APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2002, p. 20–30 Vol. 68, No. 1
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.1.20–30.2002

Grassland Management Regimens Reduce Small-Scale Heterogeneity and Species Diversity of β-Proteobacterial Ammonia Oxidizer Populations

Gordon Webster,[1] T. Martin Embley,[2] and James I. Prosser[1]*

Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD,[1] and Department of Zoology, Natural History Museum, London, SW7 5BD,[2] United Kingdom

Received 18 April 2001/Accepted 9 October 2001

The impact of soil management practices on ammonia oxidizer diversity and spatial heterogeneity was determined in improved (addition of N fertilizer), unimproved (no additions), and semi-improved (intermediate management) grassland pastures at the Sourhope Research Station in Scotland. Ammonia oxidizer diversity within each grassland soil was assessed by PCR amplification of microbial community DNA with both ammonia oxidizer-specific, 16S rRNA gene (rDNA) and functional, *amoA*, gene primers. PCR products were analysed by denaturing gradient gel electrophoresis, phylogenetic analysis of partial 16S rDNA and *amoA* sequences, and hybridization with ammonia oxidizer-specific oligonucleotide probes. Ammonia oxidizer populations in unimproved soils were more diverse than those in improved soils and were dominated by organisms representing *Nitrosospira* clusters 1 and 3 and *Nitrosomonas* cluster 7 (closely related phylogenetically to *Nitrosomonas europaea*). Improved soils were only dominated by *Nitrosospira* cluster 3 and *Nitrosomonas* cluster 7. These differences were also reflected in functional gene (*amoA*) diversity, with *amoA* gene sequences of both *Nitrosomonas* and *Nitrosospira* species detected. Replicate 0.5-g samples of unimproved soil demonstrated significant spatial heterogeneity in 16S rDNA-defined ammonia oxidizer clusters, which was reflected in heterogeneity in ammonium concentration and pH. Heterogeneity in soil characteristics and ammonia oxidizer diversity were lower in improved soils. The results therefore demonstrate significant effects of soil management on diversity and heterogeneity of ammonia oxidizer populations that are related to similar changes in relevant soil characteristics.

There is increasing concern that anthropogenic activities influence the diversity and community structure of natural microbial populations. This concern has focused mainly on the consequences of pollution, for example, global warming, contamination with recalcitrant organic compounds, and the effects of heavy metals. Different forms of land use and management regimens are also likely to exert effects on microbial community structure and, as a consequence, changes may arise which could result in a reduction in species diversity. This, in turn, may influence ecosystem function and the ability to respond to future perturbations in environmental conditions. There is evidence in some systems for maintenance of community structure following perturbations (21), particularly where populations are stabilized by attachment to particulate material. However, in more open, continuous flow systems, the relative abundances of different bacterial and archaeal groups are more susceptible to considerable fluctuation, although ecosystem function may be maintained (6).

Investigation of microbial diversity in natural communities has been made possible by the development of molecular fingerprinting techniques, in particular those based on analysis of 16S rRNA gene sequences, but the majority of studies have assessed "total" diversity, using primers which amplify 16S rRNA genes from bacterial and archaeal domains. The considerable diversity within these groups makes detection of treatment effects difficult (23) and analysis of narrower groups has been proposed (30).

Nitrification, the conversion of ammonium to nitrate via nitrite, is critical to the cycling of nitrogen in terrestrial environments, increasing nitrogen losses through leaching and denitrification of nitrate (34). This process is carried out in most ecosystems by autotrophic bacteria and is often rate limited by the activities of ammonia-oxidizing bacteria that are responsible for the oxidation of ammonia to nitrite by the enzyme ammonia monooxygenase. Ammonia oxidizers are therefore likely to be influenced by traditional grassland management regimes, such as N fertilization and soil liming, and populations of these organisms increase within managed environments (2, 20, 26).

Despite their significance in a range of environments, the slow growth and low yield of ammonia oxidizers observed during laboratory cultivation have restricted physiological and ecological studies on these organisms (34, 38). However, the application of 16S ribosomal DNA (rDNA)-based techniques circumvents many of these problems and has enabled ecological studies of ammonia oxidizer populations within a range of environments (22, 24, 27, 39, 40, 41). Comparative 16S ribosomal gene sequencing analysis has placed autotrophic ammonia oxidizers into two distinct monophyletic groups (8, 44). The first group, characterized by a limited number of marine nitrosococci (15, 49), belongs to the γ-subclass of the *Proteobacteria*. The second group, within the β-proteobacteria, includes

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, United Kingdom. Phone: 44-1224-273148. Fax: 44-1224-273144. E-mail: j.prosser@abdn.ac.uk.

most of the known isolates and contains the genera *Nitrosomonas* and *Nitrosospira* (8). Further molecular analysis, based on 16S rDNA sequences of natural populations of β-proteobacterial ammonia oxidizers, from a range of different environments, has demonstrated the existence of at least seven sequence clusters (*Nitrosospira* clusters 1 to 4; *Nitrosomonas* clusters 5 to 7) (39). The presence and relative abundance of different clusters appear to be related to environmental factors (16, 24, 32, 39, 40), but the physiological basis for the observed differences is unknown. For example, sequences representative of *Nitrosospira* cluster 2 have been found in greater relative abundance in acid agricultural soils, while cluster 3 sequences appear to be more common in neutral agricultural soil, suggesting the possibility that cluster 2 organisms are, in some way, adapted to growth at low pH. Such differences in community structure may explain the occurrence of nitrification in acid soils (3, 4, 47), while autotrophic nitrification in liquid culture rarely occurs below pH 7 (1).

The availability of techniques for analysis of natural populations of ammonia-oxidizing bacteria, their importance in biogeochemical cycling, and the potential effects of N fertilizer applications on community structure provide an ideal system for assessing the effects of anthropogenic activities on microbial diversity and heterogeneity. This was facilitated by the availability at the chosen study site of soils from three different types of British upland grass pasture: improved, semi-improved, and unimproved grasslands (37). These grasslands differ with respect to different management practices, including sowing of different grass species, N fertilization, and sheep grazing, operated over a period of 30 years. N fertilization, in particular, is likely to influence the diversity and community structure of ammonia-oxidizing bacteria. Ammonia oxidizer populations within the different soils were therefore investigated by denaturing gradient gel electrophoresis (DGGE), probe hybridization, and phylogenetic clustering of 16S rRNA and *amoA* genes (encoding the α-subunit of ammonia monooxygenase) amplified directly from extracted DNA.

MATERIALS AND METHODS

Sample site and soil collection. Soil samples were taken on 25 August 1998 and 11 May 1999 from three characteristic grassland soil sites, designated unimproved (U4a), semi-improved (U4b), and improved (MG6) grasslands by the National Vegetation Classification (37), at the Fasset Hill site (ca. 350 m above sea level), Sourhope Research Station near Kelso, Scotland, United Kingdom (map reference NT 850 205). The unimproved field plots represent natural grassland, dominated by *Festuca ovina*, *Agrostis capillaris*, and *Galium saxatile*, with no fertilizer treatment and grazed by sheep. The improved plots were *Festuca*/*Agrostis* grassland reseeded with *Lolium perenne* (perennial ryegrass) and *Trifolium repens* (white clover) between 1970 and 1985 and have received 140 kg of N ha^{-1} y^{-1} since 1990, as 50 kg of N ha^{-1} of NH_4NO_3 in March and August and 40 kg N ha^{-1} in an N, P, and K fertilizer compound (39:20:20) in May. MG6 plots were also grazed by sheep in the summer and autumn months. The semi-improved plots are essentially unimproved *Festuca*/*Agrostis* grassland with *Holcus lanatus* and *T. repens* subcommunities and receive no fertilization.

At each sampling site, three 5-by-5-m quadrats (plots) were randomly located, and five soil cores (diameter, 10 cm; depth, 5 cm) from each replicate plot were collected, pooled, and sieved (mesh size, <2 mm) to remove stones and plant material. Fresh, sieved soil was used for measurement of pH, nitrification potential, and other soil characteristics. Subsamples (5 to 10 g) of sieved soil were stored at −70°C until required for molecular analyses.

Chemical analyses. pH was measured in 1:2 (wt/vol) suspensions of soil in either 0.01 M $CaCl_2$ or distilled water. The soil water content was determined as weight loss after drying for 24 h at 105°C. Soil organic matter was determined as loss on ignition at 430°C for 24 h. Total N and total C measurements were undertaken on oven-dried soil (48) by using an automated Fison NA 1500 NCS Analyser mass spectrophotometer (Elemental Microanalysis Ltd., Oakhampton, United Kingdom). Exchangeable ammonium and nitrate were determined after 1:4 (wt/vol) extraction of soil with 2 M KCl. Ammonia and nitrate concentrations were measured colorimetrically by using an Alpkem RFA Autoanalyser (Alpkem Corporation, Clackamas, Oreg.). Soil nitrification potential was measured over 7 days by the shaken soil-slurry method (7) with an initial ammonium sulfate concentration of 50 mM and regular sampling for analysis of the nitrite and nitrate concentrations. Statistical analysis by one-way analysis of variance was undertaken on triplicate samples of each replicate plot by using the Fisher exact test.

DNA extraction. DNA was extracted from 0.5 g of soil, and samples were disrupted with a Hybaid Ribolyser Cell Disrupter (Hybaid, Ltd., Ashford, United Kingdom) (speed 4, 10 s; two times). Extractions were carried out in 2-ml screw-cap Blue Matrix Ribolyser tubes containing a mixture of ceramic and silica beads (Hybaid), 300 μl of 0.12 M sodium phosphate buffer (pH 8.0), 500 μl of Tris-buffered phenol (Fisher Scientific U.K., Loughborough, United Kingdom), and 200 μl of 1 M Tris-HCl (pH 8). After centrifugation at 13,000 rpm for 10 min, the supernatant was transferred to a 1.5-ml Eppendorf tube and reextracted with an equal volume of Tris-buffered phenol, followed by an equal volume of chloroform. The upper aqueous phase was dialyzed, to concentrate the DNA and remove humic acids by using a Microcon YM-100 centrifugal filter device (Millipore Corporation, Bedford, Mass.) with four changes of 100 μl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8). Concentrated DNA was resuspended in 50 μl of TE buffer before being further purified by standard low-melting-point agarose (1.0% [wt/vol]) gel electrophoresis with λ DNA *Eco*RI/*Hin*dIII markers (NBL Gene Sciences, Cramlington, United Kingdom). High-molecular-weight DNA (>10 kb) was removed and subsequently cleaned by using a Bio-Rad Freeze and Squeeze spin column (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene fragments. Extracted DNA was amplified by nested PCR by using the ammonia oxidizer specific, 16S rDNA primer sets βAMOf-βAMOr (22) and CTO189f-CTO654r (16) and the *amoA* specific primer sets amoA-2F (AARGCGGCSAAGATGCCGCC)–amoA-5R (TTATT TGATCCCCTC) and amoA-3F (ACCTACCACATGCACTT)–amoA-4R (GG GTAGTGYGACCACCAGTA), supplied by Karl-Paul Witzel, Max-Planck-Institut für Limnologie, Plön, Germany. The *amoA* primers 2F and 5R bind at positions 279 to 298 and positions 1065 to 1079 of the *amoA* gene sequence of *Nitrosomonas europaea* (25) amplifying a 800-bp fragment (positions 31 to 50 and 817 to 831 of the open reading frame). The 3F and 4R primers bind at positions 369 to 385 and 588 to 607 (positions 121 to 137 and 339 to 358 of the open reading frame), respectively, generating a 238-bp fragment. The primers and the PCR conditions for each primer pair are summarized in Table 1. PCR buffer (Bioline, London, United Kingdom), 1 U of Biotaq DNA polymerase (Bioline), 1 μl of each primer (final concentrations of 0.1 [*amoA*] and 0.4 pmol [16S rDNA] μl^{-1}), each deoxynucleoside triphosphate (Bioline) at a concentration of 250 μM, and 1 μl of template DNA were added to each 50-μl (total volume) reaction mixture in a 0.5-ml test tube stored on ice. Amplifications were started by placing cooled tubes into a preheated (94°C) Omn-E thermal cycler (Hybaid Ltd.), followed by the thermal cycle conditions shown in Table 1. PCR products (5 μl) were checked by standard agarose (1.2% [wt/vol]) gel electrophoresis with visualization of DNA by ethidium bromide fluorescence and Bioline HyperLadder DNA markers (Bioline) prior to analysis by DGGE. PCR amplification was also undertaken on representative 16S rDNA clones or pure cultures for each currently recognized cluster of β-subgroup ammonia oxidizers (39), for use as reference sequences for 16S rRNA gene DGGE analysis, as follows: EnvB1-8 (*Nitrosospira* cluster 1), pH4.2A/27 (*Nitrosospira* cluster 2), pH4.2A/4 (*Nitrosospira* cluster 3), pH7B/C3 (*Nitrosospira* cluster 4), EnvA1-21 (*Nitrosomonas* cluster 5), EnvC1-19 (*Nitrosomonas* cluster 6), and *N. europaea* (*Nitrosomonas* cluster 7). For *amoA* gene DGGE analysis, the following pure cultures of ammonia oxidizing bacteria were used as references: *Nitrosospira* sp. strain B6 (cluster 2), *Nitrosospira* sp. strain AV (cluster 3), *Nitrosospira* sp. strain 40KI (cluster 4), *N. europaea* (cluster 7), and *N. eutropha* (cluster 7).

DGGE analysis. DGGE was carried out as described previously (16) with modifications. PCR products (ca. 200 ng of each product) were separated using a DCode Universal Mutation Detection System (Bio-Rad Laboratories) and 1 mm-thick (16-by-16-cm glass plates) polyacrylamide gels made from a 6 to 12% (wt/vol) polyacrylamide gradient (Acrylogel 2.6 solution; acrylamide–*N,N′*-methylenebisacrylamide [37:1]; BDH Laboratory Supplies, Poole, United Kingdom) and a denaturant gradient between 25 and 75% for *amoA* analysis and between 35 and 50% for 16S rDNA (100% denaturant is defined as 7 M urea with 40% [vol/vol] formamide). Gels were poured with the aid of a Model 475 gradient delivery system (Bio-Rad Laboratories) and prepared with, and electrophoresed

TABLE 1. Summary of phylogenetic primers, functional primers, and PCR conditions used in this study

Primer set[a] (fragment length)	Target gene	Nested-PCR amplification stage	Thermocycling program
βAMOf–βAMOr (1.1 kb)	16S rRNA	Primary	5 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C; followed by a 5-min final extension at 72°C
CTO189f–CTO654r (465 bp)	16S rRNA	Secondary	2 min at 94°C; followed by 15 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C; followed by 20 cycles of 30 s at 92°C, 30 s at 55°C, and 45 s at 72°C; and 5-min final extension at 72°C
amoA-2F–amoA-5R (800 bp)	*amoA*	Primary	5 min at 94°C; followed by 20 cycles of 40 s at 94°C, 50 s at 55 to 45°C (touchdown), and 60 s at 72°C; followed by 15 cycles of 40 s at 94°C, 50 s at 45°C, and 60 s at 72°C; and 7-min final extension at 72°C
amoA-3F–amoA-4R (238 bp)	*amoA*	Secondary	5 min at 94°C; followed by 20 cycles of 40 s at 94°C, 50 s at 55 to 45°C (touchdown), and 60 s at 72°C; followed by 15 cycles of 40 s at 94°C, 50 s at 45°C, and 60 s at 72°C; and 7-min final extension at 72°C

[a] Primer CTO189f had the GC clamp CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGG (16), and primer amoA-4R had the GC clamp CGCCCGC CGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (29).

in, 1× TAE buffer (pH 8; 40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) and run at 60°C for 4 h (*amoA*) or 5 h (16S rDNA) at 200 V. Polyacrylamide gels were stained in ethidium bromide for 20 min and destained for 5 min in 1× TAE buffer. The UV gel image was then captured by using the UVP ImageStore 5000 (Ultra Violet Products, San Gabriel, Calif.).

Recovery and sequence analysis of bands from DGGE gels. Samples of individual DGGE bands were reamplified with 1 μl as a template from a suspension of the excised band resuspended in 20 μl of sterile distilled water. Reamplified PCR products of excised DGGE bands were purified by dialysis with sterile distilled water by using a Microcon YM-100 centrifugal filter device (Millipore). The purified PCR product was then resuspended in about 20 μl of sterile distilled water to give a final concentration of DNA of between 15 and 20 ng μl^{-1} as determined by standard agarose (1.2% [wt/vol]) gel electrophoresis and Bioline HyperLadder DNA markers (Bioline). Sequencing reactions were performed by using the BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Warrington, United Kingdom) with either CTO189f or amoA-2F primer, and the cycle sequencing products were analyzed with a Model ABI377 automated sequencer (PE Biosystems).

Sequence chromatographs were analyzed by using the Chromas software package (version 1.42; C. McCarthy, Griffith University, Brisbane, Queensland, Australia). Partial nucleotide sequences (272 bp for 16S rDNA and 186 bp for *amoA*) were aligned manually by using GDE version 2.2 running in ARB (Technical University of Munich, Munich, Germany) with sequences retrieved from the GenBank database (*amoA* sequences) or from ammonia oxidizer 16S ribosomal sequence databases obtained from previous studies (24, 32, 39).

Phylogenetic relationships between pairs of 16S rDNA sequences were calculated by using both distance and maximum parsimony. For distance matrix analyses, LogDet/Paralinear distances (17) of variable sites (18) was used and implemented in PAUP version 4.0b4a (43). The maximum-likelihood method was used to estimate the proportion of variable sites (10). All LogDet/Paralinear distances trees were constructed by using the minimum evolution criterion, and the data were bootstrapped 100 times.

Ammonia monooxygenase (*amoA*) nucleotide sequences were analyzed as described above by using LogDet/Paralinear distances of variable sites. In addition, LogDet/Paralinear distances were carried out with codon position 3 excluded from the phylogenetic analysis in order to compensate for potential mutational saturation and nucleotide base composition heterogeneity (11). Trees were constructed as described above, and a 50% majority rule consensus tree was calculated. Base composition data were calculated by using the function in PAUP version 4.0b4a.

Probe labeling, DNA transfer, and hybridization. Cluster-specific and genus-specific ammonia oxidizer oligonucleotide probes (40) were 3′ end labeled by using terminal transferase (50 U) in the supplied buffer with 0.05 mM digoxigenin-11-ddUTP (DIG label) at 37°C according to the manufacturer's instructions (Roche Diagnostics Ltd., Lewes, UK). DIG-labeled probes were reoptimized for specificity by using membrane-bound target ammonia oxidizer sequences and checking probe hybridization or cross-reactivity over a range of hybridization and washing temperatures. The optimized hybridization temperatures and number of mismatches to target and nontarget sequences for the DIG-labeled probes are summarized in Table 2.

DNA was transferred from DGGE gels to nylon hybridization membranes (Hybond N+; Amersham Pharmacia Biotech Ltd., Little Chalfont, Bucks, United Kingdom) with the aid of a Panther semidry electroblotter (Owl Scientific, Inc., Woburn, Mass.) according to the manufacturer's instructions, with transfer medium of 1× TAE buffer and run at 10 V for 1.5 h. After transfer, DNA was simultaneously denatured and covalently cross-linked to the membrane by incubation on a pad of 3MM filter paper (Whatman International, Ltd., Maidstone, United Kingdom) soaked in 0.4 M NaOH for 20 min, followed by washing in 5× SSC (0.75 M NaCl plus 75 mM sodium citrate; pH 7) for 2 min. Membranes were then air dried and stored at room temperature until required for hybridization.

Prehybridization and hybridization were carried out in standard hybridization buffer (5× SSC, 0.1% [wt/vol] *N*-lauroylsarcosine, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 1% [wt/vol] blocking reagent [Roche Diagnostics, Ltd.]) at the temperatures shown in Table 2 in a Hybaid Shake 'n' Stack hybridization oven (Hybaid, Ltd.). Membranes were washed free of unbound and nonspecifically bound probe by washing them in 2× SSC with 0.1% (wt/vol) SDS at room temperature (2 times; 5 min), followed by two stringency washes for 20 min each at the hybridization temperature with 0.5× SSC and 0.1% (wt/vol) SDS.

Chemiluminescent detection of the hybridised probe was carried out by first equilibrating the membrane for 1 min in maleic acid buffer (100 mM maleic acid; 150 mM NaCl; pH 7.5, 0.3% [vol/vol] Tween 20) before blocking for 30 to 60 min

TABLE 2. Oligonucleotide probes, hybridization oven temperatures, and the number of mismatches to both target and nontarget sequences for the DIG-labeled probes for analysis of ammonia oxidizer 16S rDNA fragments

Probe[a]	Target group	Oven temp (°C)	No. of mismatches to ammonia oxidizer sequences						
			EnvB1-8 (cluster 1)	pH4.2A/27 (cluster 2)	pH4.2/4 (cluster 3)	pH7B/C3 (cluster 4)	EnvA1-21 (cluster 5)	EnvC1-19 (cluster 6)	*N. europaea* (cluster 7)
β-AO233	All β-ammonia oxidizers	50.5	0	0	0	0	0	0	0
NspCL1_249	*Nitrosospira* cluster 1	48	0	3	3	3	3	4	3
NspCL2_458	*Nitrosospira* cluster 2	48	4	0	2	4	6	12	6
NspCL3_454	*Nitrosospira* cluster 3	57	3	2	0	4	4	9	10
NspCL4_446	*Nitrosospira* cluster 4	49.5	1	2	3	0	3	7	6
Nmo254a	All *Nitrosomonas* spp.	51.5	2	1	1	1	0	0	0

[a] The probes were designed by Stephen et al. (40).

TABLE 3. Characteristics of grassland soils at the time of sampling (August 1998)

Sample site	Soil type[b]	Mean (SD)[a]						
		pH[c]	Moisture content (%)	Organic matter by loss on ignition (%)	Total C (%)	Total N (%)	C/N ratio	Nitrification potential (μg of NO_3–N g [dry wt] of $soil^{-1}$ h^{-1})[d]
U4a	Brown podzol	3.35 (0.04)[1]	51.3 (1.2)[1]	28.7 (1.8)[1]	13.0 (3.7)[1]	0.87 (0.21)[1]	14.8 (0.50)[1]	0.70 (0.04)[1]
U4b	Humic brown	3.98 (0.19)[1]	34.2 (4.5)[2]	25.0 (4.7)[1]	11.4 (1.6)[1]	1.01 (0.11)[1]	11.2 (0.80)[2]	0.49 (0.26)[1]
MG6	Free-draining brown earth	5.35 (0.55)[2]	50.1 (4.5)[1]	27.9 (6.0)[1]	14.4 (3.7)[1]	1.16 (0.26)[1]	12.3 (0.66)[2]	2.24 (0.82)[2]

[a] Data are presented as means and standard deviations from three replicate plots for each grassland site. Values followed by different superscript numbers indicate significant differences between the sites at the 5% level of significance as determined by the Fisher exact test.
[b] Soil description provided by MLURI, Aberdeen, Scotland.
[c] pH of soil suspension in 0.01 M $CaCl_2$.
[d] Nitrification potential assessed over 7 days by using the shaken soil-slurry method (7).

in 1% (wt/vol) blocking reagent in maleic acid buffer. After blocking, the membrane was incubated in a 1:10,000 (vol/vol) dilution of anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Roche Diagnostics, Ltd.) for 30 min. Membranes were washed free of unbound antibody by washing the membrane twice, 15 min per wash, in washing buffer before incubation with a 1:100 (vol/vol) dilution of CSPD chemiluminescent substrate (disodium-3-(4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl)phenyl phosphate) as recommended by the manufacturer. Detection of the chemiluminescent signal was undertaken by exposure of the membrane to X-ray film (Fuji-RX X-ray film) for between 10 min and 1 h at room temperature. Membranes were stripped prior to reprobing by two washes in 0.2 M NaOH with 0.1% (wt/vol) SDS for 10 min each at 37°C, followed by rinsing in 2× SSC.

Nucleotide sequence accession numbers. Accession numbers from the GenBank database of previously determined ammonia oxidizer 16S rRNA and *amoA* gene sequences used in the analyses are as follows. For 16S rDNA the sequences are *N. europaea* (AF037106), *N. eutropha* (M96402), *N. ureae* (Z46993), *Nitrosococcus mobilis* (M96403), *N. marina* (M96400), *Nitrosospira* sp. strain 40KI (X84656), *Nitrosospira* sp. strain D11 (X84660), *Nitrosospira multiformis* (M96401), *Nitrosospira* sp. strain AV (Y10127) *Nitrosospira* sp. strain C-141 (M96397), *N. tenuis* (M96404), *Nitrosospira* sp. strain AHB1 (X90820), *Nitrosospira* sp. strain B6 (X84657), clone EnvA2-13 (Z69097), clone EnvA2-17 (Z69098), clone EnvB2-6 (Z69106), clone EnvC1-18 (Z69117), clone EnvC2-4 (Z69121), clone EnvC2-6 (Z69122), clone pH4.2A/3E (Z69155), clone pH4.2A/F2 (Z69163), clone pH4.2A/G2 (Z69164), clone pH4.2A/K2 (Z69167), clone pH4.2A/L2 (Z69168), clone pH7C/37 (Z69192), clone pH7C/53 (Z69195), enrichment culture E20_pH4/5/UP (AF178104), and enrichment culture E29_pH7/50/UL (AF178112). For *amoA* the sequences are *N. europaea* (L08050), *N. eutropha amoA1* (U51630), *N. eutropha amoA2* (U72670), *Nitrosospira* sp. strain AV *amoA1* (AF032438), *Nitrosospira* sp. strain AV *amoA2* (AF016003), *Nitrosospira* sp. strain AV *amoA3* (U92432), *Nitrosospira tenuis* (U76552), *Nitrosospira multiformis amoA1* (U91603), *N. multiformis amoA2* (U15733), *N. multiformis amoA3* (U89833), *Nitrosospira* sp. strain AHB1 (X90821), *Nitrosospira* sp. strain 39-19 *amoA1* (AF042170), *Nitrosospira* sp. strain 39-19 *amoA2* (AF016002), and *Nitrosospira* sp. strain 39-19 *amoA3* (AF006692).

Sequences of partial *amoA* and 16S rRNA gene fragments of excised DGGE bands have been deposited under accession numbers AJ308604 to AJ308622.

RESULTS

Soil properties. Soils from the three study sites originally developed from locally derived drift from andesitic lavas of Old Red Sandstone and the current differences result from changes in vegetation and management regimes. All soils were high in organic matter, as reflected by their color, texture, and high C and N content. Total organic matter and total C and N did not differ significantly in samples taken from the three grassland sites (Table 3). Despite similar C and N contents, unimproved (U4a) soil had a significantly higher C/N ratio than the semi-improved (U4b) and improved (MG6) soils and was darker. Soil pH (measured in $CaCl_2$ soil suspensions) was significantly higher in the MG6 soil (5.35) than in U4a and U4b soils (3.35 and 3.98, respectively) (Table 3). The mean potential nitrification rate was significantly higher in soil suspensions taken from the MG6 field plots than in soil samples taken from either U4a or U4b (Table 3).

DNA extraction and yield. High amounts of organic material within the grassland soils made it essential, during the DNA extraction procedure, to perform several dialysis "clean up" steps to remove contaminating humic acids. It was also necessary to purify the crude DNA extract further by agarose gel electrophoresis. Purified total soil DNA consisted of high-molecular-weight DNA fragments of about 20 kb, with mean yields of 1.31 to 4.09 μg DNA g (dry weight) of $soil^{-1}$, as estimated by agarose gel electrophoresis and DNA mass and quantification markers.

PCR amplification. DNA was thought to be sufficiently pure for molecular analysis since all DNA samples could be amplified readily with the 16S rDNA eubacterial primer set, 357f/518r (reference 29 and data not shown) However, PCR amplification performed with a single set of primers for either ammonia oxidizer-specific 16S rRNA or *amoA* genes did not consistently yield detectable PCR products. It was therefore necessary to undertake nested PCR, with primer set βAMOf-βAMOr, followed by CTO189f-CTO654r, for 16S rRNA genes and primer set amoA-2F–amoA-5R, followed by amoA-3F–amoA-4R, for *amoA* genes (Table 1). The *amoA* primer sets yielded noticeably lower PCR product concentrations with all DNA templates (ca. 12 ng μl^{-1}), assessed by ethidium bromide fluorescence, compared to yields obtained by the 16S rDNA primer sets (20 to 30 ng μl^{-1}).

16S rDNA DGGE, probe hybridization, and sequence analysis. Initial analysis was carried out on individual samples of soil obtained from replicate plots of the three grassland soil sites at Sourhope. DGGE banding patterns for ammonia oxidizer 16S rRNA genes from these sites were similar (Fig. 1), with the exception of a single U4a replicate [U4a(1)]. PCR products from all sites comigrated with products from cloned standards representing *Nitrosomonas* clusters 6 or 7 (band I) and *Nitrosospira* clusters 2 and 3 or *Nitrosomonas* cluster 5 (band II). U4a(1) generated a single band (band III) that comigrated with the *Nitrosospira* cluster 1 standard. However, comigration of certain bands from different clusters and the presence of multiple bands, which are due to the degeneracy of the reverse PCR primer (16, 24) and possibly the forward primer, prevents unambiguous identification of ammonia oxidizer clusters based on migration patterns alone. It was therefore necessary to excise and reamplify specific DGGE bands of

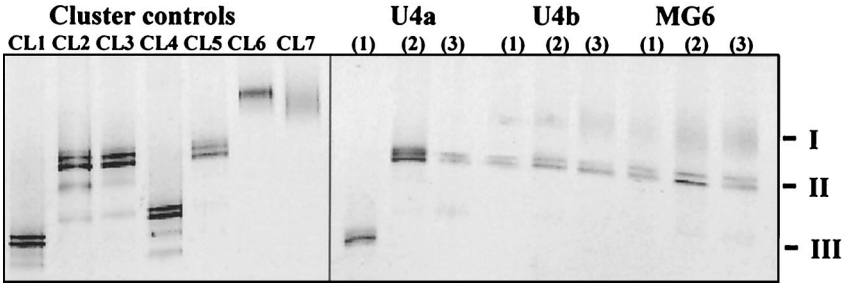

FIG. 1. DGGE analysis of 16S rDNA sequences from Sourhope grassland soil sites with representative cluster controls. Lanes 1 to 7 show β-ammonia oxidizer cluster controls: lane 1, EnvB1-8 (*Nitrosospira* cluster 1); lane 2, pH4.2A/27 (*Nitrosospira* cluster 2); lane 3, pH4.2A/4 (*Nitrosospira* cluster 3); lane 4, pH7B/C3 (*Nitrosospira* cluster 4); lane 5, EnvA1-21 (*Nitrosomonas* cluster 5); lane 6, EnvC1-19 (*Nitrosomonas* cluster 6); and lane 7, *N. europaea* (*Nitrosomonas* cluster 7). Lanes 8 to 16 show Sourhope grassland samples from triplicate plots: unimproved, U4a (lanes 8 to 10); semi-improved, U4b (lanes 11 to 13); and improved, MG6 (14 to 16). The sequences of the individual bands were obtained from the following lanes: band I, lanes 13 and 15, U4b(3) and MG6(2); band II, lanes 9 and 15, U4a(2) and MG6(2); and band III, lane 8, U4a(1)

interest for direct sequencing and phylogenetic analysis (Fig. 2). Previous 16S rRNA gene studies have shown that β-proteobacterial ammonia oxidizers are monophyletic with two genera, which can be split into seven distinct phylogenetic clusters (16, 39, 40). LogDet/Paralinear distances of variable sites produced essentially the same phylogenetic tree topology and cluster designations as previously reported (39), although the *Nitrosospira* cluster 4 in our analysis appeared to be paraphyletic and weakly supported, presumably due to the relatively short sequence used. Phylogenetic analysis confirmed the environmental sequences found in this study to be 16S rDNA of β-proteobacterial ammonia oxidizers and assigned them to *Nitrosomonas* cluster 7 [band I, sequences U4b(3)/I and MG6(2)/I], which is closely related to that of *N. europaea, Nitrosospira* cluster 3 [band II, sequences U4a(2)/II and MG6(2)/II], and *Nitrosospira* cluster 1 from the unimproved grassland site, U4a(1) [band III, sequence U4a(1)/III] (Fig. 2). In addition to sequence analysis, band identity was confirmed by DNA hybridization with specific ammonia oxidizer genus and/or cluster specific probes (40). Samples of the PCR products shown in Fig. 1 were analyzed by dot blot DNA hybridization with ammonia oxidizer cluster-specific probes NspCL2_458 and NspCl3_454 (Table 2). Positive DNA hybridization was only observed with the *Nitrosospira* cluster 3 probe (data not shown) demonstrating the absence of cluster 2 organisms in these samples.

Repeat DGGE analysis of fresh DNA extractions and PCR amplifications (Fig. 3) from the soil samples shown in Fig. 1 resulted in similar banding patterns. However, in addition to the *Nitrosospira* cluster 1 band shown in Fig. 1, plot U4a(1) showed bands that comigrated with *Nitrosospira* clusters 2 or 3 and *Nitrosomonas* clusters 6 or 7 (Fig. 3). These differences in banding patterns suggest greater heterogeneity in ammonia oxidizer populations between individual samples within the unimproved grassland plots than in the improved plots. After transfer of the DNA from the DGGE gel to nylon membranes, all of the bands visible by ethidium bromide fluorescence (Fig. 3a) were detected after hybridization with the general β-subgroup ammonia oxidizer probe, β-AO233 (data not shown). *Nitrosospira* cluster 1 sequences were confirmed in U4a(1) by hybridization with the cluster 1 probe (Fig. 3b). Bands from all samples hybridized with the *Nitrosospira* cluster 3 probe, NspCL3_454, but band intensities varied (Fig. 3c). Band intensities also varied between samples after hybridization with the all *Nitrosomonas* probe, Nmo254a, and U4a(2) and U4b(1) showed no detectable hybridization signal (Fig. 3d). No hybridization signal was observed with the *Nitrosospira* cluster 2 and 4 probes (data not shown). Hybridization therefore confirmed the presence of *Nitrosomonas* spp. and *Nitrosospira* cluster 3 and cluster 1 strains within these samples.

Impact of grassland soil management on within sample heterogeneity of ammonia oxidizer populations. To determine whether nitrogen fertilization and other management strategies influenced the heterogeneity and diversity of ammonia oxidizers within samples, DGGE analysis was undertaken on 16S rDNA PCR products from DNA extracted from replicate 0.5-g soil samples of unimproved and improved sites (Fig. 4). The unimproved site, U4a(1), showed variation in migration banding patterns between replicate 0.5-g samples (Fig. 4A), suggesting heterogeneity. Banding patterns demonstrated the presence of clusters identified in Fig. 1 and 3 (i.e., clusters 1, 2, or 3 and clusters 6 or 7), as well as a band that comigrated with the cluster 4 control [replicate U4a(1) lane D]. In contrast, replicate samples of soil from the improved site, MG6(1), showed lower ammonia oxidizer diversity with a homogeneous banding pattern. All replicates contained only *Nitrosospira* cluster 2 or cluster 3 sequences, generating the same DGGE banding pattern for each 0.5-g replicate (Fig. 4B). To discount the possibility that sequence heterogeneity within replicate 0.5-g samples of the unimproved soil was due to PCR error, replicate PCR reactions were undertaken on single DNA extractions from unimproved and improved soils. Both generated identical banding patterns for repeat PCR reactions (Fig. 5), suggesting that the variation in DGGE banding patterns for replicate samples of the unimproved soil (Fig. 4A) is due to heterogeneity in the ammonia oxidizer population at the 0.5-g sample level.

Probe hybridization of the DGGE gels shown in Fig. 4 confirmed the presence of cluster 3 sequences within four of the replicate samples of U4a(1) (Table 4), while the cluster 1 and cluster 4 probes (NspCL1_249 and NspCL4_446, respectively), each hybridized with only one replicate (Table 4). Hybridization with the all *Nitrosomonas* probe demonstrated that all replicate samples contained *Nitrosomonas* species (Table 4). No hybridization signal was observed with the cluster 2 probe in U4a(I) replicates. Replicate samples from MG6(1) were

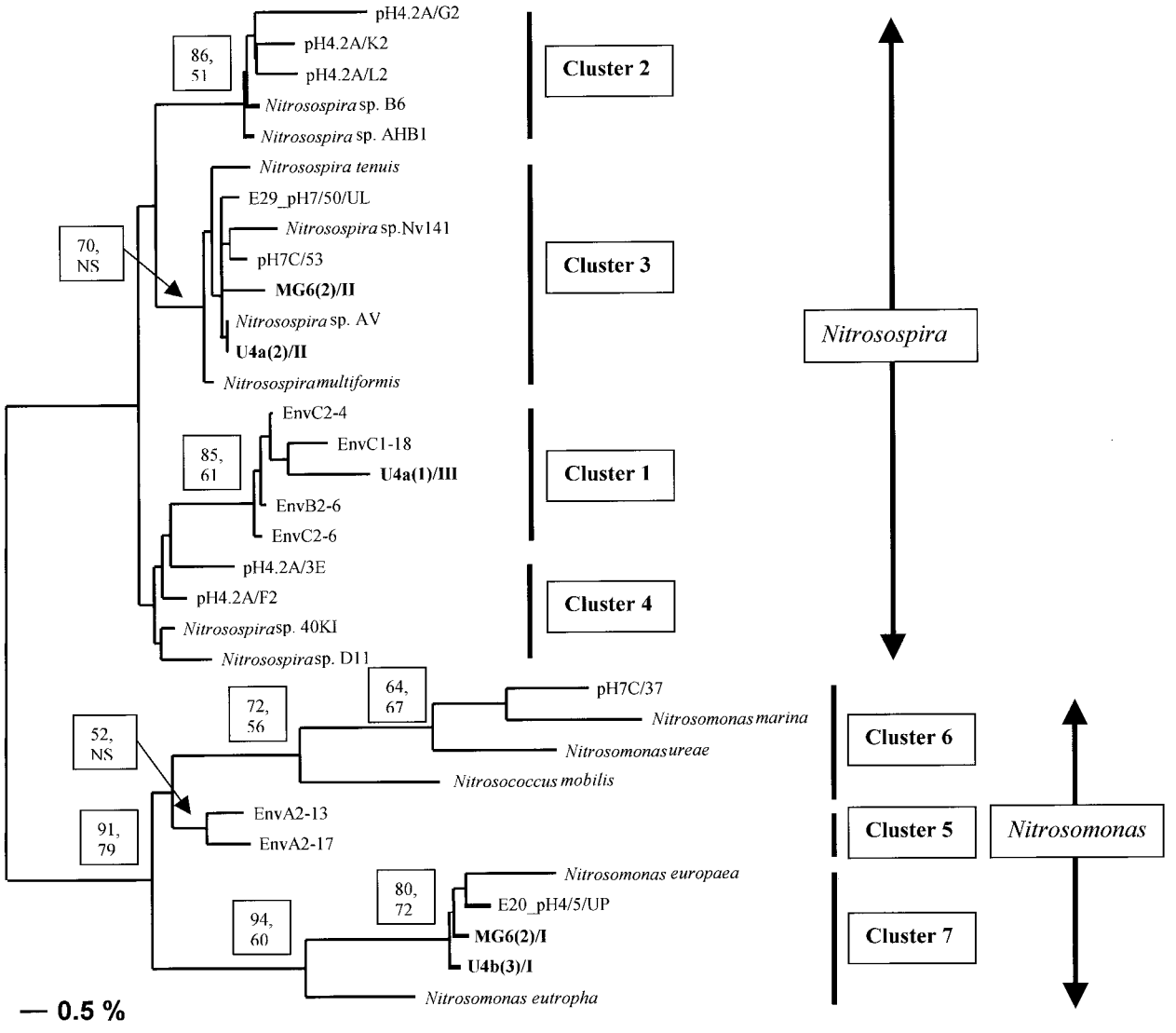

FIG. 2. Phylogenetic tree showing the relationship of the sequences derived from excised DGGE bands to environmental and pure culture ammonia oxidizer sequences based on 272 bases of aligned 16S rRNA gene sequences. Minimum evolution tree derived from LogDet/Paralinear distances of variable sites (estimated value of proportion of invariable sites = 0.659). The tree shown is one of six equally valid trees that showed minor rearrangements within clusters. Bootstrap support values over 50% (100 replicates) are shown in boxes. NS denotes support below 50%. Upper value, bootstrap derived by maximum parsimony; lower value, derived by LogDet/Paralinear distances of variable sites.

confirmed, by probe hybridization, to be less diverse in ammonia oxidizers (Fig. 4B) and contained *Nitrosospira* clusters 2 and 3 organisms but no other clusters (Table 4). These results are of interest in that *Nitrosospira* cluster 2 organisms were not previously identified within MG6(1) samples (Fig. 1 and 3).

***amoA* DGGE and sequence analysis.** After DGGE analysis of *amoA* gene sequences amplified from the three soils, similar banding patterns were found within samples from replicate plots of the improved site (MG6) (Fig. 6) and from replicates of the unimproved site (U4a). However, differences in banding patterns were found between these two sites. In contrast, DGGE banding patterns varied between replicate samples from the semi-improved site (U4b); U4b(3) exhibited a similar banding pattern to U4a, while U4b(1) and U4b(2) showed similarities to patterns observed with MG6. Sufficient PCR products for DGGE analysis were not obtained for site U4a(2), possibly through low numbers of *amoA* genes or inhibition of PCR. These results suggest that the unimproved grassland is more diverse than the improved soil in terms of DGGE banding patterns of *amoA* gene sequences; the semi-improved grassland site shares *amoA* sequences that are similar in their DGGE migration patterns to both MG6 and U4a.

Phylogenetic analysis of the sequences derived from excised *amoA* DGGE bands demonstrated the presence of both *Nitrosospira*- and *Nitrosomonas*-like sequences (Fig. 7). *Nitrosomonas*-like *amoA* sequences were also found that were closely related to the *amoA* sequence of *N. europaea*. Analysis of the nucleotide base composition (Fig. 7) suggests that the phylogeny of *amoA* sequences is complicated by a strong GC bias at codon position 3, since lower base composition values

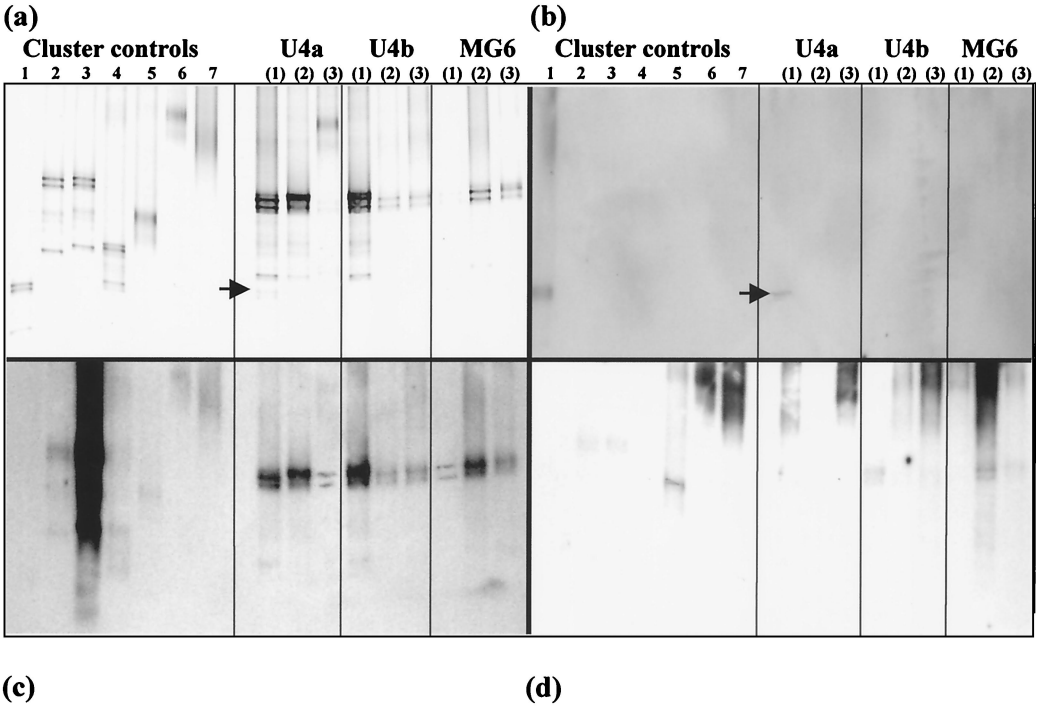

FIG. 3. Repeat DGGE analysis of 16S rDNA sequences from Sourhope grassland soil sites with representative cluster controls and DIG-labeled probing. Lanes 1 to 7 in panels a to d, β-ammonia oxidizer cluster controls (see legend for Fig. 1); lanes 8 to 16, Sourhope grassland samples from triplicate plots: unimproved, U4a (lanes 8 to 10); semi-improved, U4b (lanes 11 to 13); and improved, MG6 (lanes 14 to 16). (a) DGGE gel before membrane transfer and DNA hybridization. (b to d) Membrane hybridized with NspCL1_249 (b), NspCL3_454 (c), and Nmo254a (d).

for GC content were obtained when the third base was excluded (data not shown).

Variability of soil pH and extractable ammonium levels. To assess heterogeneity in soil physicochemical properties within unimproved and managed sites, soil pH and soil NH_4^+−N) were determined in 10 replicate 0.5-g samples from single samples of bulk soil from each site. Levels of extractable NH_4^+−N and soil pH were significantly higher in soil from the improved grassland plot MG6(1) than in the unimproved plot U4a(1) (Table 5). Variability was assessed as the coefficient of variation to standardize greater variability in assays at higher ammonium concentrations, and values were greater within the unimproved than improved soils, suggesting greater heterogeneity in the physicochemical composition of the unimproved grassland soil.

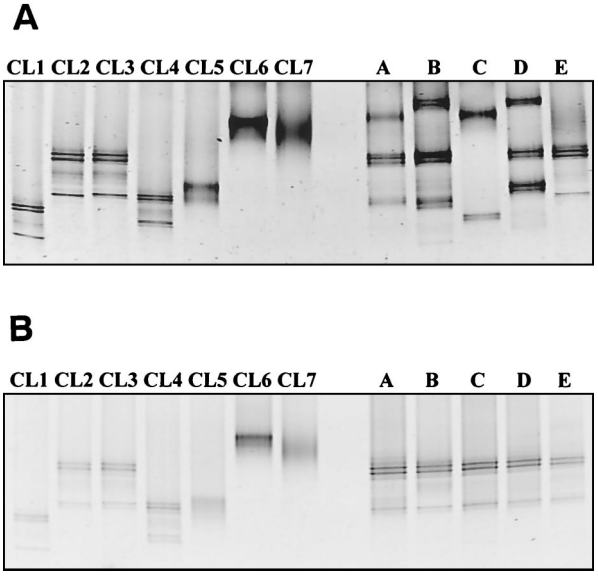

FIG. 4. DGGE analysis of 16S rDNA sequences derived from replicate 0.5-g soil samples taken from the same unimproved U4a(1) (A) and improved MG6(1) (B) grassland sites, with representative cluster controls. Lanes 1 to 7, β-ammonia oxidizer cluster controls (see legend for Fig. 1); lanes 8 to 12, replicate grassland soil samples.

DISCUSSION

Upland grass pastures provide the mainstay for grazing within the United Kingdom and are typified by acid (pH <4.0)

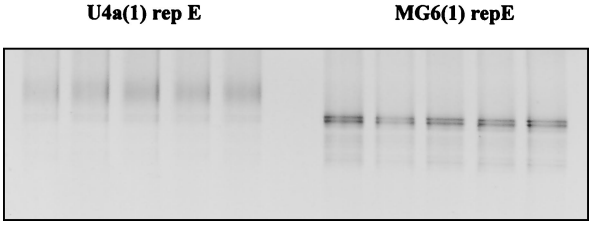

FIG. 5. DGGE analysis of 16S rDNA sequences derived from replicate PCR reactions of the same 0.5-g soil DNA extract. Lanes 1 to 5, replicate PCR reactions from soil DNA sample U4a(1) replicate E; lanes 6 to 10, replicate PCR reactions from soil DNA sample MG6(1) replicate E.

TABLE 4. Identification by probe hybridization of DGGE banding patterns of replicate 0.5-g samples from the same field plot

Sample plot and replicate	Hybridization with probe[a]:				
	NspCL1_249	NspCL2_458	NspCL3_454	NspCL4_446	Nmo254
U4a(1)					
A	−	−	+	−	+
B	−	−	+	−	+
C	+	−	−	−	+
D	−	−	+	+	+
E	−	−	+	−	+
MG6(1)					
A	−	+	−	−	−
B	−	+	−	−	−
C	−	+	+	−	−
D	−	+	+	−	−
E	−	+	−	−	−

[a] +, Positive hybridization signal; −, no hybridization signal observed.

to neutral soils, with most of the higher elevations being acidic and high in organic matter (36). Different management strategies at Sourhope Research Station enabled comparisons of β-subgroup ammonia oxidizer communities in unimproved, semi-improved, and improved grassland soil by analysis of 16S rRNA and *amoA* gene sequences. These pastures differed in their grass species and N input, and the resulting soil differed in its chemical and physical properties.

Unimproved grassland soils had the lowest pH and the lowest net nitrification potential, in comparison with improved soils, which received greater fertilizer input. Nitrate concentrations and most-probable-number estimates of ammonia oxidizers have been shown to be lower in undisturbed soils than in agricultural soils (38). Nitrification may be undetectable in climax communities, such as permanent grasslands (19, 35) and forest soils (12), where nutrient cycling is highly effective. Ammonia oxidizer populations can vary from <500 g^{-1} in acidic soils to ca. 10^5 g^{-1} in agricultural soils (2, 13, 20), and the higher nitrification potential in improved pastures may therefore result from increases in active ammonia oxidizer populations through greater N input.

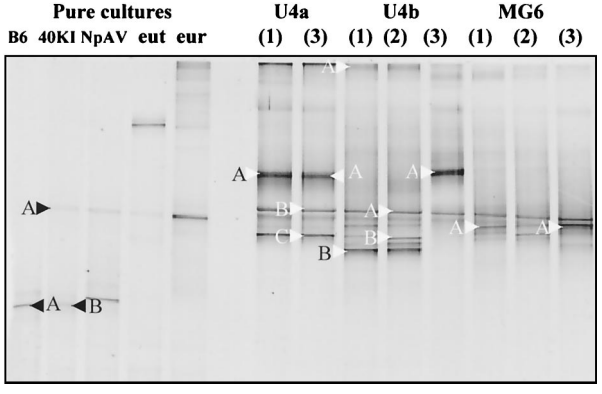

FIG. 6. DGGE analysis of *amoA* gene sequences derived from Sourhope grassland soil sites and ammonia-oxidizing bacteria pure cultures. B6, *Nitrosospira* sp. strain B6; 40KI, *Nitrosospira* sp. strain 40KI; NpAV, *Nitrosospira* sp. strain NpAV; eut, *N. eutropha*; eur, *N. europaea*. Arrows represent bands excised for sequence and phylogenetic analysis.

Amplification of ammonia oxidizer 16S rRNA gene sequences from DNA extracts of Sourhope soil required the use of a nested PCR system, which appears to be essential for samples with low numbers of ammonia oxidizers (9, 32). Nested PCR was also required for amplification of *amoA* gene sequences, and yields of PCR amplified 16S rRNA genes were generally lower in unimproved (20 ng μl^{-1}) than in improved grassland soils (30 ng μl^{-1}), possibly reflecting ammonia oxidizer cell numbers. It is also possible that differences in 16S rDNA yields may be due to differences in the levels of PCR-inhibiting compounds, such as humic acids (31, 45), although PCR amplification with the eubacterial primer set (29) resulted in the same yield of PCR product for both unimproved and improved sites (data not shown).

DGGE analysis (28) of the PCR products of both the 16S rRNA gene and the functional gene, *amoA*, were used to assess ammonia oxidizer species composition. Ammonia oxidizer 16S rDNA sequences from unimproved soils were more diverse, at the cluster level, than those from the improved site, and *Nitrosospira* cluster 1 sequences were only observed within samples from U4a. *Nitrosospira* cluster 1 sequences had previously only been detected in marine environments (24, 32, 39). However, cluster 1 sequences have recently been detected in acid forest soils from Finland and Scotland (T. Aarnio, C. J. Phillips, and J. I. Prosser, unpublished data; G. R. Campbell, unpublished data), although their frequency of occurrence within soil samples appears to be less than in marine environments. *Nitrosospira* cluster 3 and *Nitrosomonas* cluster 7 were present in all grassland types. Pure culture representatives of these clusters have been found in soil enrichments, and cluster 3 sequences often dominate in molecular studies of ammonia oxidizer diversity in soil (2, 9, 26, 33, 39, 40). *Nitrosospira* cluster 3 contains the majority of cultured ammonia oxidizers, while *Nitrosomonas* cluster 7 includes the most-studied species, *N. europaea*. Although, *N. europaea* was isolated from soil, no cluster 7 sequences have been detected in soil samples. However, sequences closely related to the *amoA* sequence of *N. europaea* were found in an agricultural soil that regularly received high levels of N fertilizer (50). We recovered sequences that were closely related to the *amoA* sequence of *N. europaea*, in addition to those related to *Nitrosospira* species. DGGE analysis of *amoA* sequences demonstrated differences in *amoA* gene diversity between unimproved and improved grass pastures, while the semi-improved site shared similarities with both.

We were able to clearly resolve differences between *amoA* sequences at the genus level. Species level relationships were poorly resolved due to a low level of sequence variation, most of which occurred at codon position three. There were marked differences between the base compositions of *amoA* sequences assigned to *Nitrosomonas* (43 to 57% G+C content) and *Nitrosospira* (67 to 83% G+C content), suggesting that they evolved under different selection pressures (14).

Although reported to be dominant in acid agricultural soils (39, 40), *Nitrosospira* cluster 2 sequences were not detected in initial experiments (Fig. 1, 2, and 3), possibly due to low abundance or spatial distribution of the organisms within these soils. However, probe hybridization (Table 4) identified cluster

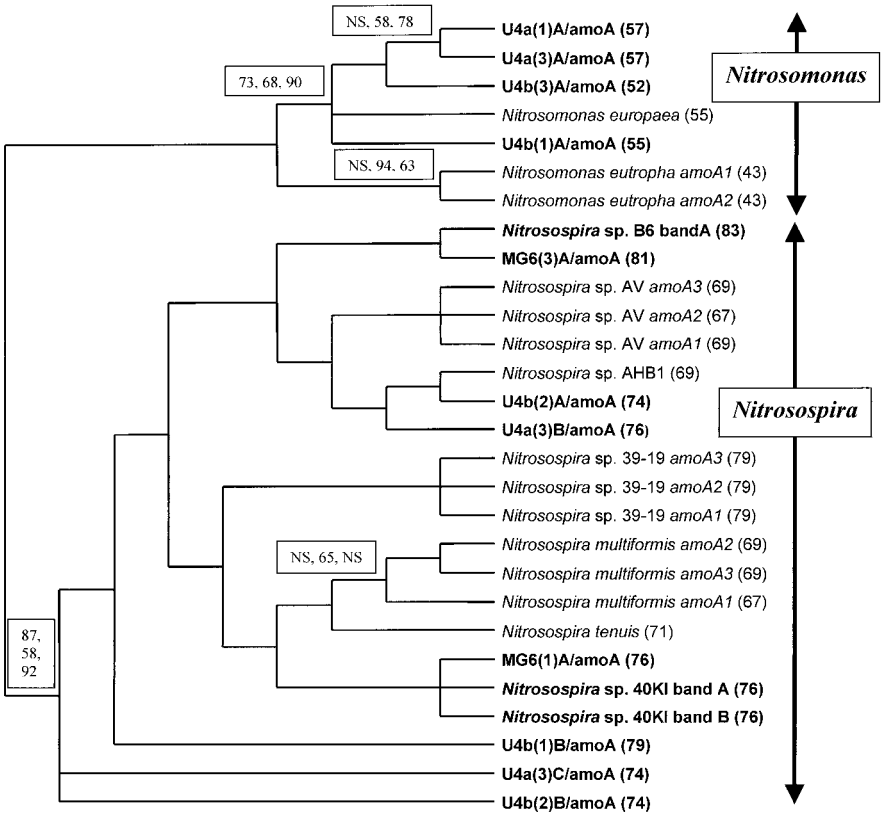

FIG. 7. Phylogenetic tree showing the relationship of the sequences derived from excised DGGE bands to pure culture ammonia oxidizer sequences based on 186 bases of aligned *amoA* gene sequences. A 50% majority-rule consensus tree (of 47 trees) derived by LogDet/Paralinear distances of variable sites (estimated value of proportion of invariable sites = 0.681) is shown. The values in parentheses represent the % G+C content of the variable sites analyzed. Bootstrap support values of >50% (100 replicates) are shown in boxes; NS denotes support below 50%. First value, bootstrap derived by maximum parsimony; second value, derived by LogDet/Paralinear distances of variable sites; third value, bootstrap derived by LogDet/Paralinear distances of variable sites with *amoA* third codon position removed.

2 sequences in DGGE bands from repeat replicate samples of the improved site, MG6(1) but not in repeated samples of U4a(1). *Nitrosospira* cluster 2 strains have been isolated from a sewage treatment works in Norway (46), suggesting physiological diversity within this group and possible selection by high ammonium concentrations.

Repeated DNA extractions, PCR amplifications of 16S rDNA genes, and subsequent analysis by DGGE demonstrated variation in migration patterns between replicate subsamples of unimproved soil, although consistently similar banding patterns were obtained from similar subsamples from the improved site. *Nitrosospira* cluster 4 sequences, not detected in earlier experiments, were also identified in one replicate sample of U4a. These results suggest that heterogeneity and diversity of ammonia oxidizer populations are greater in unimproved soils than in managed soils, possibly through selection for particular groups, as observed for α-*Proteobacteria* at this site (23). The greater diversity of ammonia oxidizer 16S rDNA sequences from unimproved grassland soils may reflect the physical and chemical heterogeneity of these undisturbed soils. Strong et al. (42), using 0.8-g-samples of soil, found substantial heterogeneity between samples, which was not compromised by the sampling scale. We observed, at a similar spatial scale (0.5 g), spatial heterogeneity in both soil pH and NH_4^+−N that was lower in improved than in unimproved soil due to regular additions of nitrogen fertilizer. Both pH and ammonium concentration are important for ammonia oxidizer growth and activity, and heterogeneity in these factors may have driven heterogeneity in ammonia oxidizer populations. Reduced diversity of ammonia oxidizer populations in fertilized soils has also been observed previously by Bruns et al (2). The present study therefore demonstrates a significant impact of soil management on the heterogeneity of soil physicochemical characteristics of relevance to ammonia oxidizer growth

TABLE 5. Heterogeneity in soil pH and ammonium concentration between different 0.5-g soil samples from the same replicate field plot bulk soil sample[a]

Sample plot	pH[b]		Ammonium concn (μg of NH_4^+−N g [dry wt] of $soil^{-1}$)	
	Mean (SD)	CV	Mean (SD)	CV
Unimproved [U4a(1)]	4.01 (0.07)[1]	1.8	11.37 (1.92)[1]	16.9
Improved [MG6(1)]	5.77 (0.04)[2]	0.8	61.55 (2.52)[2]	4.1

[a] Means and standard deviations from 10 replicate 0.5-g samples per measurement. Values followed by different superscript numbers indicate significant differences of at least the $P < 0.05$ level. CV, coefficient of variation.
[b] pH of soil suspension in distilled H_2O.

and on the diversity and heterogeneity of ammonia oxidiser populations.

ACKNOWLEDGMENTS

This study was supported by the UK Natural Environment Research Council EDGE Programme (Award GST/02/1837 to J. I. Prosser and T. M. Embley).

We thank Allison McCaig and Arjen Speksnijder for help with the phylogenetic analysis and Karl-Paul Witzel for the *amoA* primer sets.

REFERENCES

1. **Allison, S. M., and J. I. Prosser.** 1993. Ammonia oxidation at low pH by attached populations of nitrifying bacteria. Soil Biol. Biochem. **25:**935–941.
2. **Bruns, M. A., J. R. Stephen, G. A. Kowalchuk, J. I. Prosser, and E. A. Paul.** 1999. Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appl. Environ. Microbiol. **65:**2994–3000.
3. **De Boer, W., and H. J. Laanbroek.** 1989. Ureolytic nitrification at low pH by *Nitrosospira* species. Arch. Microbiol. **152:**178–181.
4. **De Boer, W., P. J. A. Klein Gunnewiek, and S. R. Troelstra.** 1991. Nitrification at low pH by aggregated chemolithotrophic bacteria. Appl. Environ. Microbiol. **57:**3600–3604.
5. **Felsenstein, J.** 1993. PHYLIP: phylogeny inference package. University of Washington, Seattle.
6. **Fernandez, A., S. Huang, S. Seston, J. Xing, R. Hickey, C. Criddle, and J. M. Tiedje.** 1999. How stable is stable? Function versus community composition. Appl. Environ. Microbiol. **65:**3697–3704.
7. **Hart, S. C., J. M. Stark, E. A. Davidson, and M. K. Firestone.** 1994. Nitrogen mineralization, immobilization, and nitrification, p. 985–1018. *In* R. W. Weaver, J. S. Angle, and P. S. Bottomley (ed.), Methods of soil analysis, part 2: microbiological and biochemical properties. Soil Science Society of America, Inc., Madison, Wis.
8. **Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders.** 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. **Hiorns, W. D., R. C. Hastings, I. M. Head, A. J. McCarthy, J. R. Saunders, R. W. Pickup, and G. H. Hall.** 1995. Amplification of 16S ribosomal RNA genes of autotrophic ammonia-oxidizing bacteria demonstrates the ubiquity of nitrosospiras in the environment. Microbiology **141:**2793–2800.
10. **Hirt, R. P., J. M. Logsdon, B. Healy, M. W. Dorey, W. F. Doolittle, and T. M. Embley.** 1999. Microsporidia are related to fungi: evidence from the largest subunit of RNA polymerase II and other proteins. Proc. Natl. Acad. Sci. USA **96:**580–585.
11. **Horner, D. S., P. G. Foster, and T. M. Embley.** 2000. Iron hydrogenases and the evolution of anaerobic eukaryotes. Mol. Biol. Evol. **17:**1695–1709.
12. **Killham, K.** 1990. Nitrification in coniferous forest soils. Plant Soil **128:**31–44.
13. **Klemedtsson, L., Q. Jiang, A. Kasimir Klemedtsson, and L. Bakken.** 1999. Autotrophic ammonium-oxidizing bacteria in Swedish mor humus. Soil Biol. Biochem. **31:**839–847.
14. **Klotz, M. G., and J. M. Norton.** 1998. Multiple copies of ammonia monooxygenase *(amo)* operons have evolved under biased AT/GC mutational pressure in ammonia-oxidizing autotrophic bacteria. FEMS Microbiol. Lett. **168:**303–311.
15. **Koops, H.-P., B. Böttcher, U. C. Möller, A. Pommerening-Röser, and G. Stehr.** 1990. Description of a new species of *Nitrosococcus*. Arch. Microbiol. **154:**244–248.
16. **Kowalchuk, G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp.** 1997. Analysis of ammonia-oxidizing bacteria of the β-subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. Appl. Environ. Microbiol. **63:**1489–1497.
17. **Lake, J. A.** 1994. Reconstructing evolutionary trees from DNA and protein sequences: paralinear distances. Proc. Natl. Acad. Sci. USA **91:**1455–1459.
18. **Lockhart, P. J., A. W. D. Larkum, M. A. Steel, P. J. Waddel, and D. Penny.** 1996. Evolution of chlorophyll and bacteriochlorophyll: the problem of invariant sites in sequence analysis. Proc. Natl. Acad. Sci. USA **93:**1930–1934.
19. **Lodhi, M. A. K.** 1982. Additional evidence of inhibition of nitrifiers and possible cycling of inhibitors produced by selected plants in a climax community. Bull. Torrey Bot. Club **109:**199–204.
20. **Martikainen, P. J.** 1985. Numbers of autotrophic nitrifiers and nitrification in fertilized forest soil. Soil Biol. Biochem. **17:**245–248.
21. **Massol-Deyá, A., R. Weller, L. Rios-Hernandez, J. Z. Zhou, R. F. Hickey, and J. M. Tiedje.** 1997. Succession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in groundwater. Appl. Environ. Microbiol. **63:**270–276.
22. **McCaig, A. E., T. M. Embley, and J. I. Prosser.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidizers. FEMS Microbiol. Lett. **120:**363–368.
23. **McCaig, A. E., L. A. Glover, and J. I. Prosser.** 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Environ. Microbiol. **65:**1721–1730.
24. **McCaig, A. E., C. J. Phillips, J. R. Stephen, G. A. Kowalchuk, S. M. Harvey, R. A. Herbert, T. M. Embley, and J. I. Prosser.** 1999. Nitrogen cycling and community structure of proteobacterial β-subgroup ammonia-oxidizing bacteria within polluted marine fish farm sediments. Appl. Environ. Microbiol. **65:**213–220.
25. **McTavish, H., J. A. Fuchs, and A. B. Hooper.** 1993. Sequence of the gene coding for ammonium monooxygenase in *Nitrosomonas europaea*. J. Bacteriol. **175:**2436–2444.
26. **Mendum, T. A., R. E. Sockett, and P. R. Hirsch.** 1999. Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the β-subdivision of the class *Proteobacteria* in arable soils to nitrogen fertilizer. Appl. Environ. Microbiol. **65:**4155–4162.
27. **Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl.** 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. **62:**2156–2162.
28. **Muyzer, G.** 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Curr. Opin. Microbiol. **2:**323–327.
29. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
30. **Nüsslein, K., and J. M. Tiedje.** 1998. Characterization of the dominant and rare members of a young Hawaiian soil bacterial community with small subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. Appl. Environ. Microbiol. **64:**1283–1289.
31. **Ogram, A.** 1998. Isolation of nucleic acids from environmental samples, p. 273–288. *In* R. S. Burlage, R. Atlas, D. Stahl, G. Geesey, and G. Sayler (ed.), Techniques in microbial ecology. Oxford University Press, Inc., New York, N.Y.
32. **Phillips, C. J., Z. Smith, T. M. Embley, and J. I. Prosser.** 1999. Phylogenetic differences between particle-associated and planktonic ammonia-oxidizing bacteria of the β-subdivision of the class *Proteobacteria* in the Northwestern Mediterranean Sea. Appl. Environ. Microbiol. **65:**779–786.
33. **Phillips, C. J., D. Harris, S. L. Dollhopf, K. L. Gross, J. I. Prosser, and E. A. Paul.** 2000. Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Appl. Environ. Microbiol. **66:**5410–5418.
34. **Prosser, J. I.** 1989. Autotrophic nitrification in bacteria. Adv. Microb. Physiol. **30:**125–181.
35. **Rice, E. L., and S. K. Pancholy.** 1972. Inhibition of nitrification by climax ecosystems. Am. J. Bot. **59:**1033–1042.
36. **Robson, M. J., A. J. Parsons, and T. E. Williams.** 1989. Herbage production: grasses and legumes, p. 1–6. *In* W. Holmes (ed.), Grass: its production and utilization, 2nd ed. Blackwell Scientific Publications, Oxford, England.
37. **Rodwell, J. S.** 1992. British plant communities, vol. 3. Cambridge University Press, Cambridge, England.
38. **Schmidt, E. L., and L. W. Belser.** 1982. Nitrifying bacteria, p. 1027–1042. *In* A. L. Page, R. H. Miller, and D. R. Keeney (ed.), Methods of soil analysis, part 2: chemical and microbiological properties. Soil Science Society of America, Inc., Madison, Wis.
39. **Stephen, J. R., A. E. McCaig, Z. Smith, J. I. Prosser, and T. M. Embley.** 1996. Molecular diversity of soil and marine 16S rDNA sequences related to β-subgroup ammonia-oxidizing bacteria. Appl. Environ. Microbiol. **62:**4147–4154.
40. **Stephen, J. R., G. A. Kowalchuk, M.-A. V. Bruns, A. E. McCaig, C. J. Phillips, T. M. Embley, and J. I. Prosser.** 1998. Analysis of β-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. Appl. Environ. Microbiol. **64:**2958–2965.
41. **Stephen, J. R., Y.-J. Chang, S. J. MacNaughton, G. A. Kowalchuk, K. T. Leung, C. A. Fleming, and D. C. White.** 1999. Effect of toxic metals on indigenous soil β-subgroup proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. Appl. Environ. Microbiol. **65:**95–101.
42. **Strong, D. T., P. W. G. Sale, and K. R. Helyar.** 1998. The influence of the soil matrix on nitrogen mineralisation and nitrification. I. Spatial variation and a hierarchy of soil properties. Aus. J. Soil Res. **36:**429–447.
43. **Swofford, D. L.** 1998. PAUP*: phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, Mass.
44. **Teske, A., E. Alm, J. M. Regan, B. E. Rittman, and D. A. Stahl.** 1994. Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria. J. Bacteriol. **176:**6623–6630.
45. **Tsai, Y.-L., and B. H. Olson.** 1992. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl. Environ. Microbiol. **58:**754–757.
46. **Utåker, J. B., L. Bakken, Q. Q. Jiang, and I. F. Nes.** 1995. Phylogenetic analysis of seven new isolates of ammonia-oxidizing bacteria based on 16S rRNA gene sequences. System. Appl. Microbiol. **18:**549–559.

47. **Walker, N., and K. N. Wickramasinghe.** 1979. Nitrification and autotrophic nitrifying bacteria in acid tea soils. Soil Biol. Biochem. **11:**231–236.
48. **Webster, G., P. R. Poulton, E. C. Cocking, and M. R. Davey.** 1995. The nodulation of micro-propagated plants of *Parasponia andersonii* by tropical legume rhizobia. J. Exp. Bot. **46:**1131–1137.
49. **Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Mackie, W. Ludwig, and E. Stackebrandt.** 1985. The phylogeny of the purple bacteria: the gamma subdivision. System. Appl. Microbiol. **6:**25–33.
50. **Yokoyama, K., H. Kimura, H. Shinozaki, and S. Tajima.** 1997. Detection of *amo* gene of chemolithotrophic ammonia-oxidizers in soil by nested PCR, p. 753–754. *In* T. Ando, K. Fujita, T. Mae, H. Matsumoto, S. Mori, and J. Sekiya (ed.), Plant nutrition for sustainable food production and environment. Kluwer Academic Publishers, Dordrecht, The Netherlands.