to 2.5%), a much lower estimation of culturability than found in terrestrial samples.

## Discussion

The aim of this study was to assess and compare the diversity of natural populations of terrestrial and marine ammonia-oxidizing bacteria by 16S rDNA analysis of enrichment cultures and environmental clones. Both approaches have limi-

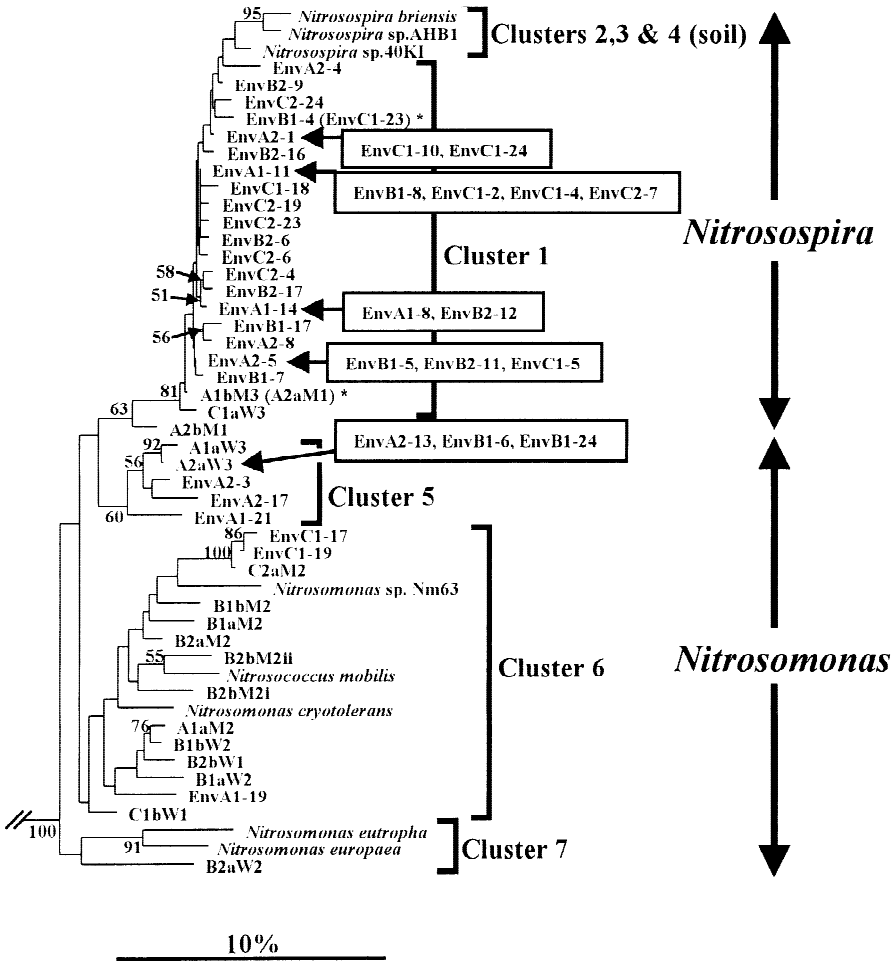


Fig. 2. Neighbor-joining tree showing the relationships between pure cultures, enrichments, and environmental clones of β-subgroup ammonia-oxidizing bacteria from marine sediment samples based on analysis of 303 bases of aligned 16S rDNA sequences. Marine clones are prefixed "Env" followed by sample site (A, B, or C for 0, 20, and 40 m from fish cage). Marine enrichments are prefixed A, B, C as for clones, the medium type (W or M), and generation number (1, 2, or 3). Clusters 1 to 7 are indicated on the tree and are based on the designations of Stephen et al. [31]. Pairs of identical sequences are indicated with an asterisk, and identical sequences belonging to groups with more than two sequences are shown in boxes.

tations. The diversity and coverage achieved in enrichment cultures is limited by the accepted limitations of methods based on laboratory cultivation, in particular the range of enrichment procedures and culture conditions employed and the presence of dormant and viable but nonculturable cells. Molecular analysis may be influenced by a number of biases (see below), but also by the length of 16S rDNA sequence used for analysis. Regions of 307 and 303 bases were used for comparison of terrestrial and marine sequences, respectively, and identity over these regions indicated similarity but not necessarily identity of the organisms from which sequences were derived. The range of enrichment conditions and length of sequence analyzed therefore contribute significantly to the resolution of the methods employed and influence interpretation of the results.

The picture of species diversity of β-subgroup proteobacterial ammonia oxidizers in both soil and marine samples changed depending on whether enrichment cultures or environmental clones were investigated. Sequences related to *Nitrosospira* cluster 3 were most abundant in enrichment cultures from soil, whereas environmental clones were distributed more evenly between *Nitrosospira* clusters 2, 3, and 4. In marine samples, sequences related to *Nitrosospira* cluster 1 were most abundant in environmental clones, whereas the majority of enrichment cultures contained sequences falling within *Nitrosomonas* cluster 6. One explanation for these differences is selection, during enrichment, for particular groups by growth media and incubation conditions to which the natural population is not well adapted. This is frequently invoked to explain the isolation of *Nitrosomonas* in laboratory culture and their apparent low relative abundance in natural environments, indicated by rare detection of sequences in environmental clones that are closely related to the type strain *Nitrosomonas europaea,* originally isolated from soil. Selection may have led to dominance of marine enrichments by nitrosomonads, while *Nitrosospira* sequences dominated clones. In contrast, the abundance of nitrosomonad sequences in both enrichments and clones from soil was low, indicating that populations in these soils may be dominated by *Nitrosospira* spp.

The high number of identical sequences belonging to *Nitrosospira* NpAV cluster 3 in independent soil enrichment cultures indicates strong selection for organisms closely related to this strain. The close similarity and, in more than one case, identity of enrichment culture sequences to those of pure cultures of *Nitrosospira* suggests that enrichment conditions favored growth of these isolates. Although representatives of *Nitrosospira* cluster 3 were found among environmental clones, they formed groups that were distinct from enrichment and pure cultures. This apparent selection was less evident in *Nitrosospira* cluster 2 representatives, where the few enrichment culture sequences grouped with those of environmental clones and two of the four cluster 2 enrichments were identical to clones and pure cultures. A *Nitrosomonas* cluster 7 sequence was obtained from a single soil enrichment culture but was not represented among clones sampled. This cluster contains *N. europaea* and other closely related sequences have previously been detected in Craibstone soils by sequence analysis or denaturing gradient gel electrophoresis [30, 31]. However, other nitrosomonad sequences obtained were from environmental clones and fell within *Nitrosomonas* cluster 6. In marine environments, previous studies of pure cultures have indicated dominance, within the β-proteobacterial ammonia oxidizers, by *Nitrosomonas.* Marine *Nitrosospira* strains had not been detected prior to application of 16S rRNA analysis [31], and no marine *Nitrosospira* strain exists in pure culture. This may be due to selective enrichment of *Nitrosomonas* as, in this study, enrichment culture sequences were dominated by *Nitrosomonas,* particularly cluster 6, whereas there was a high relative abundance of *Nitrosospira* cluster 1 sequences among clones.

Enrichment cultures were obtained using a variety of culture conditions, including differences in initial ammonium concentration and pH value and batch and continuous flow conditions. Although sample sizes are small, data summarized in Table 1 indicate that these differing growth conditions had no detectable effect on the cluster affiliations of enrichment cultures, with the exception that no enrichment culture was obtained from medium with an initial pH value of 4. The potential lack of selection of populations with greater relative abundance in the environmental samples may therefore be due to lack of identification of the most environmentally relevant factors leading to such selection. It must be emphasized, however, that this study, while demonstrating differences in relative abundance indicated by analysis of enrichment cultures and clones, does not provide definitive information on which approach better represents the natural population. It is likely that laboratory cultivation will introduce selection, but 16S rDNA-based techniques are also subject to selection, for example, due to lysis, PCR and primer biases, chance amplification events, and the retention of DNA from dead cells in natural matrices [5, 14, 22, 33]. Attempts were made to reduce the extent of such bias, but additional experimental approaches are required to discriminate between contrasting findings.

The detection of identical partial 16S rDNA sequences in clone libraries and enrichment cultures provides information on the proportion of the natural population that is culturable. Figures range from 0.1 to 10% based largely on estimates of total and viable bacterial cell population in environmental samples [2, 29, 35, 37, 38]. However, the findings of such studies on total bacterial populations are hard to extrapolate to specific groups. This study compared directly sequences from laboratory cultures and from DNA obtained from the same environmental samples. Combined data from soil and marine samples indicate that 6 of the 90 (7%) clone sequences were from organisms that are potentially culturable under the conditions described, in that they were represented in enrichment cultures. Although this is toward the upper range of previous estimates, it does highlight the dominance of clone libraries by sequences that are not present in laboratory cultures obtained using standard techniques and the consequent need for improved enrichment and isolation techniques to characterize the physiology of components of natural populations. The value of 7% is also likely to overestimate culturability, as discrimination between closely related strains was limited by the length of 16S rDNA sequence, and the use of more fine-scale molecular markers, such as *amoA* [26] or ribosomal intergenic spacers [1], may have reduced this figure further. Differences in the ratio of "culturable" to "unculturable" organisms were also found between marine and soil samples, with 2.5% and 16%, respectively, of clone sequences represented in enrichment cultures, emphasizing the sample dependence of such values.

In conclusion, molecular analysis of enrichment cultures and environmental clones from the same marine and terrestrial samples has provided quantitative information on the differences in species diversity of a specific functional group of bacteria. The two approaches provided significantly different pictures of species diversity, but additional approaches are required to determine which more correctly represents the natural population. The study also provides a value, combined for terrestrial and marine samples, which suggests that approximately 7% of the "total" sequence diversity, assessed by analysis of environmental clones, is rep-

resented in the "culturable" population, comprising cultures enriched using a very limited range of media and incubation conditions.

## Acknowledgments

This project was funded by the NERC grants GST/02/568 and GR3/8911 to JIP and TME.

## References

1. Aakra A, Utåker JB, Nes IF (1999) RFLP of rRNA genes and sequencing of the 16S–23S rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. Int J Syst Bacteriol 49:123–130
2. Bakken LR (1985) Separation and purification of bacteria from soil. Appl Environ Microbiol 49:1188–1195
3. Borneman J, Skroch PW, O'Sullivan KM, Palus JA, Rumjanek NG, Jansen JL, Nienhuis J, Triplett EW (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. Appl Environ Microbiol 62:1935–1943
4. Bruns MA, Stephen JR, Kowalchuk GA, Prosser JI, Paul EA (1999) Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appl Environ Microbiol 65:2994–3000
5. Farrelly V, Rainey FA, Stackebrandt E (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Appl Environ Microbiol 61:2798–2801
6. Felsenstein J (1993) PHYLIP: phylogeny inference package. Felsenstein, Seattle
7. Hastings RC, Ceccherini MT, Miclaus N, Saunders JR, Bazzicalupo M, McCarthy AJ (1997) Direct and biological analysis of ammonia oxidising bacteria populations in cultivated soil plots treated with swine manure. FEMS Microbiol Ecol 23: 45–54
8. Head IM, Hiorns WD, Embley TM, McCarthy AJ, Saunders JR (1993) The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J Gen Microbiol 139:1147–1153
9. Holmes AJ, Owens NJ, Murrell JC (1995) Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. Microbiol 141: 1947–55
10. Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian Protein Metabolism. Academic Press, New York, pp 21–132
11. Kowalchuk GA, Naoumenko ZS, Derikx PJ, Felske A, Stephen JR, Arkhipchenko IA (1999) Molecular analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in compost and composted materials. Appl Environ Microbiol 65:396–403
12. Kuske CR, Barns SM, Busch JD (1997) Diverse uncultivated bacterial groups from soils of the arid Southwestern United States that are present in many geographic regions. Appl Environ Microbiol 63:3614–3621
13. Liesack W, Stackebrandt E (1992) Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J Bacteriol 174:5072–5078
14. Lorenz MG, Wackernagel W (1987) Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. Appl Environ Microbiol 53:2948–2952
15. Macdonald RM, Spokes JR (1980) Selective and diagnostic medium for ammonia oxidising bacteria. FEMS Microbiol Lett 8:143–145
16. Maidak BL, Larsen N, McCaughey J, Overbeek R, Olsen GJ, Fogel K, Blandy J, Woese CR (1994) The ribosomal database project. Nucleic Acids Res 22:3485–3487
17. McCaig AE, Embley TM, Prosser JI (1994) Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiol Lett 120:363–368
18. McCaig AE, Phillips CJ, Stephen JR, Kowalchuk GA, Harvey SM, Herbert RA, Embley TM, Prosser JI (1999) Nitrogen cycling and community structure of β-subgroup ammonia oxidising bacteria within polluted, marine fish-farm sediments. Appl Environ Microbiol 65:213–220
19. Phillips CJ, Harris D, Dollhopf SL, Gross KL, Prosser JI, Paul EA (2000) Effects of agronomic treatments on the structure and function of ammonia oxidising communities. Appl Environ Microbiol 66:5410–5418
20. Phillips CJ, Paul EA, Prosser JI (2000) Quantitative analysis of ammonia oxidising bacteria using competitive PCR. FEMS Microbiol Ecol 32:167–175
21. Phillips CJ, Smith Z, Embley TM, Prosser JI (1999) Phylogenetic differences between particle-associated and planktonic β-proteobacteria ammonia oxidising bacteria in the North Western Mediterranean Sea. Appl Environ Microbiol 65:779–786
22. Polz MF, Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol 64: 3724–3730
23. Pommerening-Roeser A, Rath G, Koops H (1996) Phylogenetic diversity within the genus *Nitrosomonas.* Syst Appl Microbiol 19:344–351
24. Powell SJ, Prosser JI (1985) The effect of nitrapyrin and chloropicolinic acid on ammonium oxidation by *Nitrosomonas europaea.* FEMS Microbiol Lett 28:51–54
25. Rotthauwe JH, de Boer W, Liesack W (1995) Comparative analysis of gene sequences encoding ammonia monooxygenase of *Nitrosospira* sp. AHB1 and *Nitrosolobus multiformis* C-71 FEMS Microbiol Lett 133:131–135
26. Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl Environ Microbiol 63:4704–4712

27. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
28. Schmidt TM, DeLong EF, Pace NR (1991) Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J Bacteriol 173:4371–4378
29. Skinner FA, Jones PCT, Mollison JE (1952) A comparison of direct and plate counting techniques for the quantitative estimation of soil microorganisms. J Gen Microbiol 6: 261–271
30. Stephen JR, Kowalchuk GA, Bruns MA, McCaig AE, Phillips CJ, Prosser JI, Embley TM (1998) Analysis of β-subgroup ammonia oxidiser populations in soil by DGGE analysis and hierarchical phylogenetic probing. Appl Environ Microbiol 64:2958–65
31. Stephen JR, McCaig AE, Smith Z, Prosser JI, Embley TM (1996) Molecular diversity of soil and marine 16S rDNA sequences related to β-subgroup ammonia oxidising bacteria. Appl Environ Microbiol 62:4147–4154
32. Strunk O, Ludwig W (1999) ARB Computer program distributed by the Technical University Munich, Munich, Germany
33. Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62:625–630
34. Torsvik V, Goksoyr J, Daae FL (1990) High diversity in DNA of soil bacteria. Appl Environ Microbiol 56:782–787
35. Tunlid A, White DC (1992) Biochemical analysis of biomass, community structure, nutritional status and metabolic activity of the microbial community in soil. In: Bollag J-M, Stotzky G (eds) Soil Biochemistry, Vol 7. Marcel Dekker Inc, New York, pp 229–262
36. Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Bio Techniques 10:506–513
37. White DC, Ringelberg DB, Hedrick DB, Nivens DE (1993) Rapid identification of microbes from clinical and environmental matrices by mass spectrometry. In: Fenselau C (ed) Identification of Microorganisms by Mass Spectrometry, American Chemical Society Symposium Series 541. American Chemical Society, Washington DC, pp 8–17
38. White DC (1983) Analysis of microorganisms in terms of quantity and activity in natural environments. In: Slater JH, Whittenbury R, Wimpenny JWT (eds) Microbes in Their Natural Environments, Society for General Microbiology Vol 34. Cambridge University Press, London, pp 37–66