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Abstract approved _____
Peter J. Bottomley

In recent years considerable interest has been shown in the diversity of ammonia-oxidizing bacteria in soil communities. The majority of the research has been carried out in Northern Europe where soils have received high atmospheric inputs of nitrogen over the past two centuries. In contrast, although much work has been conducted on nitrogen cycling processes in nitrogen limited forest ecosystems in western North America, no studies have examined the characteristics of ammonia-oxidizing communities in those environments.

I was interested in measuring nitrification potential along a high-elevation temperate meadow-to-forest gradient, and characterizing the ammonia-oxidizing communities along that gradient using both molecular and culturing methods. Two experimental sites (Lookout and Carpenter) were chosen in the H. J. Andrews Experimental Forest, located in the western Cascade Range of Oregon, at elevations of approximately 1500 meters. Although nitrification potential rates (NPRs) between sites were not significantly different ($P = 0.544$), variation was observed both within and between sites for specific vegetation types. NPRs were significantly lower in forest (F) soil samples than in meadow (M) soil samples, averaging 5 and 2% of meadow NPRs at Lookout and Carpenter, respectively. In meadow soil samples, most probable number

(MPN) population densities of ammonia-oxidizers ranged from 0.6 to 2.6 x 10^4 cells gram^{-1} of oven dry soil and 0.9 x 10^3 to 1.1 x 10^5 cells g^{-1} OD soil at Lookout and Carpenter, respectively. In forest soil samples, population densities ranged from undetectable to 1.1 x 10^4 cells g^{-1} OD soil, and 0.9 x 10^2 to 2.3 x 10^3 cells g^{-1} OD soil at Lookout and Carpenter, respectively.

Microbial community DNA was amplified using primers to the ammonia monooxygenase subunit A. Terminal restriction fragment polymorphism analysis with three different restriction enzymes (*CfoI, TaqI, and AluI*) revealed community profiles dominated by *Nitrosospira* species. One fragment from *CfoI* (66 bp) and one fragment from *AluI* (392-bp) were prominent in 47 soil samples from both sites, and represented between 32 to 100% of the Genescan fragment analyses of PCR products. A full length fragment from *AluI* digests (491-bp), and three fragments from CfoI (68, 100, and 135-bp) were found sporadically in fewer soil sample T-RFLPs, and within those samples represented smaller percentages of total peak areas. The *CfoI* 135-bp fragment length was associated primarily with M and meadow/forest (M/F) soils where it was observed in approximately 58 and 100% of the respective transect locations. Eight isolates recovered from soil samples were analyzed using the same molecular methods as the field samples. The T-RFLP patterns of the isolates corresponded with many of those found in the community fingerprints. Four unique *amoA* sequences were identified among these isolates, including one that possessed the dominant T-RFLP *amoA* fingerprint in soil samples. This sequence shared 99.8% similarity with *Nitrosospira sp.* Ka4, a cluster 4 ammonia oxidizer isolated in Norway. Sequence analysis phylogenetically associated the other three isolates (with unique *amoA* sequences) near

Nitrosospira sp. Nsp1 and *Nitrosospira briensis*, both cluster 3 ammonia oxidizers. Cloning and sequencing of soil DNA confirmed that ammonia oxidizers with these *amoA* sequences were present in the soil samples. Two additional *amoA* sequences were identified in clones that were 95% similar and paraphylogenetically positioned between representatives of clusters 3 and 4. So far, these sequences have not been found in any of the isolates analyzed.

Community Profiles of Ammonia Oxidizers Across High-Elevation Forest-to-Meadow Transects

by

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Dedicated to the memory of my mother.
It was her encouragement and support
that led me here.

Community Profiles of Ammonia Oxidizers Across High-Elevation Forest-to-Meadow Transects

INTRODUCTION

For many years the process of nitrification was thought to play a minimal role in nitrogen cycling in undisturbed forest systems. Based on known physiological properties of a limited number of ammonia-oxidizing bacteria, several soil factors were considered inhibitory to ammonia oxidizers including soil acidity, high C/N ratios, and the presence of allelochemical compounds (Weber et al., 1962; Rice et al.,1972; Robertson, 1982; Myrold, 1998). With the advent of ^{15}N tracer methods for measuring gross rates of nitrogen processes in soils, it became apparent that nitrogen cycling is tightly coupled in forest soils and that little net increase in the soil NO_3^- pool size does not necessarily indicate the absence of nitrification (Davidson et al., 1992; Hart et al. 1994; Verchot et al., 2001; Merila et al., 2002).

Stark and Hart (1997) used $^{15}NO_3^-$ isotope dilution measurements to show that high rates of nitrification were occurring in western North American forest soils. Furthermore, acid-tolerant ammonia oxidizers have been sporadically isolated from forest soils in Northern European countries as well as in the United States (Martikainen et al., 1984; Schmidt et al., 1984; De Boer et al., 1989). These observations sparked interest in learning more about the nature and physiological ecology of ammonia-oxidizing bacteria in forest ecosystems (Belser et al., 1978; Hankinson et al., 1984; De Boer et al., 1989; Persson et al., 1995; Stark et al., 1996).

In recent years, considerable progress has been made into elucidating the composition of ammonia-oxidizing bacterial communities using molecular approaches primarily based on 16S rDNA technology. In general, soil populations have been shown to be dominated by the genus *Nitrosospira*, rather than by the widely-studied genus *Nitrosomonas* (Stephen et al., 1996, 1998; Bruns et al.,1998; Kowalchuck et al., 1997, 1998, 2000), and that *Nitrosospira* can be grouped into several phylogenetically distinct clusters (Stephen et al., 1996; Åakra et al., 2001b). Some studies have shown that the variation in *amoA* gene sequences which encode the catalytic subunit of ammonia momooxygenase, can be used to subdivide a population (Horz et al., 2000; Åakra et al., 2001a). It has been observed that within this monophylogenetic group, certain populations seem to be fairly ubiquitous in nature, whereas others seem to thrive in specific environments. For example, cluster 2 ammonia oxidizers seem to be associated with acidic soils, whereas those comprising cluster 3 are primarily observed in agricultural soils with high ammonium availability (Ferris et al., 1996; Rotthauwe et al.,1997; Kowalchuk et al., 2000a, 2001). Previous research involving soil ammonia-oxidizer communities has focused primarily on grasslands and agroecosystems (Belser et al., 1978; Davidson et al., 1990; Rotthauwe et al., 1997; Kowalchuck et al., 1997, 1998, 2000a, 2000b; Bruns et al., 1999; Phillips et al., 2000).

The objective of this study was to characterize the nature of the ammonia-oxidizing community in mineral soil sampled across high-elevation forest-to-meadow transects at two sites in the H. J. Andrews Experimental Forest. The meadows at these elevations contain a substantial legume component providing high quality nitrogen (N) inputs, whereas the forests are coniferous in nature, with limited understory development and presumably limited N inputs. This environment provided an opportunity to determine if differences existed among the ammonia-oxidizing communities found along transects running from meadow into forest environments. My approach involved complimentary methodologies that involved measuring nitrification potential rates (NPRs), generating T-RFLP fingerprints from soil DNA, and cloning and sequencing *amoA* genes directly from soil and from ammonia oxidizers isolated into pure culture.

MATERIALS AND METHODS

Site Description

The experimental sites were situated in the H. J. Andrews Experimental Forest, (44.2 °N latitude and 122.2 °W longitude) in the western Cascade Range of Oregon. This regional landscape is considered a classic example of a high-elevation temperate coniferous forest biome. For the past 50 years, ongoing ecological research at the Andrews has contributed to an extensive database and established infrastructure (http://www.fsl.orst.edu/lter/).

Sampling Sites

Soil samples were collected from two high-elevation (approximately 1500 m) sites, Lookout and Carpenter Mountains, situated just inside the NE and N Experimental Forest boundaries (H.J. Andrews numbers FR11, and L307, respectively). Each site was selected because of the close proximity of grassland meadows and coniferous forests. These features allowed a comparative analysis to be made of nitrification potential rates and the corresponding ammonia oxidizer communities in soils across ecological gradients. Bedrock from both sites is composed of andesite lava flows of Miocene age and of younger High Cascade rocks. Soils developed from these parent materials are primarily Inceptisols with local areas of Alfisols and Spodosols. The site at Lookout has a southwestern aspect with an approximate 40 degree slope. Thirty-year-old Douglas fir (*Pseudotsuga menziesii*) and white fir (*Abies concolor*) comprise the majority of the forest vegetation, whereas

meadow vegetation consists of leguminous and non-leguminous plants. The site at Carpenter also has a southwestern aspect with an approximate 45 degree slope. Tree coring measurements showed the predominant white fir vegetation to be about 70 y old. Meadow vegetation at Carpenter consists primarily of grasses, sedges, and Bracken fern (*Pteriadium aquilinum*). Selected soil characteristics from both experimental sites are given in Table 1.

Table 1. Soil Characteristics from Lookout and Carpenter.

Site	Vegetation	Sand	Silt	Clay	% C	% N	CEC[1]	pH
		(g per 100 g soil)			(g per 100 g soil)		(meq/100g)	
Lookout	meadow	68.7	19.4	2.1	11.24	0.89	39.5	5.6
Lookout	forest	66.6	18.6	5.4	14.98	0.94	45.1	5.1
Carpenter	meadow	66.4	18.6	5.6	9.46	0.69	39.0	5.8
Carpenter	forest	76.0	13.0	4.1	13.27	0.54	55.6	5.2

[1]CEC = cation exchange capacity

Field Site Experimental Design and Soil Sampling.

At each experimental site, three parallel transects were positioned perpendicular to the meadow-forest boundary, which was defined by the contrast between vegetation types. Transects were spaced 20-m apart with the exception of the furthest upslope transect at Carpenter being located 100-m from the middle transect to avoid a hiking trail. Each transect consisted of eight, evenly spaced 1-m diameter plots; three specific to the meadow (M), three specific to the forest (F), and two near the meadow-forest

boundary, considered the "transition zone," (M/F and F/M). Thus, for each transect at Lookout, eight plots were spaced 10-m apart, making a total of 24 plots per site. The plots at Carpenter were spaced 20-m apart, which was necessary to account for the wider transition zone existing between meadow and forest vegetation (Figure 1).

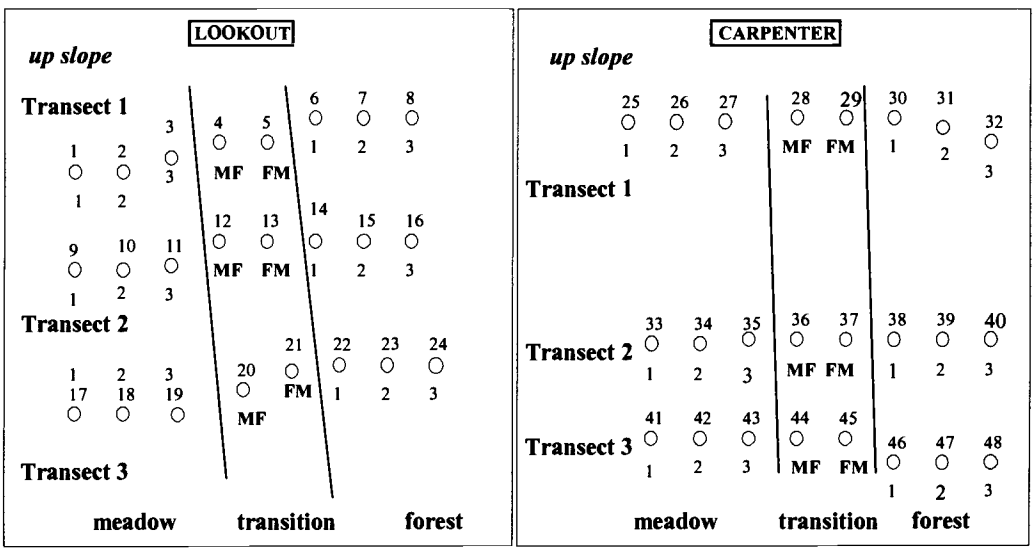

Figure 1. Lookout and Carpenter Field Design for Transects and Plots.

Five soil cores (4.7 cm diameter x 15.0 cm depth) were extracted from each plot using PVC pipe. Each core was separated into litter, humus, and mineral soil layers based on soil depth and observable soil layer characteristics. Meadow cores generally consisted of a litter layer (approximate depth 0-2.5 cm), humus or transitional layer (2.5-5.0 cm), and mineral soil layer (5.0-15.0 cm). No distinct litter layer was present in some of the meadow plots at Carpenter (transect 1). Forest plots were also sampled similarly to 15 cm depth. Forest cores consisted of litter, humus, and mineral soil layers which were

separated based on simple morphological features such as recognizable plant debris in the litter layer (needles and twigs), decomposed amorphous organic material in the humus layer, and lighter colored aggregated soil in the mineral layer. Cores taken from the transition zones were treated like meadow or forest cores, depending on the dominant vegetation type. After cores were fractionated, all five samples extracted from the same individual plot were composited into polyethylene bags, and transported in coolers to Oregon State University within 10 h of initial sampling. All samples were stored overnight at 4°C. The next day, all mineral soil samples were passed through a 4.0-mm sieve. The same soil collecting and processing protocol was followed with slight modifications for sampling done on November 16, 2000 (for MPNs).

Soil pH.

Ten g fresh mineral soil was added to 20-mL deionized H_2O and mixed for one h on a rotary shaker (180 rpm, 28°C). pH was measured using a Corning Model 10 pH Meter.

Nitrification Potential.

A shaken soil-slurry method (Hart et al., 1994) was used to measure nitrification potential rates for all 48 soil samples within 1 week of sampling. Each day, eight composite samples were randomly chosen from the larger collection of samples and triplicate 15-g sub-samples weighed into 250- ml Erlenmeyer flasks. For each sample, 100-ml of a sterile solution (pH 7.2) containing 1.5 mM NH_4^+ and 1.0 mM

potassium phosphate (KP) buffer (K_2HPO_4:KH_2PO_4) was added and slurries were incubated in an orbital shaker (180 rpm) at 28°C for 24 h. Each flask of soil slurry was sampled a total of 4 times (3, 5, 22, and 24 h) during the incubation. For each time point, a 10-mL portion of slurry was withdrawn from each flask and centrifuged at 17,000 rpm for 15 min. The supernatants were transferred to polypropylene tubes, capped, and frozen until analyzed colorimetrically for NO_3^-. At that time, the entire time series of samples from a single flask were analyzed together. Supernatants were thawed and mixed immediately prior to analysis. Aliquots (0.25 ml) of samples were mixed with 2.5 ml of Szechrome NAS (Polysciences, Inc., Warrington, Pa.), which was prepared as a 5 g/L solution in phosphoric:sulfuric acid (1:1 v/v). Color was allowed to develop for 60 min and absorbance measured at 570 nm using a Beckman Model 34 Spectrophotometer. Rates of nitrification were determined using multiple regression analysis, then log-transformed prior to performing ANOVA (S+2000, Data Analysis Products Division, MathSoft, Inc., Seattle, WA.).

Previous investigators (Stark et al., 1996) have adjusted the pH of nitrification potential soil slurries 2 h into the assay to correct for soil effects on the slurry pH. We noticed within the first hour of incubation that the pH dropped from pH 7.2 to pH 5.7. This drop was corrected by adding 0.25 mM KOH dropwise to the slurries during the first 2 and 4 hours of the assay to bring the pH back to neutral. pH adjustments were found to have no significant effect on the nitrification potential rates. Therefore, we chose to conduct the assays without pH adjustments.

Acetylene Block.

Triplicate samples (2.0 g) of soil from Lookout and Carpenter were dispensed into 74-ml sterile glass vials along with 13.3-ml portions of KP buffer (pH 7.2) and 1.5 mM NH_4^+. Vials were plugged with butyl stoppers and crimped with aluminum seals. Treatments for each site included two vials without acetylene and vials with 1% and 10% (v/v) acetylene, respectively. All samples were inverted and shaken on a darkened rotary shaker at 28°C for 12 h at 180 rpm. Aliquots (10 ml) of slurry were withdrawn from each vial and analyzed (as described above) for NO_3^-. Nitrification was eliminated from both Lookout and Carpenter meadow soils treated with both concentrations of acetylene, suggestive of the chemolithoautotrophic nature of ammonia-oxidation. Forest soil samples were not evaluated due to the low NPRs observed.

MPN Enumeration.

This assay was restricted to four plots along each transect (M_2, M/F, F/M, F_2) reflecting the sampling scheme of November 16, 2000. Two cores were extracted and composited from each plot, and 4 g of fresh soil was vortexed in 36-ml of a general ammonia oxidizer mineral medium (Table 2) containing 20 mM NH_4^+, 4.0 mM KP buffer, 4.0 mM K_2CO_3, trace elements, and adjusted to pH 7.2 (Schmidt et al., 1982; Donaldson et al., 1989). Ten-fold dilution series were prepared from each soil suspension and quadruplicate 1-ml samples from each dilution were inoculated into 9-ml portions of the same medium. Dilutions were carried out to 10^{-7} and incubated at 28°C in the dark. Spot checks for NO_2^- were performed after 1, 2, and 4 months of

incubation using modified Griess-Ilosvay reagents (Schmidt et al.,1994). Estimates of the ammonia-oxidizing populations were determined from terminal NO_2^- positive dilutions cross-referenced to tables generated from MPNES software (Woomer, 1994), and corrected for soil moisture content.

Table 2. Standard Ammonia Oxidizer Mineral Medium.*

	liter^{-1} dH_2O	mM
$(NH_4)_2SO_4$	1.32 g	10.00
K_2CO_3	0.54 g	4.00
KH_2PO_4	0.41 g	3.00
$MgSO_4$	0.18 g	
K_2HPO_4	0.17 g	1.00
$CaCl_2$	29.40 mg	
NaH_2PO_4	9.20 mg	0.10
EDTA	6.20 mg	
$FeSO_4$	2.70 mg	
$MnCl_2$	197.90 µg	
$CuSO_4$	166.00 µg	
$ZnSO_4$	100.64 µg	
Na_2MoO_4	96.80 µg	
$CoCl_2$	1.90 µg	

*adjusted to pH 7.2

MPN population (Figure 2) densities of ammonia oxidizers in M soil samples ranged from 0.6 to 2.6 x 10^4 cells g^{-1} OD soil and 0.9 x 10^3 to 1.1 x 10^5 cells g^{-1} OD soil at Lookout and Carpenter, respectively. In F soil samples, population densities ranged from undetectable to 1.1 x 10^4 cells g^{-1} OD soil and 0.9 x 10^2 to 2.3 x 10^3 cells g^{-1} OD soil at Lookout and Carpenter, respectively.

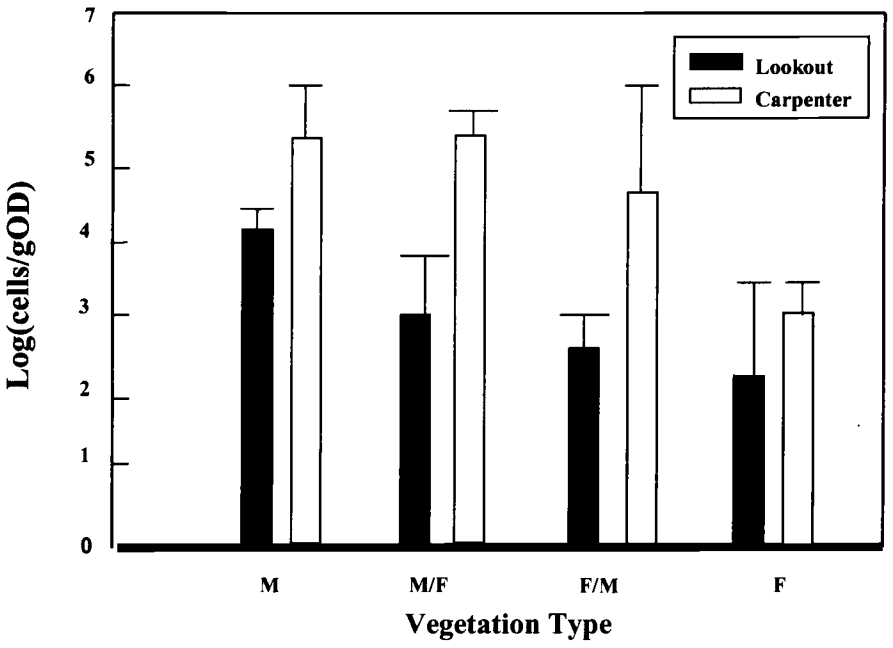

Figure 2. Most Probable Number Enumerations of Ammonia Oxidizers.

Pure Culture Isolation of Ammonia Oxidizers.

Terminal MPN dilutions that were positive for NO_2^- were incubated until NO_2^- concentrations reached 0.5 mM. Ten fold serial dilutions of the terminal positive dilutions were repeated as above and 1-ml samples inoculated into 9-ml of ammonia oxidizer mineral medium. Tubes were again incubated at 28°C in the dark and checked for NO_2^- production until no higher dilutions tested positive. From the terminal NO_2^- positive dilutions, 1 ml inocula were backtransferred to 9 new tubes of fresh medium and incubated for another month. Tubes containing 9-ml of heterotrophic media (1.5 g glucose, 0.75 g yeast extract, and 0.75 g Bactotryptone in 1 L de-ionized H_2O) were inoculated with 1-ml samples of tubes that became positive for NO_2^-. Contamination checks that did not show signs of turbidity after 3 weeks incubation suggested the

possibility of pure cultures. Two additional contamination checks were carried out in Czapek-Dox medium for fungi (Parkinson, 1994) and "Medium #3" which was reported to detect microaerophilic heterotrophs contaminating cultures of ammonia oxidizers (Åakra et al., 1999).

Isolates were also obtained from enrichments that began as nitrification potential slurries in ammonia oxidizer mineral medium. Flasks were allowed to shake for 24 h under similar conditions for nitrification potentials followed by serial dilutions and inoculation into growth medium (as described above for MPNs). Enrichments were incubated for approximately 1 month at 28°C in the dark, tested for the presence of at least 0.5 mM NO_2^-, then subjected to the same serial dilution isolation protocol described above.

DNA Extraction from Soils and PCR with *amoA* Primers.

Soil DNA was extracted using a FastDNA Spin kit for soils (Bio 101, Inc., Carlsbad, CA), which is designed to extract PCR-ready genomic DNA from microorganisms, plants, and animals. Cells were lysed using a combination of detergents, silica, ceramic beads, and a specialized homogenizing instrument designed by the manufacturer. Subsequent steps removed proteins and other contaminants. Purified soil DNA was subjected to PCR using the *amoA* primers of Hortz et al., 1999. The forward primer (*amoA*-1F; 5'- GGGGGTTTCTACTGGTGGT) targets positions 332 to 349 and the reverse primer (*amoA*-2R; 5'-CCCCTCKGSAAAGCCTTCTTC [K = G or T; S = G or C] corresponds to positions 802 to 822 of the open reading frame

published for the *amoA* gene sequence of *Nitrosomonas europaea* (McTavish et al., 1993).

For T-RFLP analysis, amplification was carried out using the forward primer (*amoA*-1F) 5'- labeled with the dye 6-FAM (Genset, La Jolla, CA). PCR was carried out in a total volume of 50 µl in 0.2-ml Eppendorf tubes, using an ABI GeneAmp DNA thermocycler (model 2400). A hot-start procedure was used to reduce nonspecific amplification. Each reaction mixture contained either 200 or 250 ng of soil template DNA in a solution containing 1X PCR buffer (75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, and 0.1% w/v Tween 20), 3.0 mM $MgCl_2$, 0.20 mM of each deoxynucleoside triphosphate, 0.16 µM of each primer, 0.04% w/v bovine serum albumin (BSA), 2.5 U of *Taq* DNA polymerase (Fermentas, Inc, Hanover, MD), topped with sterile mineral oil (Sigma, St. Louis, MO). Positive controls contained 20 ng of genomic DNA prepared from cultures of *Nitrosomonas europaea* ATCC19178 and *Nitrosospira* AV. Negative controls contained either no DNA or 50 ng *Escherichia coli* genomic DNA as template. The thermal profile for the amplification of *amoA* target sequences was optimized as follows: 5 min at 94°C; pause to add *Taq* polymerase; then 30-35 cycles (depending on whether DNA genomic concentration was 250 or 200 ng, respectively) of 60 s at 94°C (denaturation), 60 s at 60°C (annealing), and 90 s at 72°C (elongation). PCR cycling was completed by a final elongation step consisting of 7 min at 72°C. Aliquots (25 µl) of the PCR products were electrophoresed and visualized in 1.2% (w/v) low melt agarose gels using TAE buffer and standard electrophoresis procedures (Sambrook et al., 1989).

T-RFLP Analysis.

The fluorescently labeled PCR products (491-bp) were gel extracted and purified using the Wizard® DNA Purification system (Promega Corp., Madison, Wis.), and quantified using a Bio Spec-1601 DNA/protein/enzyme analyzer (Shimadzu). A portion of each sample (30 ng) was digested with one of three restriction enzymes; *TaqI* (recognition site: T/CGA), *CfoI* (recognition site: GCG/C) and *AluI* (recognition site: AG/CT). 5U of each enzyme were added to a total volume of 20 µl which included 0.1 µl acetylated BSA (10 µg/ml) and 1X buffer (Promega Corp.). Incubation temperature for *CfoI* and *AluI* digests was maintained at 37°C, whereas *TaqI* digests were incubated at 65°C. Following overnight restriction digests, fragments were purified by adding 40-µl filter-sterilized 95% EtOH and stored at -20°C for 4 h. Samples were centrifuged for 40 min at 12,900 rpm, the supernatant withdrawn, 100 µl filter-sterilized 95% EtOH added, and samples centrifuged again. The pelleted restriction fragments were allowed to air dry before being resuspended in sterile water. Fourteen fmol of *amoA* restriction fragments were submitted to the Central Services Laboratory (Center for Gene Research and Biotechnology, Oregon State University, Corvallis) where they were resolved on a Long Ranger polyacrylamide gel (FMC, Rockland, ME) on an ABI 377 Prism DNA sequencer using GeneScan software (ABI, Inc., Freemont, CA). The internal size standard, GeneScan 1000-ROX (ABI, Inc.), was loaded in each lane. Fragment lengths were estimated using the Local Southern Method in GeneScan software v. 2.1 (ABI, Inc). T-RFLP community fingerprints were determined from the presence of fragments of specific lengths and by determining the

contribution of individual peak areas to the total peak area of all fragments in a sample digest.

Cloning and Sequencing.

Soil DNA from Carpenter plot #45 (shown to contain a variety of *amoA* T-RFLP patterns) was selected for cloning. The PCR protocol described above was followed with the exception that nonlabeled forward primers were used. PCR products were electrophoresed, gel extracted, purified and quantified as described previously. They were ligated into the pGEM®-T Easy vector using T4 ligase (Promega Corp), and transformed into competent *E. coli* cells according to manufacturer's recommendations. After plating 100-µl aliquots of each transformed culture onto LB/amp/IPTG/X-Gal medium, plates were incubated at 37°C for 24 h. White colonies were streaked for isolation on LB/amp plates and incubated overnight at 37°C. Single colonies were selected from each isolated clone and inoculated into vials containing 3 mL LB/amp broth. Cells were allowed to grow overnight with shaking (150 rpm) at 37°C. Plasmid DNA was extracted using the QIAprep® Spin Miniprep kit (QIAGEN Inc, Valencia, CA) and quantified. Approximately 2.0 µl of each plasmid prep was reamplified using the 6-FAM forward *amoA* primer and unlabeled reverse *amoA* primer. Protocols for electrophoresis, gel purification, and restriction digests were followed as described above. Purified terminal restriction fragments were submitted to GeneScan (CSL) for fragment analysis. Specific clones were selected for sequencing based on the similarity of their T-RFLP patterns to those observed in the soil community profiles. Double-stranded cycle sequencing was performed using 400 ng plasmid DNA and 12.0 pmols

M-13 forward and reverse primers while following Taq dye terminator chemistry using an ABI cycle sequencer (CSL).

Genomic DNA Extraction from Cultures of Ammonia Oxidizers.

One of two procedures for extracting genomic DNA from isolates was followed depending on the cell density of the culture. Cultures that easily achieved turbidity were harvested by centrifugation (8,000 rpm for 30 min). Otherwise, if cell densities were too sparse to pellet by centrifugation, cells were gently filtered onto 0.2-μm (25 mm) polycarbonate membrane filters. Each filter was placed into a 2.0-ml Eppendorf tubes with 200 μl of a 5 M guanidine thiocyanate solution (30 ml 0.5 M EDTA, 7.5 ml 10% N-lauroylsarkosine, and 88.6 g GITC in 150 ml H_2O). Samples were vortexed for 1 min, centrifuged for another min at 15000 rpm, then allowed to sit on the benchtop for 10 min. Additional steps for genomic DNA extraction and purification were done using the DNeasy™ Tissue kit (QIAGEN Inc.) using a spin filter column and a series of wash solutions. As little as 200 ng genomic DNA could be recovered from a 10-ml aliquot of culture.

16S rDNA PCR and Sequencing.

Primers used for 16S rDNA amplification were initially designed as probes for ammonia-oxidizing β-proteobacteria (Mobarry et al., 1996), but found to work well as primer sets (Purkhold et al., 2000). The forward primer (Nso190; 5' - CGATCCCCTGCTTTTCTCC) targets positions 190-208 of the *E.coli* 16S rDNA gene, and the reverse primer (Nso1225; 5' - CGCGATTGTATTACGTGTGA)

corresponds to the *E. coli* 16S rDNA positions 1225-1244. The PCR protocol described above was followed except that 100-µl reaction volumes were used that included 2.5 mM $MgCl_2$ and 40-ng purified isolate genomic DNA as template. Thermal cycling conditions differed from those used for *amoA* by implementing a lower and longer annealing step (55°C for 90 s) for optimizing primer binding. Post PCR methods for electrophoresis, gel extraction, purification, and quantification were as described above for *amoA* PCR products. Double-stranded cycle sequencing of the 16S rDNA fragment utilized Nso190 and Nso1225 primers for the forward and reverse reads, respectively. Approximately 700-bp from each strand could be unambiguously read from the sequence output. Both strands of the 491-bp *amoA* product amplified from pure cultures were also sequenced using *amoA*-1F and *amoA*-2R.

Phylogenetic Analysis.

Six unique *amoA* sequences were obtained, four from pure culture isolates and two from clones that represented T-RFLP patterns observed in the soil community profile but not found in pure cultures. Sequences were submitted to a Blast search and representative sequences in the GenBank database sharing the greatest similarity to each new sequence were imported into the BioEdit Sequence Alignment Editor for Windows 95/98/NT. Thirty sequences were aligned manually before using Clustal W for multiple sequence alignment. Masking reduced the sequences to 394 characters, and phylogenetic analysis was performed in Paup* 4.0 using Neighbor Joining method with a Kimura 2-parameter distance measure. Bootstrap support values were generated from

100 parsimony-maximum-likelihood analyses of the aligned sequences to determine the confidence values of tree branches.

Statistical Analysis.

Analyses were performed using S+2000 software (MathSoft, Inc., Seattle, WA.). One-way analysis of variance (ANOVA) was used to determine all differences between M and F soils. The categorical variable for all ANOVAs was vegetation with four levels (M, M/F, F/M, and F). Linear regression models were constructed to calculate NPRs. Variance checks indicated that there was increasing variation with the means from data obtained from NPRs and MPNs, which necessitated the need to log-transform the response variables. All values reported as means are actual means, rather than back-transformed means. Spatial autocorrelation was tested using the Durbin-Watson method, since the limited number of data points prohibited the use of the more sensitive semi-variograms statistical procedure. T-RFLP data were analyzed according to relative peak areas generated from Genescan electropherograms. Indicator species analysis of *amoA CfoI/AluI* T-RFLPs was calculated with the method of Dufrene et al., 1997. A Monte Carlo test of significance calculated the tendency for fragment indicator potential by combining relative abundance with relative frequency for the *CfoI* 135-bp fragment. Differences between comparisons involving only two variables were done with t- tests.

RESULTS

Soil Chemical and Physical Properties.

At both sites, mineral soils (< 2 mm) were sandy loams with low clay contents. The forest soils were slightly more acidic (pH 5.2) than the meadow soils (pH 5.7). Soils at both sites, and in both meadow and forest locations, contained high organic C and N contents. The C/N ratios in meadow soils were lower than the forest soils at both Lookout (12.6 versus 16.0, respectively) and Carpenter (13.7 versus 24.6, respectively).

Nitrification Potential Rates (NPRs).

NPRs differed substantially between meadow (M) and forest (F) soil samples at each site. On average, NPRs obtained from Lookout and Carpenter M samples were approximately 19 and 64 times greater than F samples (0.394 μg NO_3^--N g^{-1} OD soil hr^{-1} versus 0.021 μg NO_3^--N g^{-1} OD soil hr^{-1}), and (0.382 μg NO_3^--N g^{-1} OD soil hr^{-1} versus 0.006 μg NO_3^--N g^{-1} OD soil hr^{-1}) respectively. Mean NPRs obtained from meadow/forest (M/F) transition zone soil samples were similar to mean rates obtained from M samples at both sites (0.263 μg NO_3^--N g^{-1} OD soil hr^{-1} and 0.394 μg NO_3^-N g^{-1} OD soil hr^{-1}, and 0.335 μg NO_3^--N g^{-1} OD soil hr^{-1} and 0.382 μg NO_3^--N g^{-1} OD soil hr^{-1} for Lookout and Carpenter, respectively). In contrast, a clear distinction could be made between NPRs of M/F and F/M at both sites ($P = 0.042$). At Lookout, NPRs obtained from two of the three forest/meadow (F/M) transition zone samples were below the lower limit of detection (LLD). At Carpenter, NPRs obtained from the three

F/M samples were similar (mean value = 0.024 μg NO_3^--N g^{-1} OD soil hr^{-1}) and about 14 times lower than NPRs of the M/F zone. At Carpenter, the NPRs of the F/M soil samples were generally greater than NPRs of F soil samples. NPRs for three of nine F samples were < LLD, and the mean NPR of the remainder (0.006 μg NO_3^--N g^{-1} OD soil hr^{-1}) was about 25 % of F/M values. At Lookout, the NPRs of two of nine F soil samples were < LLD, and the remainder (mean value = 0.021 μg NO_3^--N g^{-1} OD soil hr^{-1}) averaged about 6% of M and M/F values, respectively. Furthermore, they were about 3.5 times greater than the NPRs of the F soil samples from Carpenter.

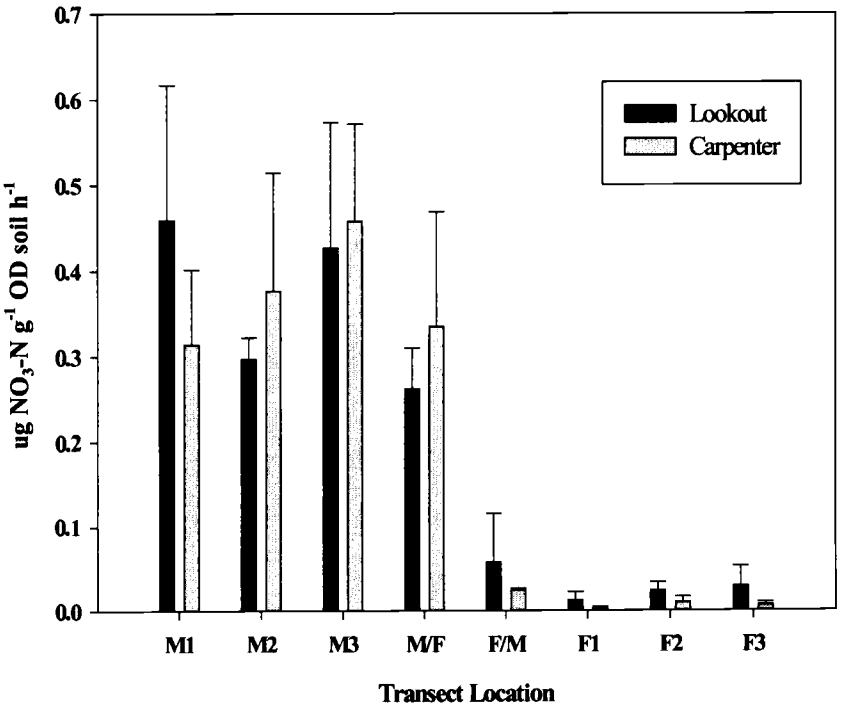

Figure 3. Nitrification Potential Rates Averaged Across Transects.

Molecular Analysis.

All but one of the 48 soil DNA samples (Carpenter plot #30) were successfully amplified using *amoA* primers, and generated the expected 491-bp full-length band size fragment of *amoA*. T-RFLP analysis using *TaqI* generated a single peak (283-bp) in all 47 soil DNA samples suggesting that *Nitrosospira* and/or *Nitrosospira*-like organisms predominated throughout both experimental sites. Based on sequence analysis, a 219-bp fragment length would have indicated the presence of *Nitrosomonas* species (Horz et al., 2000). T-RFLP analysis using *AluI* produced a dominant peak (392-bp) from 45 samples. Two samples failed to give measurable fragments perhaps resulting from the occurrence of extremely short fragments that fell outside the range of accurate detection using the size standard GS1000ROX. The 392-bp fragment made a major contribution in all T-RFLP fragment analyses (Figure 4), ranging from 32 to 100% of PCR products, with the latter value representing 29 of 45 soil samples.

A full length PCR product (with no discernable *AluI* cut site) was observed in 8 samples from each site. Three-fourths of these 8 samples at Lookout and one half at Carpenter comprised $\geq$ 20% of the total products. This fragment was distributed among four of nine M soil samples at each site. This fragment was observed only in two Carpenter forest plots existing along the same transect (#38 and #40) where it comprised 36 and 14% of the total peak area calculated for each of these plots.

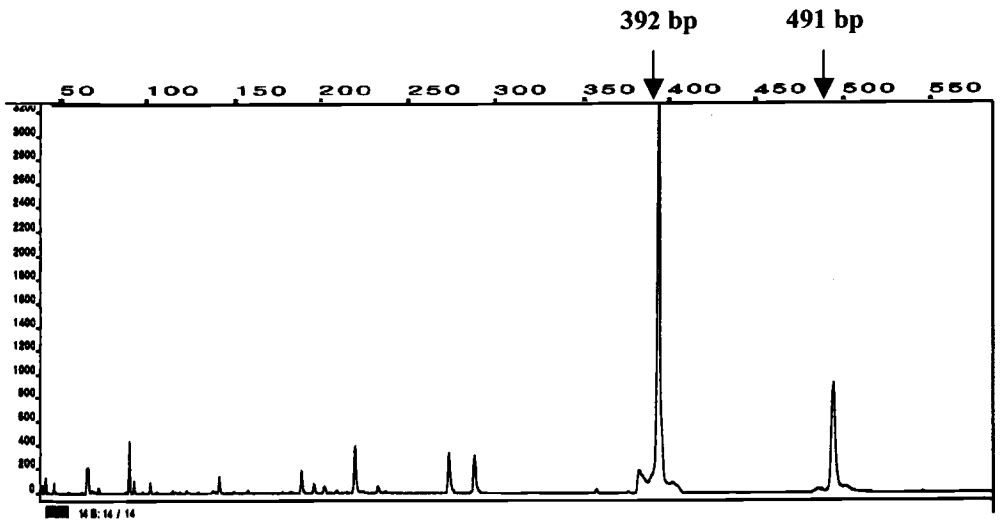

Figure 4. Electropherogram of Soil *amoA* PCR Products Restricted with *AluI*.

T-RFLP analysis using *CfoI* also produced a consistently prominent peak (66-bp) in all 47 samples. Based upon the sequences we analyzed using the full-length PCR *amoA* products, this fragment could occur in at least four different *Nitrosospira* species or strains (Table 3). Three additional peaks, representing fragments 68, 100, and 135-bp in length, were observed in 5, 6, and 24 soil DNA samples, respectively. The 100-bp fragment appeared in M and M/F soils at Lookout, and in F/M and F soils at Carpenter. The relative peak areas of the 68 and 100-bp fragments averaged less than 14% of the total peak areas in the 11 samples in which they were found. Only in two plots did the 68 and 100-bp fragments account for > 20% of the total peak area (Carpenter #35 and #45, respectively).

The 135-bp fragment length was distributed similarly in M and M/F samples at both locations (55 and 61% of M and 100% of M/F samples; see Figure 5). A lower occurrence of this fragment was found in forest soil samples at both sites (8 to 13%). A Monte Carlo test of significance showed the tendency for this fragment to be an indicator for M and M/F soils in 60% of 1000 permutations. Sequencing was performed on a single clone that exhibited a 135-bp fragment from T-RFLP data. The sequence analysis associated the 135 *CfoI* bp fragment with a full length PCR fragment without an *AluI* site. Further analysis of all T-RFLP data collected from the 47 soil DNA samples restricted with *CfoI* and *AluI*, found unrestricted full length *AluI* fragments associated with *CfoI* 135-bp fragments in 13 of the 24 samples (Figure 5). The most plausible explanation for this observation is the existence of at least one other *amoA* sequence that pairs the *CfoI* 135-bp fragment with the *AluI* 392-bp fragment. Further analysis of the clone library is required to verify this hypothesis.

Even though the 135-bp fragment was widely distributed in M and M/F samples at both locations, its relative abundance was variable. In six M samples from Lookout, this fragment contributed between 20 and 40% of the total peak area per Genescan fragment analysis. At Carpenter, this fragment was observed in five M samples, but its contribution was more variable, ranging from 9 to 47% of total peak area per sample analysis. In forest soil samples, the relative abundance of the 135-bp fragment was lower than in M samples, ranging between 3 and 18% at Lookout, and only 3% at Carpenter.

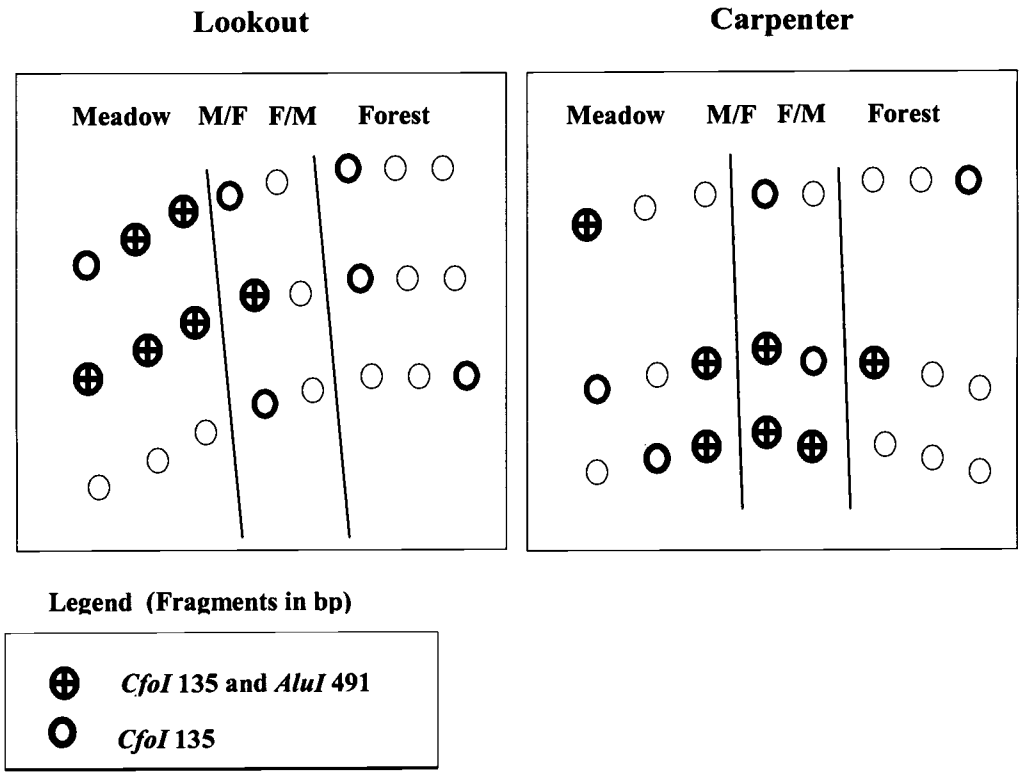

Figure 5. Distribution of the *CfoI* 135-bp T-RFLP Fragment.

Isolates and Sequencing.

Enrichments and back-transfers from MPN positive dilutions produced NO_2^- producing cultures free of heterotrophic contaminants. There was no pattern to the locations from where isolates were successfully recovered because the process of purification occurred randomly. Nevertheless, sequence information obtained from the isolates provided further insight into the nature of the ammonia oxidizer community. T-RFLP patterns from 16 of these isolates matched those obtained from the community fingerprints (Table 3). Sequencing *amoA* from 8 isolates representing the 4 different T-RFLP patterns (shown in Table 3) produced four unique sequences (Appendix A2)

which were phylogenetically placed in a Neighbor Joining tree (Figure 5) relative to *Nitrosospira* clusters 3 and 4 (*Nitrosospira sp.* Nsp1, and *Nitrosospira sp.* Ka4, respectively). Cloning identified 2 additional *amoA* sequences not present in the isolates but observed in the T-RFLP community profiles. They included the 100 and 135-bp fragments restricted with *CfoI*. The phylogeny of these cloned ammonia oxidizers remains somewhat unresolved, but share greatest similarity with *Nitrosospira sp.* Ka4 and *Nitrosospira sp.* III7 (94%). Sequencing of the 16S rDNA gene from the isolate (CT2F) that represented the most widespread and dominant T-RFLP pattern at both Lookout and Carpenter confirmed that its closest relative was Ka4, because the sequences were identical.

Table 3. T-RFLP Patterns Observed in Isolates and Clones.

Site of Origin	No. of Isolates	Fragment Lengths (bp) *CfoI*	*AluI*	*TaqI*
CT1Ma, CT1M/Fb, CT1F, CT3Mb, CT3M/F CT2F(enrichment)*	6	66	392	283
LT1F/Ma,*d,*e,* LT1Fd,* LT2M/Fa(enrichment)*	5	66	491	283
LT1F/Mf*	1	66	34	283
LT1M, LT2Md, LT2Fb*	3	66 and 68	491	283
soil clone #26		100	392	283
soil clone #14		135	491	283

*amoA was sequenced from these isolates

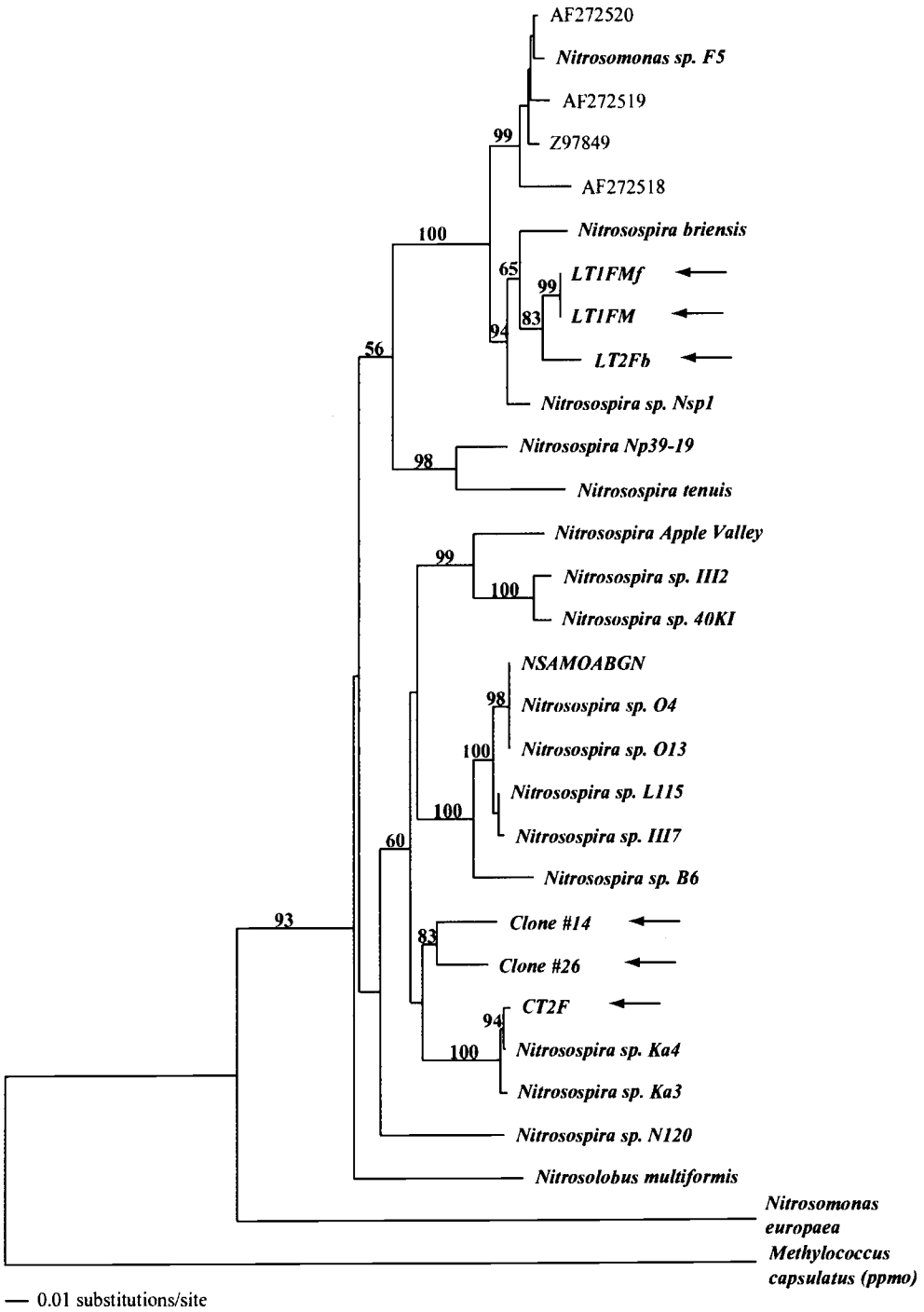

Figure 6. Neighbor Joining *amoA* Phylogenetic Tree.

DISCUSSION

Characterizing the communities of ammonia oxidizers indigenous to this high-elevation temperate forest ecosystem was facilitated by the experimental layout at both Lookout and Carpenter. Sampling across meadow-to-forest transects enabled me to examine the possibility that functional diversity exists within these populations both between adjacent plots and across regions of dissimilar soil and vegetation types.

Differences between the nitrification characteristics of the high-elevation M and F soils were clearly illustrated at both sites by the differences observed between their NPRs and net N mineralization characteristics. Other more subtle differences between the sites were highlighted by the NPRs and net NH_4^+ mineralization rates in the F/M transition zone being significantly greater than in F samples at Carpenter. No transitional zone effects were apparent at Lookout despite this being a younger forest than Carpenter (30 vs. 60 y). Both larger NPRs and net NH_4^+ mineralization rates were observed in F soil samples at Lookout than at Carpenter. Although the overall differences between M and F sites were anticipated based upon findings of other comparative forest and non-forest studies (Schimel et al., 1989; Killham, 1990; Tietema et al, 1992; Stienstra et al., 1994; De Boer et al., 1996; Pederson et al., 1999; Priha et al., 1999; Ste-Marie et al., 1999; Zhu et al., 1999; Brierly et al., 2001), these differences in N processes provide a backdrop for examining if community composition differences among ammonia-oxidizing bacteria can be linked to N processes.

Because I was interested in the possibility that community profiles might change along gradients connecting different soil environments, I chose to use *amoA* PCR primers known for their fine-scale resolution of closely related ammonia oxidizers (Rotthauwe et al., 1997; Purkhold et al., 2000). Because these primers target the catalytic subunit of the functional ammonia monoxygenase gene, *amoA*, amplified sequences provide an effective tool for exploiting differences between species of closely related ammonia oxidizers.

T-RFLPs of all samples treated with *TaqI* produced a single peak representing a 283-bp fragment, indicating the predominance of *Nitrosospira* and/or *Nitrosospira*-like organisms at both sites (Horz et al., 1999). This finding is in accord with many studies that show the ubiquity of this genus in terrestrial ecosystems (Stephen et al., 1996; Hiorns et al., 1995; Kowalchuck et al., 1997; Mendum et al., 1999). It has also been shown that these *amoA* primers are not biased towards *Nitrosospira* over *Nitrosomonas* species (Rotthauwe et al., 1997), supporting the findings that a single genus of ammonia-oxidizing bacteria inhabited the mineral soil from both experimental sites.

T-RFLP patterns provided evidence for the presence of a homogenous core community at both sites and evidence for the presence of other types of ammonia oxidizers at various locations across the transects. Information that was complementary to the T-RFLP patterns came from sequencing the *amoA* 491-bp product obtained from isolates. Sequence analyses from 4 isolates and 2 clones showed them to be distinct from *amoA* sequences in the Genbank database. One of these sequences (analyzed from an isolate) contained the T-RFLP pattern found in the dominant member distributed throughout both sites (*CfoI* 66 and *AluI* 392). Phylogenetically, this ammonia oxidizer

shared 99.8% similarity to *Nitrosospira sp.* Ka4, originally isolated from a lead contaminated soil in Norway (Åakra et al., 1999), and is often characterized as a cluster 4 ammonia oxidizer (Purkhold et al., 2000). Interestingly, the other three isolates that were sequenced all shared greatest similarity to *Nitrosospira sp.* Nsp1 (94 and 97%), a cluster 3 ammonia oxidizer first isolated from soil in Sardinia (Koops et al., 1985).

Because we recovered representatives of Ka4 from terminal soil dilutions of MPN assays, we assume this lineage is a major component of the ammonia oxidizer community in these environments. It is intriguing to speculate that Ka4 is a major contributor to nitrification in these soils. Several studies have measured gross rates of nitrification in soils under coniferous forests of varying ages using ^{15}N isotope dilution. Gross rate values of 100 mg-N/ m^2/d have been routinely reported in the literature (Hart et al., 1994). Such a rate equates to about 40 ng g^{-1} soil h^{-1} which is similar in magnitude to the NPRs of 6 and 20 ng g^{-1} OD soil h^{-1} measured in forest soil samples at Carpenter and Lookout, respectively. If I assume a rate of ammonia-oxidation by *Nitrosospira* sp. of about 10 x 10^{-15} mol $cell^{-1}$ h^{-1} (Schmidt and Belser, 1980; Jiang and Bakken, 2001) the above-mentioned gross rates of nitrification could be supported by populations of about 4 x 10^4 cells g^{-1} OD soil. This value is approximately 10-fold greater than the average MPN values obtained for forest soil samples (see materials and methods). A similar calculation carried out on M soil samples gave a population estimate of about 3 x 10^6 cells g^{-1} OD soil. Again, this value is about 10-fold greater than the MPN estimate reported in this manuscript. Both Phillips et al. (2000) and Kowalchuk et al. (2000b) have shown that the MPN underestimates by 10-500 times the population size of ammonia-oxidizing bacteria

estimated using quantitative PCR. It is possible that other populations are involved in nitrification in these soils. In this context, however, I have observed that representatives of the Ka4 lineage grow very slowly in the medium used for isolation and for determining MPNs. Therefore Ka4-like organisms may be the major contributors to ammonia-oxidation in these soils, and their populations are simply underestimated by the traditional MPN procedure because they can not achieve a density sufficient to produce measurable NO_2^-. Indeed, Jiang and Bakken (2002) showed that *Nitrosospira* AF produced NO_2^- at a similar rate per cell as other nitrosospirads despite having a generation time that is twice as long as isolates (B6, 40KI, LII5) used in that study.

The identification of clone #14 and cluster 3 type ammonia oxidizers with T-RFLP patterns similar to those patchily distributed across the sites raises issues about their role in nitrification. Typically, cluster 3 organisms are found to dominate in soils (Hiorns et al., 1995; Stephen et al., 1996, 1998; Bruns et al., 1999; Mendum et al., 1999; Phillips et al., 2000), and make up the majority of cultured *Nitrosospira* representatives (Phillips et al., 2000; Webster et al., 2002). In three grassland studies, population shifts from predominantly cluster 3 to cluster 4 ammonia oxidizers occurred in response to prolonged periods without fertilizer use (Bruns et al., 1999; Kowalchuck et al., 2000a, 2000b). These shifts seemed to correspond with increased plant diversity and decreased N mineralization. It was hypothesized that reduced concentrations of available ammonia may select for cluster 4 ammonia oxidizers which must compete effectively with heterotrophs and plant roots for limited substrate (Woldendorp et al., 1989; Kowalchuck et al., 2001). In the context of this hypothesis it should be noted that the Ka4 type of *Nitrosospira* was prevalent throughout both M and F environments

that were widely different in N availability. For example, N mineralization from M soils of Carpenter and Lookout proceeded rapidly to NO_3^- at rates of about 1-2μg NO_3^--N/g/d, while a much lower net rate of NH_4^+ production accompanied by undetectable net NO_3^- accumulation occurred over 25 d in Lookout F samples. Zero net production of both NH_4^+ and NO_3^- occurred over 25-35 d in Carpenter F samples (Heichen and Cromack, unpublished observations). Obviously, N availability is not the only factor accounting for the widespread occurrence of Ka4 across these sites, nor is N limitation the sole reason for restricted distribution of group 3 nitrosospirads. Further work needs to be done to determine the relative population densities of Ka4 and the group 3 representatives in the locations where they occur together, and their relative contributions to the gross rates of nitrification in these environments.

Finally, the fingerprint of the Ka4 lineage was found throughout F soil samples where NPRs were < LLD, and where net NO_3^- accumulation was barely detected after 80 d incubation. These data indicate that Ka4 possesses superior persistence and survival abilities under conditions sub-optimal for nitrifying activity. Several studies have shown that physiological differences exist among isolates of ammonia-oxidizing bacteria (Belser et al., 1978; Åakra et al., 1999; Jiang et al.,1999; Koops et al., 2001). Further research is needed to determine what physiological attributes might account for the persistence of Ka4 in F soils at these sites.

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APPENDIX

A1. *CfoI* and *AluI* T-RFLP Distribution for Soil *amoA* PCR Products.

			Fragment Lengths in Base Pairs											
			CfoI						*AluI*					
Lookout Carp		Vegetation	66		68		100		135		392		491	
1	25	M	L	C					L	C	L	C		C
2	26	M	L	C			L		L		L	C	L	
3	27	M	L	C					L		L	C	L	
4	28	M/F	L	C			L		L	C	L	C		
5	29	F/M	L	C							L	C	L	C
6	30*	F	L						L		L			
7	31	F	L	C							L	C		
8	32	F	L	C		C				C	L	C		
9	33	M	L	C			L		L	C	L	C	L	
10	34	M	L	C					L		L	C	L	
11	35	M	L	C		C			L	C	L	C	L	C
12	36	M/F	L	C					L	C	L	C	L	C
13	37	F/M	L	C				C		C		C		
14	38	F	L	C		C			L	C	L	C		
15	39	F	L	C								C		
16	40	F	L	C							L	C		C
17	41	M	L	C							L	C		
18	42	M	L	C						C	L	C		
19	43	M	L	C		C				C	L	C		C
20	44	M/F	L	C					L	C	L	C		C
21	45	F/M	L	C	L			C		C	L	C	L	C
22	46	F	L	C				C			L	C		
23	47	F	L	C							L	C		
24	48	F	L						L		L	C		

*No PCR products obtained.

A2. *amoA* Sequences for Novel Ammonia-Oxidizing Isolates and Clones.

CT2F enrichment (pH 6.2, 10 mM $NH4^+$) 5′→3′ *CfoI* 66 bp *AluI* 392 bp

GGGGTTTCTACTGGTGGTCGCACTACCCCATCAACTTCGTCTTTCCCTCCAC
CATGATACCTG**GCGC**CCTCATCATGGACACCGTCCTGCTGCTCACCCGTAA
CTGGATGATCACCGCACTGGTCGGCGGCGGCGCATTCGGCCTGCTGTTCTAC
CCGGGCAACTGGCCCATTTTTGGACCGACGCACCTGCCACTGGTAGCCGAA
GGCGTATTGCTCTCCCTGGCTGACTACACCGGCTTCCTGTATGTACGCACCG
GCACGCCTGAATACGTACGGCTGATCGAACAAGGCTCGCTGCGCACCTTTG
GCGGCCACACCACCGTCATCGCGGCCTTCTTCTCCGCCTTCGTCTCCATGCT
CATGTTCTGCGTCTGGTGGTACTTTGGCA**AGCT**CTACTGCACCGCCTTCTAC
TACGTCAAAGGCCCCCGCGGCCGAGTCACCATGAAGAACGACGTCACCGCC
TACGGCGAAGAAGGCTTTCCCGAGGGG

LT1FM a,d,e, LT1Fd, LT2MFa enrichment (pH 6.2, Ø NH4+) *CfoI* 66 bp *AluI* 491 bp

GGGGT TTC TACT GGTGGT CGCACTACCCCATCAACTTCGTCTTCCCC
TCCACCATGATACCCG**GCGC**CC TCGTCATGGACACCGTCATGCTGCTCA
CCCGCAACTGGATGATCACAGCCCTGGTTGGTGGCGGCGCATTCGGACT
CCTGTTCTACCCGGGTAACTGGCCCATCTTCGGCCCCACCCACCTGCC
GCTGGTAGCCGAAGGCGTACTGTTGTCGGTTGCCGACTACACCGGCTTCCT
GTACGTTCGCACCGGCACCCCCGAGTACGTACGCAACATCGAACAAGGCTC
ACTCAGAACCTTTGGCGGACACACCACCGTCATCGCCTCATTCTTTGCCGCC
TTCGTCTCCATGCTCATGTTCTGCCTGTGGTGGTACTTCGGCAAACTTTAC
TGCACCGCATTCTTCTACGTCAAAGGGGCCCGTGGCCGCGTCACCATGAA
AAACGACGTCACCGCATTTGGCGAAGAAGGCTTTCCCGAGGGG

LT1FMf *AluI* 34 bp *CfoI* 66 bp

GGGGTTTCTACTGGTGGTCGCACTACCCCATC**AGCT**TCGTCTTCCCCTCCA
CATGATACCCG**GCGC**CCTCGTCATGGACACCGTCATGCTGCTCACCCGCAA
CTGGATGATCACAGCCCTGGTTGGTGGCGGCGCATTCGGACTCCTGTTCTAC
CCGGGTAACTGGCCCATCTTCGGCCCCACCCACCTGCCGCTGGTAGCCGAA
GGCGTACTGTTGTCGGTTGCCGACTACACCGGCTTCCTGTACGTTCGCACCG
GCACCCCCGAGTACGTACGCAACATCGAACAAGGCTCACTCAGAACCTTTG
GCGGACACACCACCGTCATCGCCTCATTCTTTGCCGCCTTCGTCTCCATGCT
ATGTTCTGCCTGTGGTGGTACTTCGGCAAACTTTACTGCACCGCATTCTTCA
CGTCAAAGGGGCCCGTGGCCGCGTCACCATGAAAAACGACGTCACCGCATT
TGGCGAAGAAGGCTTTCCCGAGGGG

LT2Fb 5′→3′ *CfoI* 66 and 68 bp *AluI* 491 bp

(GGGGTTTCTACTGGTGGT.....TT)
CCCCTCCACCATGATACCGG**GCGCGC**TCGTCATGGACACCGTCATGCTG
CTCACCCGCAACTGGATGATCACAGCCTTAGTCGGCGGAGGCGCATTCG
GACTCCTGTTCTACCCGGGTAACTGGCCCATCTTCGGCCCCACCCACCTG
CCGCTGGTAGCCGAAGGCGTACTGTTGTCGGTTGCCGACTACACCGGCTT
CCTGTACGTTCGCACCGGCACCCCCGAGTACGTACGCAACATCGAACAA
GGCTCGCTCAGAACCTTTGGCGGTCACACCACCGTCATCGCCTCGTTCTT
TGCCGCCTTCGTCTCCATGCTCATGTTCTGCCTGTGGTGGTACTTCGGCAA
ACTTTACTGCACCGCATTCTTCTACGTCAAAGGGGCCCGTGCCGCGTCAC
CATGAAAAACGACGTCACCGCATTTGGCGAAGAAGGCTTTCCCGAGGGG

Clone #14R *CfoI* 135 bp *AluI* 491 bp

GGGGTTTCTACTGGTGGTCGCACTACCCCATCAACTTCGTCCTCCCCTCCAC
CATGATCCCGGGTGCCCTCATCATGGACACCGTCCTGCTGCTCACCCGCAAC
TGGATGATCACCGCCCTGGTGGGAGGTG**GCGC**GTTCGGCCTGCTGTTCTAC
CCGGGTAACTGGCCCATATTTGGACCGACGCACCTGCCGCTGGTAGCCGAA
GGCGTATTGCTCTCCCTGGCTGACTACACCGGCTTCCTCTATGTACGCACGG
GCACCCCTGAGTACGTACGGCTGATCGAACAAGGGTCGTTACGCACCTTTG
GCGGGCACACCACCGTCATTGCCGCGTTCTTCTCCGCCTTCGTCTCCATGCT
CATGTTCTGCGTCTGGTGGTACTTTGGCAAACTCTACTGCACCGCCTTCTTC
TACGTCAAAGGACCCCGCGGCCGAGTCACCATGAAGAACGACGTCACCGCC
TACGGCGAAGAAGGCTTTCCAGAGGGG

Clone #26 *CfoI* 100 bp *Alu*I 392 bp

GGGGTTTCTACTGGTGGTCGCACTACCCCATCAACTTCGTCTTCCCCTCCAC
CATGATACCGGGTGCCCTCATCATGGACACCGTCCTGCTGCTCAC**GCGC**AA
CTGGATGATCACCGCCCTGGTGGGAGGCGGCGCCTTCGGCCTGCTGTTCTAC
CCGGGCAACTGGCCCATTTTTGGACCGACGCACCTGCCGCTGGTAGCCGAA
GGCGTATTGCTCTCCCTGGCTGACTACACCGGCTTCCTCTATGTACGCACGG
GCACCCCTGAGTATGTACGGCTGATCGAACAAGGCTCCTTGCGCACCTTTG
GCGGCCACACCACCGTCATTGCCGCATTCTTCTCCGCGTTCGTCTCCATGCT
CATGTTCTGCGTGTGGTGGTATTTTGGCA**AGCT**CTACTGCACCGCCTTCTTC
TATGTCAAAGGTCCCCGTGGCCGAGTCAGCATGAAGAACGACGTTACCGCG
TACGGCGAAGAAGGCTTTCCCGAGGGG

A3. Lookout and Carpenter ANOVAs for NPRs Measured in June 2000.

Site	Vegetation	Rates*	SE
Lookout	M	0.389	0.103
	M/F	0.263	0.047
	F/M	0.059	0.056
	F	0.021	0.010
Carpenter	M	0.383	0.098
	M/F	0.334	0.134
	F/M	0.024	0.002
	F	0.007	0.001

*rates = ($\mu g\ NO_3\text{-}N\ g^{-1}$ OD soil h^{-1})
n = 3

ANOVA Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.57349608	0.39337402	22.38	< 0.001
Error	43	0.75592621	0.01757968		
Corrected Total	47	2.32942228			

R-square	Coeff Var	Root MSE	NP Mean
0.675488	66.72442	0.132588	0.198710

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Region	3	1.57318853	0.52439618	29.83	< 0.001
Site	1	0.00030755	0.00030755	0.02	0.8954

Scheffe's Test for Nitrification Potential Rates.

Alpha	0.05
Error Degrees of Freedom	43
Error Mean Square	0.01758
Critical Value of F	2.82163

Comparisons significant at the 0.05 level are indicated by ***.

Region Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
M - M/F	0.10829	-0.07356	0.29014
M - F/M	0.36181	0.17996	0.54365 ***
M - F	0.38956	0.26097	0.51814 ***
M/F - M	-0.10829	-0.29014	0.07356
M/F - F/M	0.25352	0.03080	0.47623 ***
M/F - F	0.28127	0.09942	0.46312 ***
F/M - M	-0.36181	-0.54365	-0.17996 ***
F/M - M/F	-0.25352	-0.47623	-0.03080 ***
F/M - F	0.02775	-0.15410	0.20960
F - M	-0.38956	-0.51814	-0.26097 ***
F - M/F	-0.28127	-0.46312	-0.09942 ***
F - F/M	-0.02775	-0.20960	0.15410

ANOVA Results for Additional Analyses.

1. No significant site-to-site differences:
   F-statistic = .3745 two-sided p-value = .5437

2. No significant differences between transects (combined sites);
   F-statistic = .0031 two-sided p-value = .9560

2. No significant site:transect interactions:
   F-statistic = .9925 two-sided p-value = .3245

3. No significant site:region interactions:
   F-statistic = 1.273 two-sided p-value >.1000

4. Significant differences between regions:
   F-statistic = 7.991 two-sided p-value = .0006