The Role of Methane- and Ammonia-Oxidizing Bacteria in the Emission of Greenhouse Gases from Agricultural Soils

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Summary

Nitrous oxide production from N-impacted soils is highly influenced by rates of inorganic nitrogen metabolized by the soil microbial community. The processes of nitrification and denitrification convert soil nitrite into nitrous oxide either directly or via nitrate. Although methane-oxidizing bacteria (MOB) are predominantly involved in converting methane to carbon dioxide, their evolutionary linkage to ammonia-oxidizing bacteria (AOB) makes them dynamic players in the oxidation of ammonia and in the production of nitrous oxide. Little to nothing is known about the metabolism of inorganic nitrogen by MOB. Furthermore, ammonia oxidation by AOB has been extensively characterized in only a single bacterial strain. To understand the function of these bacteria in producing or consuming greenhouse gases in soils, techniques must be developed and utilized to directly link the native microbial population structure with its function. Through this project, we completed three objectives to close these gaps in knowledge: 1) we characterized the rates of ammonia and hydroxylamine oxidation to nitrite and the concomitant production of nitrous oxide by pure MOB cultures, 2) we characterized hydroxylamine oxidoreductase genes and rates of ammonia oxidation in two AOB species, and 3) we optimized and applied molecular tools and traditional techniques to link diversity, abundance, and function of AOB in native, N-impacted, forest soils. Our results demonstrated that each of four MOB species, even those with close taxonomic relationships, metabolized nitrogen differently. The two AOB species studied had similar hydroxylamine oxidoreductase gene structure, but not surprisingly, their metabolisms were highly tuned to their optimal growth conditions. Last, we demonstrated our ability to correlate AOB community structure with function in native N-impacted soils using 16S rDNA gene clone libraries, quantitative PCR, and nitrification activity assays. Accomplishing these objectives has brought us a large step forward towards understanding and quantifying the specific roles of MOB and AOB in soil nitrogen transformations. Using the techniques mastered and knowledge generated through this study, MOB and AOB populations and their roles in greenhouse gas production and consumption in soils can be directly targeted.

Objectives

The original goal of this project was to determine the mechanism of greenhouse gas production by methane- oxidizing bacteria (MOB) and ammonia-oxidizing bacteria (AOB) in agricultural soils. However, we quickly found difficulties with our experimental design and interpretation because little to nothing was known about how these bacteria actually processed inorganic

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nitrogen. Thus, our research focus changed from greenhouse gas production to simply inorganic nitrogen metabolism by these two bacterial groups. We accomplished three objectives:

Objective 1: We characterized the physiological responses of pure MOB and AOB cultures to changes in methane (for MOB), ammonia and hydroxylamine concentrations.

Objective 2: We optimized molecular tools for determining AOB population diversity and abundance within complex soil communities.

Objective 3: We applied activity measurements and molecular tools to samples of native N-impacted soils to correlate AOB population diversity, abundance, and activity with differing levels of soil nitrogen and rates of nitrification.

Approach and Procedures

Physiology of Cultivated Bacterial Cells

1. *Methane oxidizing bacteria*. Batch cultures (100ml) of *Methylomicrobium album* (Type I), *Methylomonas rubra* (Type I), *Methylosinus trichosporium* (Type II) and *Methylosinus sporium* (Type II) were grown in nitrate mineral medium at pH 6.8 under 50-50% air-CH4 at 30˚C. The cells were harvested in mid-log phase by centrifugation (8,000 g, 8 min), washed in HEPES buffer (10 mM, pH 6.8) and resuspended in the same buffer (1 ml). Cell numbers were determined by light microscopy using a Petroff-Hausser counting chamber. A total of 109 cells were added to HEPES buffer to 1 ml final volume in 10 ml glass vials, which were sealed with butyl rubber stoppers and aluminum crimp seals. The appropriate amount of substrate was added to the liquid phase and methane was added to the headspace where required. Vials were incubated at 30˚C with shaking (200 rpm). Nitrite production from ammonia and/or hydroxylamine oxidation was measured colorimetrically after 24 h (Hageman and Hucklesby 1971). Rates of N_2O production were measured after 0, 30, 60, 120 and 180 min by gas chromatography.
2. *Ammonia oxidizing bacteria*. *Nitrosospira multiformis* (ATCC 25196) and *Nitrosomonas europaea* (ATCC 19718) were grown in defined mineral media (250 ml). Cells were harvested at mid- or late-log phase of growth by centrifugation, washed with sodium phosphate buffer (50mM NaH_2PO_4, 2mM $MgCl_2$, pH 7.5 for *Ns. multiformis* and pH 8 for *Nm. europaea*), resuspended in the same buffer (1 ml), and placed on ice prior to measuring activities. Cells were enumerated by light microscopy with a Petroff-Hausser counting chamber. Known numbers of cells were placed into the reaction chamber of a Clark-style oxygen electrode containing sodium phosphate buffer at selected pH and temperature. Ammonium- and hydroxylamine-dependent oxygen consumption activities were measured in the electrode chamber as described elsewhere (Stein and Arp 1998). In vitro hydroxylamine oxidation activity was measured as described previously (Frear and Burrell 1955). Genomic DNA from cells of *Ns. multiformis* and *Nm. europaea* was digested, blotted, and probed for hao genes using standard protocols (Sambrook and Russell 2001).

Ecology of AOB in Native Soils

1. *Population diversity*. Soil samples (250 g ea.) were collected at three sites along a well-documented gradient of nitrogenous pollutants within a mixed coniferous forest of the San Bernardino Mountains (Fenn and Bytnerowicz 1993). The sites were Camp Paivika (CP), Strawberry Peak (SP), and Dogwood (DW) and the dates of collection were March 2002, September 2002, and February 2003. Composite samples were made for each site on each collection date and sieved to 2 mm. DNA was extracted directly from the soil using a kit. The DNA was purified further using an additional column from the kit and the resulting product was diluted 1:10 with NANO-Pure water. For positive controls, genomic DNA was extracted from pure cultures of *Nitrosomonas europaea* and *Nitrosospira multiformis* using a genomic DNA isolation kit. Polymerase chain reaction (PCR) of 16S rRNA genes was performed with βAMOf and βAMOr primers (McCaig et al. 1994). PCR products were cloned into *E. coli* TOPO10 cells. Insert-containing colonies were selected and screened using a second PCR reaction with internal primers (CTO189f-GC and CTO654r) (Kowalchuk et al. 1997). Clones yielding positive amplification were classified by gene sequencing and/or oligonucleotide hybridization. For oligonucleotide hybridization, purified plasmid was transferred onto membranes and probed with *Nitrosospira* cluster 2-, 3-, or 4-specific oligonucleotides (Stephen et al. 1998).
2. *Population abundance*. Quantitative PCR (qPCR) was carried out using the primer and probe set developed previously (Hermansson and Lindgren 2001). The annealing temperature of the assay was optimized using both *N. multiformis* and *N. europaea* genomic DNA on the gradient function of the iCycler to maximize specificity of the reaction and ensure that the majority of ß-Proteobacteria AOB target was detected in the samples. Soil DNA extracted using a kit was further purified by gel filtration on Sephadex G-100 columns. Total DNA was quantified immediately prior to performing qPCR by comparing the intensity of ethidium bromide stained bands on agarose gels to a dilution series of 1 kb ladder. Quantification of autotrophic AOB abundance in each sample was determined in replicate reactions against a standard curve of 150 fg-1.5 ng *Nitrosospira multiformis* genomic DNA. The detection limit of the assay was 500 fg, or ca. 0.001% of total extracted DNA. Three to four separate soil DNA preparations were analyzed in triplicate from each site. In order to rule out PCR inhibition by material in the sample, a known concentration of genomic DNA from *N. multiformis* was mixed with one replicate soil DNA preparation for each experiment. Due to the generally low numbers of autotrophic AOB in the soil DNA preparations, some reactions failed to generate a threshold cycle even through the control sample spiked with *N. multiformis* DNA amplified with high efficiency (>80%). For these reactions, we assumed that the lower limit of detection was an accurate representation of the population and included this number in calculations of the mean percentage autotrophic AOB out of total DNA extracted from each soil sample.
3. *Population activity*. Six replicate soil samples (5 g) for each site were placed into glass bottles (125 ml), washed with sodium phosphate buffer (1mM; pH 7.2) and collected by centrifugation to remove soluble nitrate. The washed soils were resuspended in 50 ml of the same buffer containing NH_4Cl (1.5 mM). The slurries were shaken continuously in the dark at 25˚C for three to five days. The pH was adjusted to 7 with NaOH every day over the course of the experiment. Half of the samples were treated with 1% (vol/vol) acetylene to

inactivate autotrophic ammonia oxidation activity (Hyman and Wood 1985). Aliquots of slurry (2 ml) centrifuged and analyzed for nitrite plus nitrate ions. Ammonia concentrations were measured at time 0 and after 30 days for triplicate (50 g) composite soil samples from each site collected in September 2002. The samples were amended with 200 mg kg^{-1} $(NH_4)_2SO_4$ and maintained at field capacity moisture in foil-covered glass flasks.

Results

Inorganic Nitrogen Metabolism by Methane-Oxidizing Bacteria

Methanotrophs and ammonia-oxidizers harbor evolutionarily related monooxygenase enzymes, particulate MMO (pMMO) and AMO respectively, and both bacteria oxidize methane and ammonia (Holmes et al. 1995). We investigated the ability of four methanotrophic strains to oxidize ammonia to nitrite both in the presence and absence of methane. In the presence of methane, ammonia is presumably oxidized cometabolically, i.e., reductant required for the process is derived from the oxidation of methane. In the absence of methane, reductant for ammonia oxidation must come from other sources, e.g., NADH. All of the strains were capable of oxidizing ammonia to varying degrees; however *M. album* and *M. trichosporium* oxidized significantly more ammonia in the absence of methane than *M. rubra* or *M. sporium* (*fig. 1*). The relatively large quantity of ammonia oxidized by *M. trichosporium* in the absence of methane indicated that this strain had a larger pool of endogenous reductant to support ammonia oxidation. The inclusion of methane resulted in a competition with ammonia for access to pMMO. With 1mM ammonia, methane was preferentially metabolized, but with 5mM ammonia, there was significant ammonia oxidation by all of the strains except for *M. rubra*. Incidentally, *M. rubra* had low ammonia oxidizing activity under all conditions tested, indicating that this strain either did not have an ammonia uptake mechanism or a pathway to metabolize it.

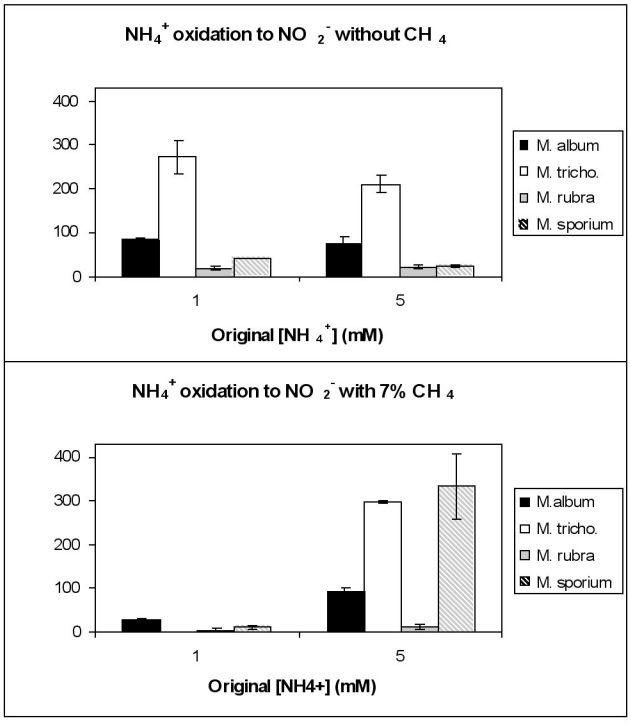

Figure 1. Comparison of nitrite production from lmM or 5mM ammonia by four species of methanotrophic bacterial after 24h incubation at 30^{oC} in HEPES buffer (10nM, pH 6.8)

Next, we compared the rates of hydroxylamine oxidation to nitrite by the four methanotrophic strains since hydroxylamine is an intermediate in the oxidation of ammonia to nitrite. Based on the ammonia oxidation results, we expected only M. rubra to be incapable of hydroxylamine oxidation; however, *M. sporium* was also incapable of this metabolism (*fig. 2*). This result

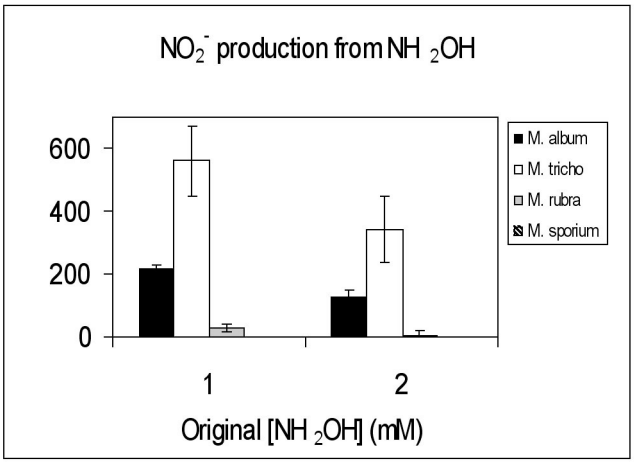

Figure 2. Comparison of nitrite production from ImM hydroxylamine by four species of methanotrophic bacteria after 24h incubation at 30^{oC} in HEPES buffer (10nM, pH 6.8)

the presence of 1mM hydroxylamine and assaying for nitrous oxide production over a 3h time course. *M. album* and *M. trichosporium* produced substantial quantities of nitrous oxide, although *M. album* produced more than *M. trichosporium*, the opposite trend of their ammonia and hydroxylamine oxidizing activities (*fig 3*). This result is logical because nitrous oxide production allows for the removal of toxic hydroxylamine from the cell. By this argument, more nitrous oxide would be produced by *M. album* than *M. trichosporium* from hydroxylamine because its oxidation by *M. album* was significantly slower. In contrast, *M. rubra* and *M. sporium* produced little to no nitrous oxide, matching their inability to oxidize hydroxylamine. These results indicated that hydroxylamine metabolism by methanotrophic bacteria is directly linked to their production of nitrous oxide, similar to that found in ammonia oxidizing bacteria. Furthermore, the ability of each methanotrophic strain to metabolize inorganic nitrogen appears to be quite different and does not follow taxonomic relationships. suggests that either the levels of hydroxylamine used were toxic especially to *M. sporium* or, like *M. rubra*, the nitrogen metabolism of this strain is different. Expectedly, hydroxylamine oxidation by *M. album* and *M. trichosporium* was consistent with their abilities to oxidize ammonia.

Nitrous oxide production during ammonia oxidation in ammonia-oxidizing bacteria is thought to be a secondary product from hydroxylamine oxidation (Hooper et al. 1997). Therefore, methane-oxidizing bacteria should also produce nitrous oxide in the presence of substantial quantities of hydroxylamine. We tested this hypothesis by incubating the four methanotrophic strains in

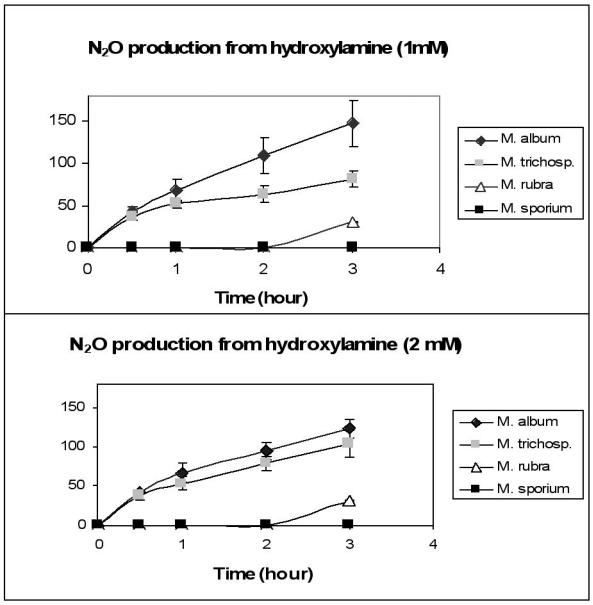

Figure 3. Comparison of rates of nitrous oxide production by four strains of methanotrophic bacteria from ImM or 2mM hydroxylamine. Cells were incubated at 30^{oC} in HEPES buffer (10nM, pH 6.8)

Comparison of Central Metabolic Activity of Nitrosomonas europaea *and* Nitrosospira multiformis

The primary O_2-consuming activities in ammonia oxidizers, as measured by oxygen electrode, include ammonia oxidation to NH2OH by ammonia monooxygenase (AMO) and reduction of ½ O_2 to H_2O by AMO and the terminal oxidase (Wood 1986). Upon addition of allylthiourea, oxygen consumption in both *Nm. europaea* and *Ns. multiformis* ceases due to loss of AMO activity and does not resume until a substrate for hydroxylamine oxidoreductase (HAO) is added (Bédard and Knowles 1989). Thus, NH_4^+-dependent oxygen consumption represents the activities of AMO and the terminal oxidase, while NH_2OH-dependent oxygen consumption represents mainly terminal oxidase activity. Resting cell assays (i.e., non-growth conditions) were used in the following experiments to tightly control environmental conditions and subsequent physiological responses of the cells. This approach provided immediate and accurate measures of the responses of *Nm. europaea* and *Ns. multiformis* cells to environmental stimuli without continuous changes in growth phase, pH, substrate availability, and product accumulation that occur in batch cultures.

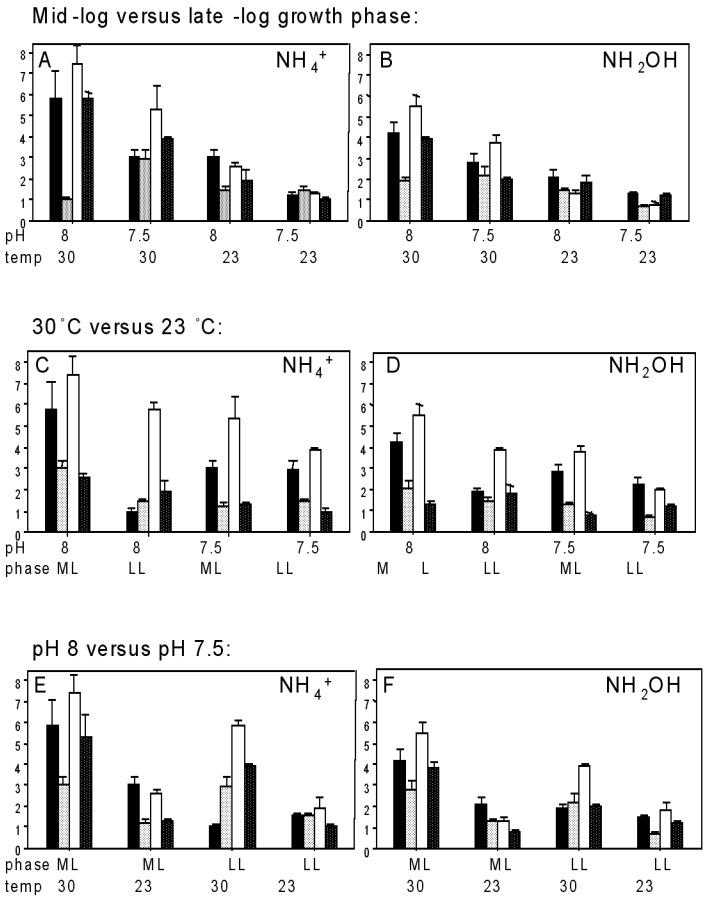

Fig. 4. Comparisons of NH_4^+- and NH_2OH-dependent oxygen consumption rates by cells exposed to different environmental conditions. Rates on y-axis are nmol oxygen consumed $\cdot min^{-1} \cdot 10^8$ cells $^{-1}$. Comparison of NH_4^+- dependent oxygen consumption activities (panels A, C, E) and NH_2OH-dependent oxygen consumption activities (panels B, D, F) are shown at indicated pH, temperature, and/or growth phase of: (■ *Ns multiformis* cells in mid-log growth (A&B), at 30 C (C&D), and at pH 8 (E&F); ( ) ■ *Ns. multiformis* cells in late-log growth (A&B), at 23 C (C&D), and at pH 7.5 (E&F); ( ) □ *Nm. europaea* cells in mid-log growth (A&B), at 30 C (C&D), and at pH 8 (E&F); and ( ) ■ *Nm. europaea* cells in late-log growth (A&B), at 23 C (C&D), and at pH 7.5 (E&F). ML = mid-log, LL = late-log phase of growth.

Cultures of *Nm. europaea* grow optimally at 30˚C and at an initial pH of 8, whereas *Ns. multiformis* grows optimally at 23˚C (they can not be maintained at 30˚C) and no higher than pH 7.5. Despite the differences in optimal culturing conditions, both *Ns. multiformis* and *Nm. europaea* cells responded similarly to each test condition of different pH, temperature, and growth phase, although the responses were of different magnitudes (*fig. 4*). Generally, both genera exhibited decreased activity levels at: 1) late- versus mid-log phase of growth (*fig. 4 A&B*), 2) 23˚C versus 30˚C (*fig. 4 C&D*), and 3) pH 7.5 versus pH 8 (*fig. 4 E&F*). *Ns. multiformis* was most affected by growth phase and had the greatest incremental loss of activity when comparing mid- to late-log phase cells measured at pH 8 and 30˚C ($P<0.05$ by t-test) (*fig. 4 A&B*). In

contrast, the activity of *Nm. europaea* cells were most affected by temperature, showing significant decreases in activity from 30˚C to 23˚C regardless of growth phase or pH ($P<0.05$) (*fig. 4 C&D*). Some exceptions to these general trends were evident, for example NH2OH-dependent oxygen consumption rates were slightly higher for late- than mid-log phase cells of *Nm. europaea* at 23˚C (*fig. 4B*), and late-log phase cells of *Ns. multiformis* had higher NH4+-dependent oxygen consumption activity at 23 versus 30˚C at pH 8 (*fig. 4C*) and at pH 7.5 versus pH 8 at 30˚C (*fig. 4E*). Because the measurement of in vivo oxygen consumption activities involved multiple enzymatic processes, we also measured the in vitro activity of HAO in mid- and late-log phase cells to confirm whether NH_2OH-dependent rates of oxygen consumption in vivo reflected realistic trends in activity levels of the cells. Cells of both genera in mid-log phase of growth had similar levels of in vitro NH_2OH-oxidizing activity. However, *Ns. multiformis* cells in late-log phase had significantly less activity (18% of mid-log values) than cells of *Nm. europaea* (35% of mid-log values).

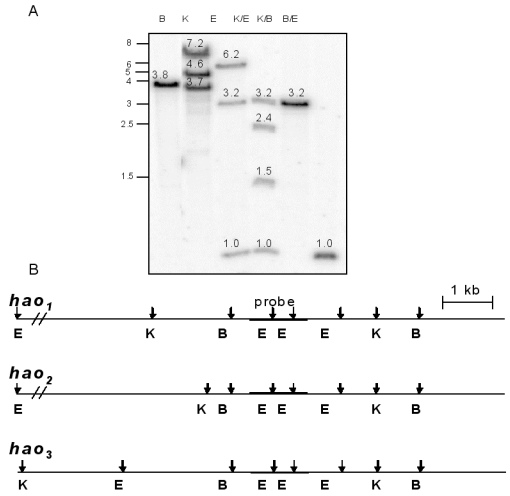

Fig. 5. (A) Southern blot hybridizations of radiolabeled probe specific to *hao* genes of *Ns. multiformis* hybridized to genomic DNA of *Ns. multiformis* digested with one or two restriction enzymes. B = *Bam*HI, K = *Kpn*I, or E = *Eco*RI. Ladder is in kilobase pairs. (B) Map of radiolabeled bands from panel (A) showing restriction sites for the 3 putative copies of the *hao* gene. Double slashed lines represent c.a. 1 kilobase pair.

The general metabolic similarities of *Ns. multiformis* and *Nm. europaea* cells stimulated our examination of hao genes, which are present in three nearly identical copies in *Nm. europaea* and encodes hydroxylamine oxidoreductase, the second enzyme in the pathway for oxidizing ammonia to nitrite (Hommes et al. 2002). Southern blot hybridization with a probe for the *Ns. multiformis* hao gene and mapping of restriction patterns near the probe site revealed that *Ns. multiformis* also harbors three highly similar copies of the hao gene. (*fig. 5*). Based on the position of restriction sites, it appeared that regions upstream of the hao probe sequence were less well conserved than downstream regions among all three copies (*fig. 5B*).

Ecology of Ammonia-Oxidizing Bacteria in an N-Saturated Forested Ecosystem

Previous studies showing high nitrification rates in N-saturated forest soils of the San Bernardino Mountains (Fenn et al. 1996) prompted our investigation of the diversity of autotrophic AOB in this ecosystem. We hypothesized that acid-tolerant AOB (Nitrosospira cluster 2) or those generally found in high-ammonium content soils (Nitrosospira cluster 3) would preferably occupy the more highly N-impacted site at Camp Paivika (CP) relative to the less impacted site at Dogwood (DW). Because the available primer sets for amplifying ß-Proteobacteria AOB from environmental samples are not highly specific, we used a nested primer set to obtain a high proportion of autotrophic AOB gene clones. We first PCR-amplified and cloned 1.1 kb fragments of 16S rRNA genes from CP, SP, and DW soils sampled in March 2002 (McCaig et al. 1994) and then re-amplified the cloned products with internal primers (Kowalchuk et al. 1997). The proportion of cloned genes conferring positive re-amplification with the internal primers

from two separate soil DNA extractions ranged from 20-50% at CP, 70-80% at SP and 50-80% at DW. One hundred and eighty four clones that re-amplified with internal ß-Proteobacteria AOB primers were selected at random for additional classification by either direct sequencing or oligonucleotide hybridization. For the sequenced genes, all but 3 of 45 1.1 kb sequences shared 98-100% homology to cultivated Nitrosospira isolates from soils and affiliated with clusters 2, 3 or 4. More than half of the sequences were affiliated with Nitrosospira cluster 4 isolate Ka3. None of the sequences clustered with Nitrosomonas or Nitrosomonas-like species. Oligonucleotide hybridization with probes specific to Nitrosospira clusters 2, 3, and 4 (Stephen et al. 1998) allowed classification of the remaining clones. The combination of gene sequence and oligonucleotide hybridization classification of the cloned genes revealed the relative distribution of Nitrosospira clusters among the three sampled sites (*table 1*). Only 20% of the clones analyzed were related to Nitrosospira cluster 2 and were evenly distributed among the sites despite significant differences in soil pH. There appeared to be an equal distribution of cluster 3 and 4 related sequences in CP soils suggesting a possible trend towards cluster 3-like sequences when compared to clone distribution from the other two soils. The clones unrelated to Nitrosospira clustered outside of the AOB group but within the β-Proteobacteria as determined by partial or complete gene sequencing (data not shown).

Table 1. *Affiliation of cloned 16S rRNA genes with Nitrosospira spp. gene clusters* [a].

Study Sites	Total [b]	Cluster 4 (%) [c]	Cluster 3 (%)	Cluster 2 (%)	Non-AOB [d]
Camp Paivika	53	24 (45.3)	23 (43.4)	5 (9.4)	1 (1.9)
Strawberry Peak	73	42 (57.5)	16 (21.9)	11 (15.1)	4 (5.5)
Dogwood	58	37 (67.3)	15 (27.3)	4 (7.3)	2 (3.4)

[a] Cloned PCR products were either hybridized with oligonucleotides corresponding to the three dominant terrestrial clusters of *Nitrosospira* spp. and/or directly sequenced.

[b] Total number of clones derived from each soil sample conferring positive PCR amplification signal.

[c] Values in parentheses represent the percent out of the total number of gene clones for that site.

[d] Non-Nitrosospira species were all within the ß-Proteobacteria as determined by partial or complete sequencing.

Due to the limited number of cloned genes examined per soil and the non-quantitative nature of clone library construction and analysis, statistical differences in autotrophic AOB diversity among the three sites could not be inferred. Thus, we investigated the abundance of autotrophic AOB as a possible mechanism for differences in nitrification rates previously measured in CP versus DW soils (Fenn et al. 1996, Korontzi et al. 2000). We chose to estimate the abundance of autotrophic AOB at each site by real-time quantitative PCR using a probe and primer set for 16S rDNA developed previously (Hermansson and Lindgren 2001). The measurements had very large ranges and revealed no significant differences in autotrophic AOB abundances amongst the CP, SP, and DW soils (*table 2*).

Table 2. *Abundance of autotrophic AOB in each soil as determined by qPCR.*

	% of Total [a]		**Total # of Reactions : # BDL [b]**	
Study Sites	Mar 2002	Feb 2003	Mar 2002	Feb 2003
Camp Paivika				
Mean	0.39	0.11	12:4	10:3
Std.dev.	0.68	0.11		
Range	0.001-2.2	0.001-0.3		
Strawberry Peak				
Mean	0.03	0.01	12:3	9:3
Std.dev.	0.05	0.01		
Range	0.001-0.11	0.001-0.03		
Dogwood				
Mean	0.10	0.13	8:0	11:1
Std.dev.	0.19	0.14		
Range	0.004-0.54	0.003-0.45		

[a] Data represent the percentages of genomic autotrophic AOB DNA out of the total extracted soil DNA (ng autotrophic AOB DNA/ng soil DNA) based on standard curves generated with Nitrosospira multiformis genomic DNA as described in Materials and Methods. Only reactions in which control samples spiked with N. multiformis DNA had >80% recovery and also correlated to standard curves with > 90% amplification efficiency are reported. Outliers are not included in the data set.

[b] BDL = below detection limit. Values for these samples are included in the mean and range values reported for each site as 0.001% of the total soil DNA, i.e., the lower limit of detection.

Since no obvious differences in autotrophic AOB diversity or abundance were observed amongst the sites, we directly measured the specific contribution of autotrophic AOB activity to gross nitrification potential rates in the three soils collected in both September 2002 and February 2003. The soils were washed prior to incubation to remove 70-90 % of the nitrate such that the amount remaining prior to starting the experiments was c.a. 4, 2, and 0.5 mg · kg dry soil-1 for CP, SP, and DW soils collected in Sept 2002, and 6.2, 3, and 0.8 mg · kg dry soil-1 for the same soils collected in February 2003. Optimal nitrification conditions for autotrophic AOB, i.e., plentiful ammonia by controlling solution pH at 7 and oxygen availability by constant shaking, were maintained in the slurries. The activity of autotrophic AOB was inactivated in replicate soil slurries by the inclusion of acetylene, a specific inactivator of ammonia monooxygenase (Hyman and Wood 1985). Nitrite plus nitrate accumulation from acetylene treated and untreated slurries was measured over three to five days to determine the rates of PNA (*table 3*). Significantly more nitrite plus nitrate accumulated over time in CP and SP slurries compared to DW (c.a. 4- to 10-fold difference) both with and without acetylene treatment. As a positive control for native populations of autotrophic AOB activity in a field soil, triplicate slurries of sandy soils from an N-fertilized, irrigated, turfgrass ecosystem produced nitrite plus nitrate at over 10 times the rate of any of the forest soils and produced no nitrite or nitrate in the presence of acetylene (data not shown).

Table 3. *Change in nitrite plus nitrate accumulation in soil slurries over time and change in ammonium concentrations over 30 days in static soils.*

Study Sites	PNA Rates in Soil Slurries[a]						ΔNH_4^{+b}
	September 2002			February 2003			
	-acetylene	+acetylene	AOB PNA[c]	-acetylene	+acetylene	AOB PNA[c]	
Camp Paivika	0.46 (0.09)	0.33 (0.10)	0.13	2.68 (0.09)	2.68 (0.07)	0.004	+3.30 (2.1)
Strawberry Peak	0.71 (0.09)	0.51 (0.05)	0.20	3.02 (1.35)	2.73 (0.20)	0.29	+2.90 (0.6)
Dogwood	0.12 (0.02)	0.05 (0.01)	0.06	0.74 (0.34)	-0.11 (0.06)	0.85	- 0.90 (0.4)

[a]Rate of accumulation: mg soluble NO_2^- plus NO_3^- · kg $soil^{-1}$ day^{-1} with standard deviation in parentheses. Rates determined over 5 days (September 2002) or 3 days (February 2003) in pH-controlled soil slurries containing ammonium and with or without acetylene.

[b]Change in mg NH_4+ · kg $soil^{-1}$ day^{-1} with standard deviation in parentheses. Soils (3 replicates) collected in Sept. 2002 were incubated for 30 days with 200 mg NH_4+ and maintained at field capacity moisture, as described in Materials and Methods.

[c]Contribution of autotrophic AOB to PNA rates in mg soluble NO_2^- plus NO_3^- · kg $soil^{-1}day^{-1}$. Rates were calculated as the difference between PNA in slurries without acetylene minus PNA in slurries with acetylene.

The contribution of autotrophic AOB to total potential nitrification activity was calculated by subtracting the rate of PNA in acetylene treated from untreated slurries (*table 3*). The contribution of autotrophic AOB to overall nitrification rates was low and not significantly different among the three soils collected at either sampling date; however, over both sampling dates the average relative contribution of autotrophic AOB to PNA was 14, 20, and 83% for CP, SP, and DW soils, respectively. The change in ammonium content in CP, SP, and DW soils amended with 200 mg ammonium · kg $soil^{-1}$at native pH and field moisture content was also measured (*table 3*). The accumulation of ammonium in CP and SP soils indicated higher rates of N-mineralization over ammonia oxidation, whereas DW soil had a net loss of ammonium. These results suggested that inorganic ammonia was not necessarily the substrate for nitrite plus nitrate accumulation in CP or SP slurries, but was a likely substrate in DW slurries. This assumption is consistent with the relative contribution of autotrophic AOB to overall nitrification activity in the three soils.

Discussion

AOB and MOB are extremely important organisms in relation to both the nitrogen and carbon cycle in soils. This Kearney project investigated inorganic nitrogen metabolism in both MOB and AOB species and developed a suite of techniques to link the structure and function of AOB populations in complex soil ecosystems. The information and techniques developed through this

study has enabled further exploration of the structure and function of MOB and AOB communities in soils and their roles in greenhouse gas fluxes.

In our first objective, we characterized ammonia and hydroxylamine oxidation and nitrous oxide production by four strains of methanotrophic bacteria. *M. trichosporium* and *M. sporium* are closely related to each other by 16S rRNA phylogeny as are *M. rubra* and *M. album* (Hanson and Hanson 1996). However, the ability of the strains to metabolize inorganic nitrogen was not shared between the close relatives. *M. sporium* and *M. rubra* did not respond as expected based on what is known about pMMO and cometabolism in methanotrophs. These strains may have different forms or regulation of pMMO, or lack the ability to take up ammonia. Alternatively, *M. sporium* harbors soluble monooxygenase (sMMO) as well as pMMO. Perhaps sMMO was responsible for oxidizing ammonia in the present of methane in this strain, and the production of nitrite did not require a hydroxylamine intermediate. This would be a radical departure from the characterized physiology of other methanotrophs and certainly merits further investigation. Together, the results from this part of our study showed that even closely related methanotrophic strains have evolved different mechanisms for metabolizing inorganic nitrogen. Future studies relating the activity of MOB to greenhouse gas fluxes in soils must proceed with caution since closely related MOB species obviously have different physiological pathways.

Next, we compared metabolism and hao genes of *Nitrosomonas* and *Nitrosospira* species of ammonia-oxidizing bacteria. We showed through rates of substrate-dependent oxygen consumption that whole cells of *Nm. europaea* and *Ns. multiformis* responded similarly, but at different magnitudes, to environmental stimuli and that these trends were generally consistent with in vitro hydroxylamine oxidation rates by cell-free extracts. We also demonstrated for the first time that *Ns. multiformis*, like *Nm. europaea*, maintains three similar copies of the hao gene. This study demonstrated that *Ns. multiformis* and *Nm. europaea* share similar physiological responses to environmental stimuli and similar hao gene organization and structure. However, the fine details of cellular physiology and genetic homology remain somewhat divergent between the two bacteria, suggesting a mechanism for niche specialization among *Nitrosomonas* and *Nitrosospira* genera. To fully understand ecological roles among the ammonia oxidizers, further comparative physiological and genetic studies must be accomplished.

Last, we optimized molecular tools for determining AOB population diversity and abundance within complex soil communities. Then we applied both activity measurements and molecular tools to samples of native N-impacted soils to correlate AOB population diversity, abundance, and activity with differing levels of soil nitrogen and rates of nitrification. This study is the first to investigate the diversity, abundance, and activity of autotrophic AOB in a U.S. forest soil heavily impacted by dry deposition of atmospheric nitrogenous pollutants. We showed that differences in soil nitrogen content and pH did not select for particular species of autotrophic AOB nor was there an enrichment of autotrophic AOB populations in response to increased soil nitrogen concentrations. We also demonstrated that potential nitrification activity in N-saturated forest soils was not highly driven by the activity of autotrophic AOB, whereas, less impacted soils with much lower potential nitrification rates did support autotrophic AOB activity. The activity of a representative autotrophic AOB species of Nitrosospira cluster 3 was inhibited when inoculated into sterile forest soils, indicating that the soils were not ideal environments for this particular bacterial group. However, the relatively high rates of potential nitrification activity not

attributable to autotrophic AOB indicated that other microorganisms and processes are thriving in the N-saturated soils.

Although the diversity and abundance of autotrophic AOB were not significantly different across the N-saturation gradient, the PNA rates were significantly higher in the CP and SP soils than in the DW soils, whereas the potential contribution of autotrophic AOB was highest in the DW soils. Together, our observations indicate that other microbial populations or other processes besides those involving autotrophic AOB were likely responsible for relatively high rates of PNA in the CP and SP N-saturated soils, whereas lower rates of activity in the less N-impacted DW soil were predominantly catalyzed by autotrophic AOB. Similar observations were made in forest soils of California's Sierra Nevada, where nitrification activity was also only partially inhibited by acetylene (Pedersen et al. 1999). This study used 15N pool dilution techniques to demonstrate that inorganic ammonia was not the substrate for nitrate accumulation in mature forest soils.

Besides autotrophic AOB, acidic soils can support the activity of heterotrophic nitrifiers (De Boer and Kowalchuk 2001). Heterotrophic nitrifiers include bacteria and fungi that oxidize both organic and inorganic ammonia while utilizing organic carbon as an energy source (De Boer and Kowalchuk 2001). Evidence for heterotrophic nitrification in CP and SP soils is supported by the accumulation of nitrite plus nitrate in the presence of acetylene in ammonium-amended soil slurries, and the increase in soluble ammonium concentrations in ammonium-amended static soil incubations. The inferred dominance of heterotrophic over autotrophic nitrification activity in the N-saturated, low pH, forest soils is in sharp contrast to other N-impacted soils in which autotrophic AOB apparently dominate (De Boer and Kowalchuk 2001, Kowalchuk et al. 2000, Laverman et al. 2001, Pennington and Ellis 1993). The ecology of heterotrophic nitrifiers is highly understudied in the field of microbial ecology. The present study demonstrated a unique N-impacted environment in which heterotrophic nitrification may dominate over autotrophic nitrification, and also expanded the biogeographical range of several *Nitrosospira* gene clusters.

The nitrogen cycle has been wildly altered from anthropogenic inputs, especially from agriculture and industry. A long-term goal for my laboratory is to determine how soil bacteria involved in nitrogen transformations are impacted by human activities, specifically in their production and consumption of greenhouse gases. Our research indicates that unique genetic content of ammonia- and methane-oxidizing species translates into altered rates of activity. Thus, the creation of unifying assumptions regarding populations of these bacteria and their activities in the environment is becoming more confounding. We are narrowing the possibilities for how AOB and MOB metabolize nitrogen to generate greenhouse gases in N-impacted soils and are rapidly developing a suite of traditional and molecular techniques to correlate identity, abundance and function of these bacteria in soils. So far, our results have changed our initial assumptions regarding how these microbes actually function in nature.

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