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Differences between Betaproteobacterial Ammonia-Oxidizing Communities in Marine Sediments and Those in Overlying Water

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To assess links between betaproteobacterial ammonia-oxidizing bacteria (AOB) in marine sediment and in overlying water, communities in Loch Duich, Scotland, were characterized by analysis of clone libraries and denaturant gradient gel electrophoresis of 16S rRNA gene fragments. *Nitrosospira* cluster 1-like sequences were isolated from both environments, but different sequence types dominated water and sediment samples. Detailed phylogenetic analysis of marine *Nitrosospira* cluster 1-like sequences in Loch Duich and surrounding regions suggests the existence of at least two different phylogenetic subgroups, potentially indicative of new lineages within the betaproteobacterial AOB, representing different marine ecotypes.

Lithoautotrophic nitrification, the microbial conversion of NH_4^+ to NO_3^-, is central to biogeochemical transformation of nitrogen and in well-mixed waters of coastal marine environments is traditionally associated with open oxic waters and the uppermost oxic benthic sediment (19), where high ammonia concentrations are sustained by sedimentation of particulate organic nitrogen. In benthic sediments, oxygen rarely penetrates more than a few millimeters and nitrification is assumed to be negligible. Ammonia-oxidizing bacteria (AOB) perform the first, often rate-limiting step of nitrification, the oxidation of ammonia to nitrite, and 16S rRNA and *amoA* gene sequences associated with betaproteobacterial AOB have been detected in marine sediments and in open waters. *Nitrosospira* cluster 1-like (1, 5, 14) and *Nitrosomonas* cluster 5- (1, 5) and 7-like (1, 14) sequences have been found in Arctic, Antarctic Ocean, and Mediterranean waters. In addition, identical or closely related *Nitrosospira*-like (3, 7, 10, 13) and *Nitrosomonas*-like (10) sequences have been isolated from Pacific Northwest, Scottish, and Netherland coastal sediments. These findings suggest ubiquity of *Nitrosospira* cluster 1-like AOB in marine environments, and published phylogenies indicate close evolutionary relationships of sequences present in open waters and sediments (2, 3, 5), whereas the contrasting characteristics of these two environments might be expected to select for different ecotypes, with adaptation to different ammonia concentrations and oxygen tensions. Previously (3), we reported *Nitrosospira* cluster 1-like sequences within anoxic sediments of a marine loch, Loch Duich, suggesting that they represented AOB adapted to this environment and therefore likely to be less abundant in the overlying water. An alternative hypothesis is that these sequences are derived from settling and sedimentation of cells from the water column. To test these hypotheses, we have analyzed 16S rRNA gene sequences, amplified from nucleic acids extracted from water and sediment samples at Loch Duich, by sequence analysis of clone libraries and denaturing gradient gel electrophoresis (DGGE) and assessed the evolutionary relationships of sequences obtained from sediment and water samples by detailed phylogenetic analysis.

Sample location and collection. Surface sediment samples were retrieved in July 2001 from a water depth of ca. 120 m at Loch Duich (57°15.46′N, 5°30.26′W) and depths of 50 to 90 m at three adjacent sites (Narrows of Raasay, 57°20.19′N, 6°05.13′W; Sound of Raasay, 57°24.57′N, 6°08.26′W; Inner Sound, 57°26.00′N, 6°00.40′W) as described previously (3, 11). Two replicate water samples were collected at the Loch Duich site with a Niskin water sampler at 0-, 5-, 15-, 50-, 70-, and 90-m water depths by filtering 1.5 liters of water through 0.22-μm-pore-size sterile polycarbonate filters (Millipore), which were stored at −20°C until use.

Community analysis of ammonia-oxidizing bacteria. Polycarbonate filters were cut in half, and nucleic acids were extracted separately from each half-filter and from sediments as described by Griffiths et al. (4), with half-filters cut into small pieces prior to disruption of cells. In order to compensate for PCR drift (18) and to include the dominant sequences from different sampling depths, cloning inserts of 1.1 kb were created by pooling several amplicons generated with the βAMO161f-βAMO1301r primer set (9) from Loch Duich water samples taken at different depths. PCR fragments were purified by agarose electrophoresis, excised DNA bands were cleaned using Qiaquick columns (QIAGEN, Hilden, Germany), and clone libraries were constructed with the pGEM T-vector system (Promega Ltd.) and XL1-Blue MRF Kan supercompetent *Escherichia coli* cells (Stratagene, Inc., Cambridge, United Kingdom). To screen sequence inserts, 150 clones containing a 1.1-kb insert were reamplified with the CTO189f-CTO654r primers (7), and products were reamplified with the 357f-GC-518r primer set and examined by DGGE for migration patterns that were identical to those of amplicons from environmental samples. Inserts of clones corresponding

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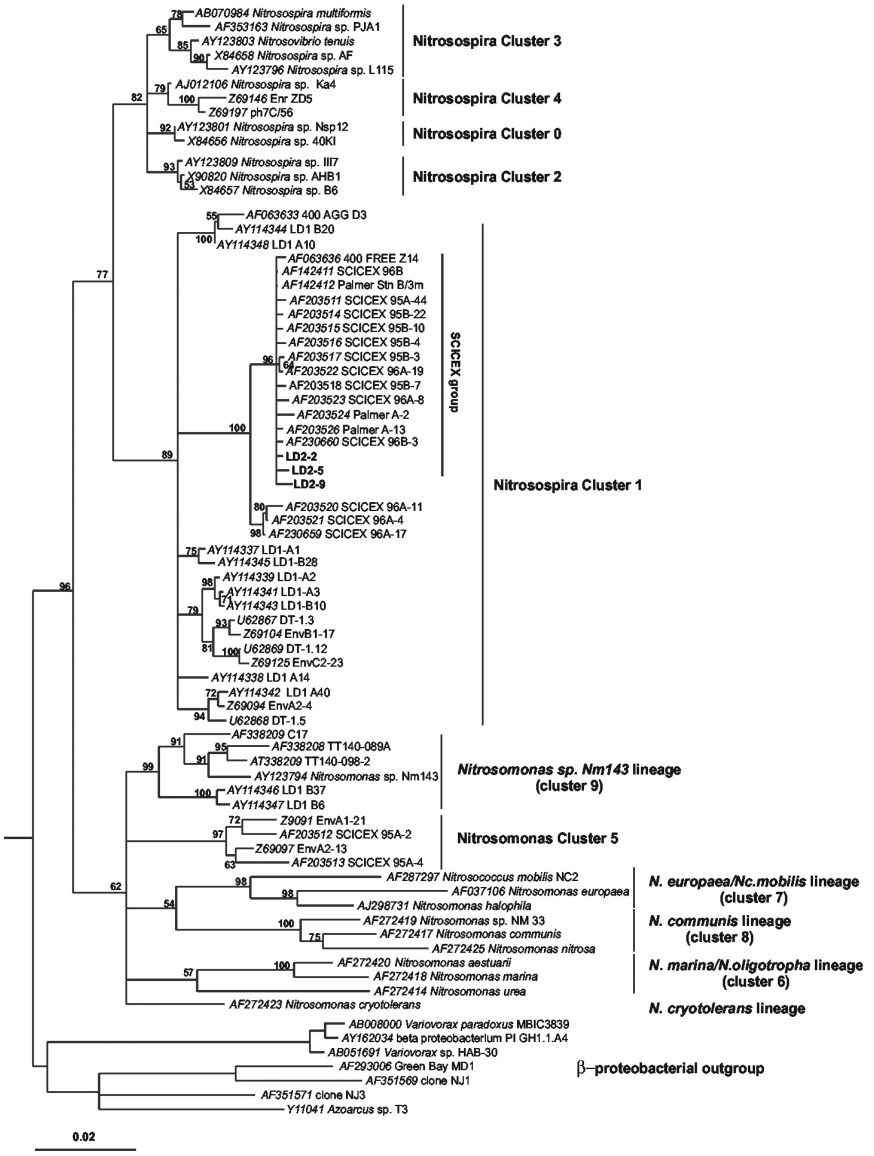

FIG. 1. Evolutionary distance dendrogram showing the positions of environmental 16S rRNA gene 1.1-kb clone sequences recovered from Loch Duich water samples (in bold) in relation to representative members of the betaproteobacterial ammonia oxidizers. The tree is based on results of neighbor-joining analysis of LogDet paralinear distances and maximum-parsimony analysis. The multifurcation connects branches for which a relative order could not be determined unambiguously in the majority of bootstrap resamplings (1,000 replicates) of both treeing methods. Conservative bootstrap values of maximum parsimony or LogDet paralinear distances are indicated. The scale bar indicates an estimated 0.02 change per nucleotide position. Lineage and cluster designation are as suggested by Purkhold et al. (15, 16). GenBank accession numbers are shown in italics. *Nc*., *Nitrosococcus*.

to the dominant bands in DGGE profiles of all water samples were sequenced. Clones of sequences showing identical migration patterns were also selected for replicate sequencing, and clone sequences were aligned with those of excised DGGE bands to determine the degree of sequence identity. 16S rRNA secondary structure of sequences was confirmed manually by alignment with *E. coli* 16S rRNA gene sequence. Clone sequences were aligned to closely related sequences retrieved from the GenBank database by using the BLASTn algorithm (1). Assembly and manual refinements of alignments were carried out by using the Sequencer 4.1 program (Gene Codes Corporation, Ann Arbor, Mich.). Phylogenetic relationships between 16S rRNA gene sequences were calculated as LogDet paralinear distances, as implemented in PAUP 4.0b10 (8), and demonstrated using the neighbor-joining method as the 50% bootstrap consensus tree (1,000 resamplings). Phylogenetic relationships were additionally inferred by maximum parsimony (Phylip 3.62). For DGGE analysis, 16S rRNA genes were amplified by nested PCR with the CTO189f-GC-CTO654r primer set and reamplified with the eubacterial 357f-GC-518r primers (12), and DGGE was carried out as described previously (7). Individual DGGE bands were excised, DNA was eluted and reamplified, and PCR products were analyzed by DGGE to ensure purity and correct migration and sequenced with the

518r primer. Partial clone sequences have been deposited in the GenBank database under accession numbers AY461518 to AY461520.

Sequence analysis of betaproteobacterial ammonia-oxidizing bacteria. Clone libraries of Loch Duich water samples, constructed from 1.1-kb amplicons generated with the semispecific βAMO primer set (9), were dominated (61%) by sequences that incorporated the CTO 16S rRNA gene motif specific for betaproteobacterial AOB (7, 16). Screening of AOB clone sequences by DGGE migration patterns of fragments spanning the highly variable V3 region demonstrated dominance of three different sequence types, two of which (LD2-2 and LD2-5) were retrieved at a high frequency (98%) in nearly equal proportions from the clone libraries. The third sequence (LD2-9) was present in only two clones with identical migration patterns. Sequencing of replicate clones with matching DGGE migration patterns confirmed 100% sequence identity over the entire insert. A BLASTn GenBank database comparison of the clone sequences suggested betaproteobacterial AOB 16S rRNA genes highly similar to *Nitrosospira*-like sequences. Phylogenetic analysis placed the sequences within a clade of sequences which have all been isolated from Arctic and Antarctic Ocean (2, 5) as well as Mediterranean (14) water samples (Fig. 1), forming a deep-branching subgroup of nearly identical sequences (SCICEX subgroup) within a clade of sequences previously described as *Nitrosospira* cluster 1-like sequences. The deep-branching tree topology of the cluster 1 sequences within *Nitrosospira* and of the SCICEX subgroup within the cluster 1 clade was retrieved with both treeing methods applied. In contrast to the deep-branching cluster 1 SCICEX subgroup, which consisted entirely of clone sequences isolated from water samples (2, 5, 14; this study), the other cluster 1 subgroups, except for one sequence isolated from marine aggregates (400 AGG D3 [14]), contained sequences isolated exclusively from marine sediments (3, 7).

Inferring the AOB tree topology based on 1.1-kb 16S ribosomal DNA sequences with maximum parsimony and the generally more conservative LogDet paralinear distance suggested placement of the cluster 1 sequences as a highly diverse additional lineage within the nitrosospiras. This was also indicated by relatively low average similarities (95.8%) between the 1.1-kb 16S rRNA gene sequences of recognized betaproteobacterial AOB representing the main *Nitrosospira* clusters and all cluster 1 clone sequences (Table 1). The close relationships of all other recognized *Nitrosospira* clusters and the consequent traditional grouping of all nitrosospiras into a single lineage (6, 15, 16) are supported by higher average sequence identities (98.3%). The deep-branching tree topology within the cluster 1 clade is also reflected by the low sequence similarities of the cluster 1 subgroups (e.g., 97.3% sequence similarity between SCICEX and EnvB1-17 subgroups), while considerably more variable sites were found when aligning 1.1-kb consensus sequences generated from all cluster 1 sequences than when aligning those from recognized nitrosospiras (96 and 56 variable sites, respectively). 16S rRNA secondary structure analysis of 100%-consensus sequences constructed from recognized *Nitrosospira* and cluster 1 sequences indicated that no single motif was responsible for group discrimination but that distinctive nucleotide substitutions were located throughout all domains, in particular within the more conserved stem areas (e.g., *E. coli* 16S rRNA gene positions 585, 681, 744, 771, 808, 1002, and 1040).

TABLE 1. Similarities of 1.1-kb 16S rRNA gene sequences

Nitrosospira strain	16S rRNA gene sequence similarity (%)		
	LD2-2	LD2-5	LD2-9
Nitrosospira multiformis	95.1	94.7	95.2
Nitrosospira sp. strain Ka4	95.6	95.2	96.0
Nitrosospira sp. strain 10	95.1	94.9	95.0
Nitrosospira sp. strain 12	95.5	95.1	95.6
Nitrosovibrio tenuis	95.0	94.8	95.1
Nitrosospira sp. strain III7	95.5	95.1	95.6
Nitrosospira sp. strain PJA1	94.6	94.2	94.7
Nitrosospira sp. strain AHB1	95.4	95.0	95.6
Nitrosospira sp. strain AF	94.6	94.4	94.7
Nitrosospira sp. strain L115	94.1	93.9	94.2
Nitrosospira sp. strain 40KI	95.2	94.8	95.2
Nitrosospira sp. strain B6	95.0	94.6	95.0
Nitrosospira sp. strain 57	95.9	95.5	96.0
Nitrosospira sp. strain 65	94.9	94.7	94.9

DGGE migration patterns. DGGE profiles of AOB communities in Loch Duich water samples, except for the surface water samples, were dominated by two distinctive bands, with identical profiles in replicate water filter samples with the exception of the surface water samples (Fig. 2). Bands comigrated with clone sequence fragments, indicating sequence identity, which was confirmed by alignment of clone sequences with those generated from excised bands. Sequences of the upper and lower dominant bands were identical with those of the LD2-2 and LD2-5 clones, respectively, discriminated by nucleotide substitutions at only two base positions. Surface water sample 0B contained the LD2-5-associated band but not the LD2-2-associated band, and surface water sample 0A contained an additional band, comigrating with clone sequence LD2-9. DGGE profiles derived from Loch Duich sediment samples (S1 and S2) showed more-complex banding patterns, with at least 10 clearly distinguishable bands and profiles identical to those from samples obtained in the previous year (S/2000) (3). Banding patterns of Loch Duich and Raasay Sound sediment samples (NR, SR, and IS) differed slightly. Some bands that have previously been associated with the new *Nitrosomonas* sp. strain Nm143 lineage (3) were only faintly visible or were absent, and one of the dominant Loch Duich *Nitrosospira* cluster 1 bands (LD1-A3) was present at lower relative intensity. Comparison of clone library and water profiles with Loch Duich sediment profiles indicates that no sequence comigrating with dominant water sequences (LD2-2, LD2-5, and LD2-9) was generated in sediment amplicons.

Differences between DGGE profiles derived from water and sediment samples provided no evidence for links between benthic and pelagic AOB in Loch Duich. Phylogenetic analysis clearly discriminated sequences that were present in DGGE profiles almost exclusively from either water or sediment samples into different phylogenetic groups. These may represent different ecotypes, and the results indicate that pelagic AOB introduced into sediments by sedimentation and mixing processes or sediment AOB brought into the water column by bottom currents or bioturbation may be present only at considerably lower cell numbers than the indigenous AOB. How-

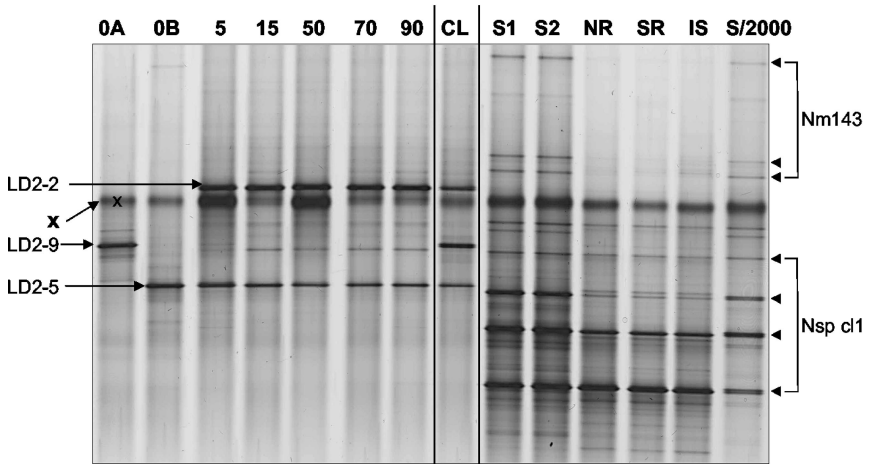

FIG. 2. DGGE analysis of nested 160-bp betaproteobacterial ammonia oxidizer 16S rRNA gene sequences amplified from DNA extracted from Loch Duich water and sediment samples from Loch Duich and neighboring Raasay Sounds. Amplification products of water samples and sediments were obtained using primers CTO189f-GC plus CTO654r (7) nested with 357f-GC and 518r (12) PCR. Clone library (CL) sequences were assembled from βAMO161f plus βAMO1301r amplicons of Loch Duich water samples and reamplified with 357f-GC plus 518r for DGGE analysis. Lanes: 0A to 90, from Loch Duich surface (0A and 0B) and subsurface (depth, 5 to 90 m [lanes 5 to 90]) water samples; S1 and S2, Loch Duich surface sediment samples at 120-m water depth; NR, SR, and IS, Raasay Sounds sediment samples (NR, Narrows of Raasay; SR, Sound of Raasay; IS, Inner Sound); S/2000, Loch Duich surface sediment sample sampled the previous year. Clone sequences shown in lane CL are (from top to bottom) LD2-2, LD2-9, and LD2-5, as marked by arrows (left) indicating comigrating sequences in water samples. Sequences of DGGE bands in sediment samples that had been identified previously (3), as indicated by arrows (right), belonged to the *Nitrosomonas* sp. strain Nm143 lineage (LD1B6, LD1-B37, and LD1-EB5, from top to bottom) or were associated with *Nitrosospira* cluster 1 (LD1-A3, LD1-A2, LD1-A1, and LD1-A10, from top to bottom). X marks an artifact band that generated no sequence when excised and reamplified.

ever, seasonal sampling is required to determine whether sediment sequences reflect AOB present in water samples at different times of the year.

No cultured representative of the cluster 1 sequences has been described, and their association with nitrosospiras and the ability to oxidize ammonia therefore require confirmation. Supportive evidence is provided, however, by the high observed nitrification rates (1.6 μM ml^{-1} day^{-1} [J. Barnes, personal communication]) in Loch Duich sediments, where AOB sequences belong predominantly to cluster 1 (3), and cluster 1 sequence fragments have been isolated from nitrifying enrichment cultures (17). We recently predicted the existence of a new lineage within the nitrosomonads based on analysis of 16S rRNA gene clone sequences (3), and this has now been supported by analysis of a cultured strain (*Nitrosomonas* sp. strain NM143 lineage) (15, 16). The deep-branching tree topology, low 16S rRNA gene similarities, and conserved nature of the base substitutions in the cluster 1 sequence alignments suggest that cluster 1 is evolutionarily distant from the other *Nitrosospira* clusters. It further indicates that it belongs to a clade within the betaproteobacterial AOB that may share a common ancestor with the other *Nitrosospira* clusters but otherwise represents an independent *Nitrosospira* lineage. Additionally, the presence of *Nitrosospira* cluster 1 only in marine environments suggests adaptation to and possible requirement for high salt concentrations within its environment. This adaptation is usually also reflected by high evolutionary distances that justify the classification into independent lineages (e.g., *Nitrosomonas cryotolerans* and *Nitrosomonas marina* lineages) (6). There is a similar degree of discrimination within *Nitrosospira* cluster 1, providing evidence for evolutionarily distant benthic and pelagic *Nitrosospira* cluster 1 ecotypes. This is indicated by the deep-branching tree topology and the phylogenetic distance of the SCICEX subgroup (together with the three additional SCICEX sequences 96A-4, 96A-11, and 96A-17) from sediment *Nitrosospira* cluster 1 sequences. Care must be exercised when proposing divergent phylogenetic groups based solely on clone sequences, but *Nitrosospira* cluster 1 is strongly supported by numerous replicate and highly similar sequences.

In conclusion, geochemical conditions within marine open waters and benthic sediments appear to select for distinct microbial communities of betaproteobacterial AOB consisting of different *Nitrosospira* cluster 1 ecotypes. Additionally, this study provides further evidence for the global distribution of *Nitrosospira* cluster 1 in marine environments, in particular with the SCICEX subgroup (2, 5) in marine waters and in temperate marine sediments in general. The widespread distribution of the *Nitrosospira* cluster 1 organisms in marine environments suggests an important role within the marine nitrogen cycle.

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