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By

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ABSTRACT

NUCLEIC ACID ANALYSIS OF AUTOTROPHIC AMMONIA-OXIDIZING BACTERIA IN SOILS

By

Mary Ann Bruns

Nitrification, the microbial oxidation of ammonium to nitrate, leads to significant nitrogen (N) losses from soils. Nitrification rates in soils have been related to population sizes of ammonia-oxidizing bacteria, but population structure effects are poorly understood. This study employed nucleic acid-based methods to determine how ammonia oxidizer populations differed in cultivated, never-tilled, and successional soils. Hybridizations were conducted between genomic DNA from pure cultures and probes from *Nitrosomonas europaea* genes for ammonia monooxygenase and hydroxylamine oxidoreductase. These probes produced 24 to 80% less hybridization signal in genomic DNA from four other ammonia oxidizers under low-stringency conditions. This indicated that *N. europaea* functional probes would not provide quantitative size estimates for heterogeneous populations. Sizes of ammonia oxidizer populations in soils were determined by Most Probable Number (MPN) enumeration in media containing 10, 100, or 2000 ppm NH_4-N. MPN counts were consistently one-tenth lower in never-tilled soils than in cultivated soils. Population structures were compared by using PCR to amplify 16S rRNA genes of ammonia oxidizers from bacterial community DNA, cloning the PCR products, and comparing cloned sequences for percent similarity over a 318-base region. The 16S rRNA gene sequences obtained from never-tilled soils were four times more diverse than sequences obtained from cultivated soils. A phylogenetic tree showed that sequences from cultivated soils fell within one distinct cluster of *Nitrosospira*; sequences from never-tilled soils fell into two clusters of *Nitrosospira* and one of *Nitrosomonas*. Denaturing gradient gel electrophoresis (DGGE) patterns differed between samples from never-tilled and cultivated soils; patterns from samples from replicate plots of each treatment were very similar in each of two successive years. Probe hybridizations of DGGE blots showed that sequences from the single subgroup of *Nitrosospira* spp. predominated in samples from cultivated or fertilized successional soils; these were not detected in samples from never-tilled or unfertilized successional soils. Differences in populations were reflected in mean NH_4-N concentrations that were tenfold higher in never-tilled soils than in cultivated soils. Different influential factors in these soils include pH, allelopathy, microsite distribution, or direct effects of cultivation and fertilization.

DEDICATION

This dissertation is dedicated to the **Center for Rural Affairs**, a private, nonprofit, rural advocacy organization in Walthill, Nebraska. The Center for Rural Affairs' research and education efforts have given me professional guidance which I hope has shaped my perspective as a soil scientist.

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Chapter One

OVERVIEW

Nitrification, the microbial oxidation of ammonia to nitrate, can lead to significant nitrogen (N) losses from ecosystems. Nitrification occurs as a two-step process carried out by two different groups of autotrophic bacteria: ammonia-oxidizing bacteria first convert ammonia to nitrite; then nitrite oxidizers convert nitrite to nitrate. Both groups oxidize inorganic N as their principal source of energy and use CO_2 as their principal source of C (Bock et al., 1989). Autotrophic nitrifiers must be distinguished from heterotrophic nitrifiers, which include some soil fungi and soil bacteria such as *Arthrobacter* spp. (Kurokawa et al., 1985). The heterotrophic nitrifiers oxidize reduced inorganic N in cometabolic reactions, but not as a means of generating energy (Killham, 1986). Heterotrophic nitrification constitutes only a small fraction of total nitrification in most soils (Kuenen and Robertson, 1988).

Virtually all soils containing NH_4-N are subject to nitrification, and nitrification rates vary widely. Nitrification rates are typically low in undisturbed soils, such as grasslands and some forest soils, compared to cultivated soils which receive N fertilizer and undergo disturbance (Clark and Paul, 1970; Schmidt and Belser, 1982). Nitrification rates are controlled principally by NH_4-N concentration, but other environmental factors such as pH, temperature, oxygen content, and moisture also play important roles (Robertson, 1990). The size of the autotrophic nitrifier population is related to nitrification activity in soils (Belser and Mays, 1982), but the influence of population structure on nitrification rates is poorly understood.

The autotrophic nitrifiers represents a critical link in N cycling within microbial and plant communities. In acid and neutral soils nearly all ammonia is protonated. When N is in the form of ammonium (NH_4^+), it is retained in the soil as an exchangeable cation bound to negatively charged sites on mineral or organic soil components (Young and Aldag, 1982). Ammonium can also be retained as a nonexchangeable cation held in clay mineral interlayers or organic matter complexes (Nommik and Vahtras, 1982). In the intracellular environment of ammonia-oxidizing bacteria, the substrate that is oxidized is ammonia. When nitrifying bacteria convert ammonia to nitrate, N in the anion form is no longer held at negatively charged sites on soil colloids. Nitrate is mobile and susceptible to loss from the system by leaching into groundwater or by denitrification as nitrogen gases into the atmosphere. The activity of nitrifying bacteria is a major factor in determining whether the soil retains or loses nitrogen. Ammonia oxidizers are the more functionally important group of nitrifying bacteria, since they carry out the first, rate-determining step in nitrification (Koops and Moller, 1992).

The objective of this study was to determine whether soils with different disturbance histories select for different populations of autotrophic ammonia oxidizers (AAOs). Soils for this study were obtained from replicated, 1-ha plots at the Long-Term Ecological Research (LTER) site at Kellogg Biological Station near Kalamazoo, Michigan. This site was established in 1989 to study ecological interactions, nutrient availability, and biotic diversity of cropped, native, and successional ecosystems that are representative of the Upper Midwest U.S.A (Robertson et al., 1996). The two treatments with the most distinctive disturbance histories at LTER are Treatment 1 (high-input, conventionally tilled rotation of corn and soybeans) and Treatment 8 (never-tilled, native successional). Prior to 1989, Treatment 1 plots had been cropped in corn and soybeans for at least 40 years. These plots have been moldboard plowed, disked, and cultivated every year since 1989, and they have been more intensively fertilized than all other treatments at LTER. Treatment 8 plots, which were cleared of native deciduous forest in 1959, have never been cropped or fertilized. A third treatment, which has a disturbance history intermediate between Treatment 1 and Treatment 8, is Treatment 7 (historically tilled successional). Treatment 7 plots had also been under cultivation before 1989, but since that year have been undisturbed, unfertilized, and left to revegetate with native successional flora. Treatment 7 plots contain 5x5 m microplots which have been fertilized every year. These three main treatments plus the microplots are described in the following chapters as cultivated, never-tilled, unfertilized successional, and fertilized successional treatments, respectively.

The most common method for studying AAO populations in soils has been to estimate numbers of AAOs per gram of soil with Most-Probable-Number (MPN) analysis (Schmidt and Belser, 1982). MPN media tend to select for nitrifiers that grow fastest under the conditions provided, however, and recovery efficiency is poor (Belser and Mays, 1982). MPN analysis is uninformative about *in situ* population structures of AAOs in soils. Although fluorescent antibody analysis has been used to study AAO population diversity in soils (Belser and Schmidt, 1978), fluorescent antibodies can only be prepared for AAO strains that have already been cultured, so that uncultured strains are not detected. In one study, antibodies developed against all isolates from one soil did not cross-react with half of the isolates obtained from a different soil. AAO diversity thus appears to be too great for fluorescent antibody analysis to provide reliable characterizations of population structures in soils from diverse locations.

Nucleic acid-based methods offer significant opportunities for the study of *in situ* populations of AAOs. These methods, which are based on the study of bacterial DNA rather than the bacteria themselves, do not require the organisms to be cultured. The first steps in nucleic acid analysis of bacterial communities are to extract community DNA from soils by lysing the cells and purifying the DNA that is released. Appendix A at the end of this dissertation describes the development and evaluation of the DNA extraction and purification methods used in this study. AAO populations (typically 10^4 to 10^5 per gram) represent less than 0.01% of total bacterial numbers in soils, which typically range from 1-5 x 10^9 per gram (Schmidt and Belser, 1982). Thus, the total amount of bacterial community DNA from soil will contain only a small fraction of ammonia oxidizer DNA. Despite proportionally small populations of AAOs in soils, specific genes or gene fragments from their DNA can be copied in the polymerase chain reaction (PCR) with oligonucleotide primers that are complementary to their genes (Schmidt et al., 1991). Gene fragments in the PCR product mixtures can be separated by inserting individual fragments into clones of *Escherichia coli*; these can then be analyzed for their DNA sequences or for cross-reactivity to other nucleic acid probes (Schmidt et al., 1991). Entire PCR mixtures can also be analyzed by denaturing gradient gel electrophoresis (DGGE), which is now considered to be more efficient and informative than previous cloning and sequencing methods (Muyzer et al., 1993). DNA sequence variation in these gene fragments should reflect variation in the population which gave rise to them, assuming there is negligible bias in the molecular techniques used (Suzuki and Giovannoni, 1996).

Two types of genes could be useful in analyzing variation within AAO populations. The first type are functional genes that code for enzymes in the ammonia oxidation pathway. DNA sequences for the genes for ammonia monoxygenase and hydroxylamine oxidoreductase, the two principal enzymes in this pathway, were made available by McTavish et al. (1993) and Sayavedra-Soto et al. (1994), respectively. These sequences were obtained from cultures of *Nitrosomonas europaea,* which is the most extensively studied AAO strain. Much of what is known about *N. europaea* has been generalized as being representative of all AAOs in the environment, although this may not be a valid generalization. Two distinct groups of terrestrial AAOs are now recognized on the basis of phylogenetic variation: 1) the *Nitrosomonas* group, which includes all strains of *Nitrosomonas* and some strains of *Nitrosococcus*; and 2) the *Nitrosospira* group, which includes strains of *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio.* All terrestrial AAOs identified to date belong to the β-subgroup of Proteobacteria (Woese et al., 1984), although some marine AAOs (*Nitrosococcus* spp.) belong to the gamma subgroup.

The second type of gene that would be useful in characterizing population diversity of AAOs are phylogenetic genes coding for ribosomal RNA (Figure 1.1). Bacterial rRNA genes are 1500 bases long and contain signature regions that are specific for various bacterial groups. These regions offer specific binding sites for oligonucleotide primers or probes for group-specific PCR. The DNA sequences for the small subunit (16S) rRNA genes of 13 AAO strains are available in the Ribosomal Database Project (RDP, Maidak et al., 1994). McCaig et al. (1994), in the laboratory of Jim Prosser at the University of Aberdeen, Scotland, used the RDP sequences to develop primers specific for AAOs and their close relatives in the β-Proteobacteria. These primers were used in PCR reactions on community DNA from a wide variety of environmental samples. The PCR products were used to generate a database of 110 partial 16S rRNA sequences. The region for which these partial sequences were obtained was 318 bases long and is indicated in Figure 1.1. This region

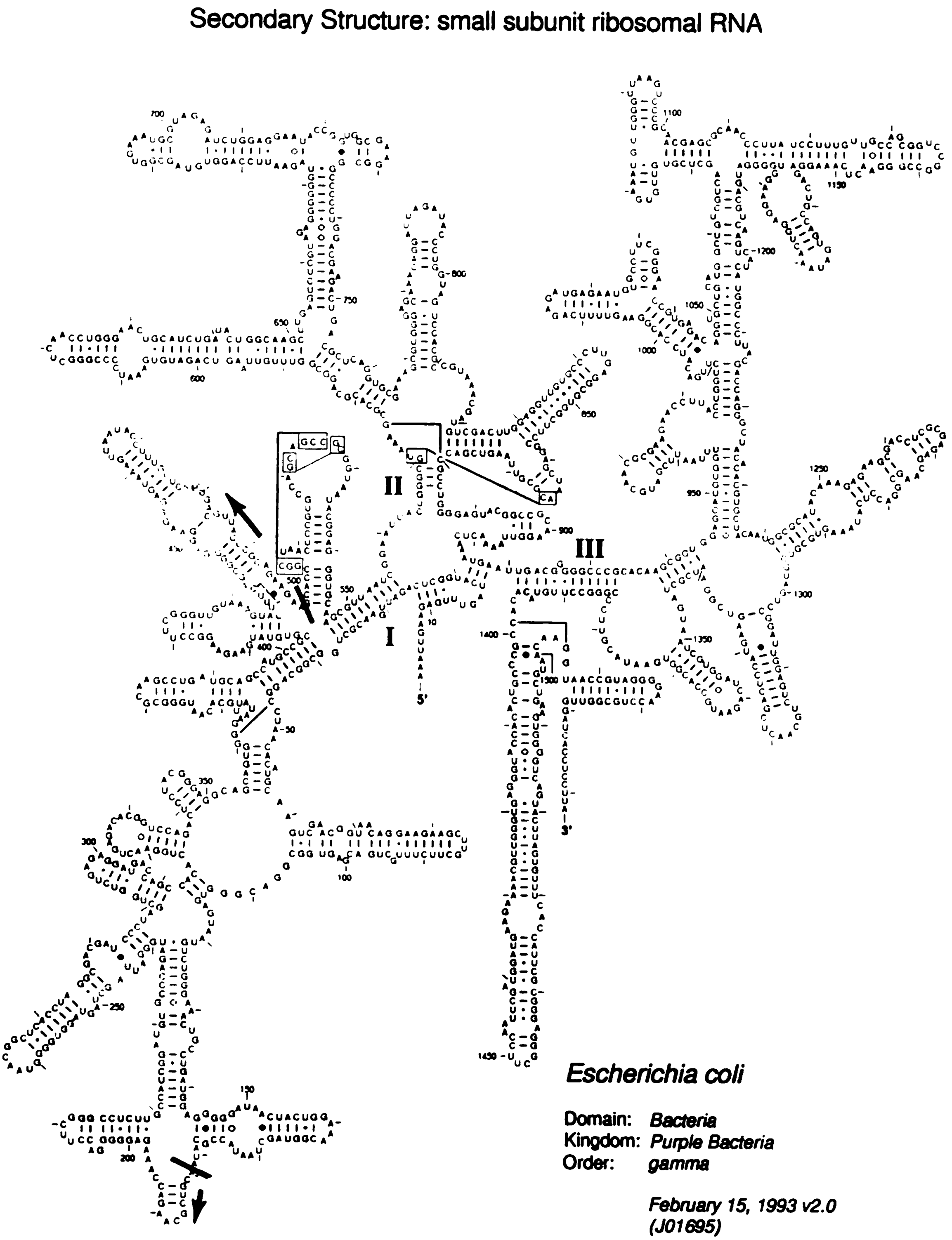

FIGURE 1.1. Structure of 16S rRNA molecule of Escherichia coli. (From the Ribosomal Database Project (Maidak et al., 1994).

corresponds to positions 382 to 500 of the *E. coli* rRNA molecule.) This database represented a much greater breadth of gene sequence diversity among AAOs than previously available in the RDP. The database enabled Stephen et al. (1996a) to identify four distinct clusters of *Nitrosospira* and two clusters of *Nitrosomonas*. The greater number of rRNA gene sequences thus made it possible to obtain a more detailed characterization of genetic diversity among AAOs in the β-Proteobacteria.

My dissertation project represents one of the first applications of nucleic acid-based techniques to the study of AAOs in soils. This dissertation was undertaken as part of two larger community analysis research efforts. The first effort was that of the Community Diversity Thrust Group in the Center for Microbial Ecology (CME), a National Science Foundation (NSF) Science and Technology Center. The research goals of this thrust group are to determine temporal and spatial patterns of microbial diversity, identify environmental influences on community structure, and demonstrate effects of community structure on ecosystem function (CME, 1993). The second research effort, also funded by NSF, was the LTER project at Kellogg Biological Station, which integrates the study of above- and below-ground biotic diversity in agricultural and native ecosystems.

My approach to studying microbial soil community structure was to focus on AAOs, because of their unique functional role in nitrogen cycling and nutrient availability. By examining diversity within one bacterial group and relating it to soil biochemical measurements, I hoped to generate meaningful insights into the relationships between soil microbial diversity and ecosystem function. I believed that this approach could circumvent some of the technical and interpretive problems involved in the study of whole soil microbial communities, which are characterized by vast diversity and complexity.

A grant from the U. S. Department of Education for environmental microbiology education enabled me to work for five months in Dr. Prosser's laboratory. During that time, I learned the cloning, sequencing, and probing techniques used in this laboratory (Embley, 1991). In Aberdeen I also had access to the partial 16S rDNA sequence database and identified eight oligonucleotide probe sequences for different AAO subgroups (Stephen et al., 1996b). After I returned to the U. S., John Stephen and George Kowalchuk performed DGGE analysis on DNA samples from LTER soils at the Netherlands Institute of Ecology in Heteren.

My dissertation research involved three phases, each of which corresponds to a chapter in this dissertation: 1) culturing known AAOs and developing more reliable cultural methods for AAOs from LTER soils; 2) evaluating nucleic acid probes for functional genes by using genomic DNA from pure AAO cultures; and 3) developing rRNA gene probes for AAO subgroups and analyzing diversity of rDNA sequences retrieved from bacterial community DNA. Each chapter presents the rationale, methods, and results for one of these research phases. The final section of the last chapter contains a discussion of the main conclusions from this study. The appendix at the end of this dissertation contains a published article which describes methods development for bacterial DNA extraction from soils. Key findings from the following chapters and appendix are summarized as follows.

Chapter Two, Cultural Methods for Studying Autotrophic Ammonia-Oxidizing Bacteria, describes the performance of cultural methods for enumerating and isolating AAOs from LTER soils. Key findings in this chapter were: 1) use of lower substrate concentrations in most-probable-number (MPN) media (10 ppm vs. 100 ppm NH_4-N) gave higher recovery of AAOs from LTER soils; 2) NH_4-N concentrations in LTER soils ranged from 1 to

10 ppm; this may explain why more AAOs were recovered at the lower NH_4-N concentration; 3) MPN counts of AAOs from never-tilled soils were consistently one-tenth lower than MPN counts from cultivated soils during a three-year period; 4) ratios of MPN counts obtained at 10 ppm vs. 2000 ppm NH_4-N were twentyfold higher for AAO populations from never-tilled soils than for populations in cultivated soils; and 5) use of a lower NH_4-N concentration in soil enrichments was an important factor in the isolation of a novel *Nitrosospira* strain from never-tilled soil.

Chapter Three, Evaluating *Nitrosomonas europaea* functional gene probes in hybridization tests with *Nitrosospira* DNA, is a feasibility study for using *N. europaea* functional gene probes to detect homologous genes from other AAOs in the pool of DNA from the rest of the microbial community. Key findings in this chapter were: 1) probe hybridization signals from genomic DNA varied enough among the AAOs tested to require that probe hybridization tests be performed under conditions of low specificity, which would increase the risk of nonspecific probe binding to DNA from other bacteria; 2) hybridization signals with genomic DNA using a gene probe for the hydroxylamine oxidoreductase enzyme were less variable across the AAOs tested; this means that the probe would be more specific and reliable than other functional probes for estimating AAO numbers; and 3) gene sequence variability and differences in gene copy number make functional probes less reliable for giving absolute estimates of the sizes of mixed AAO populations; relative estimates are feasible if AAO numbers are sufficiently high to produce detectable signal.

Chapter Four, Use of 16S rDNA Analysis to Compare Diversity of Autotrophic Ammonia Oxidizer Populations in Soils, describes the use of PCR-based 16S rDNA analysis to compare AAO population structures in cultivated, never-tilled, and successional soils at LTER. Key findings were 1) AAO gene sequences from never-tilled soils were more diverse than AAO sequences from cultivated soils; 2) sequences of Cluster 3 *Nitrosospira*, a phylogenetically distinct group identified by Stephen et al. (1996a), predominated in samples from cultivated and fertilized successional soils but were not detected in samples from cultivated and fertilized successional soils; 3) DGGE banding patterns were spatially reproducible over an area of 1 km2, with replicate plots for the same treatments giving identical patterns in two successive years.

Chapter Four's final section discusses the study's main conclusions, relates them to CME and LTER research goals, and interprets them in the contexts of microbial ecology and agronomy. Conclusions are also based on data for relevant soil properties that have been obtained as part of the LTER data collection program. The main conclusions of this study are: 1) sizes of AAO populations in never-tilled soils are significantly smaller than AAO populations in cultivated soils by a factor of ten; 2) AAO gene sequences in cultivated soils exhibit less diversity than AAO sequences in never-tilled soils, with Cluster 3 *Nitrosospira* sequences predominating in cultivated soils; 3) predominance of Cluster 3 *Nitrosospira* sequences appears to be associated with nitrogen fertilizer application, because these sequences were also observed in fertilized but not unfertilized successional soils; 4) effects of AAO population differences on nitrogen cycling in LTER soils may be reflected in the consistently higher NH_4-N levels observed in never-tilled plots; and 5) although different AAO populations in cultivated and never-tilled soils exhibit similar net nitrification rates during in-field nitrification tests, differences in population function may still be exhibited by the lower percentages of NH_4-N converted to NO_3-N during the measurement periods.

Appendix A, DNA Recovery from Soils of Diverse Composition, describes the performance of DNA extraction and purification methods with eight physically and chemically distinct soils, including soils from the cultivated and never-tilled plots at the KBS LTER site (CK and NK soils, respectively). Key findings were 1) bacterial cell lysis efficiency in soils using the high-NaCl/SDS/heat method varied from 26 to 92%, indicating that this extraction method recovers the majority of bacterial DNA from some, but not all, soils; 2) crude DNA yields from cultivated and never-tilled soils were within the expected ranges based on direct counts of bacteria prior to extraction; this extraction method thus recovered the majorityof bacterial DNA from these soils; 3) coefficients of variation for crude and purified DNA yields from cultivated and never-tilled soils were high (about 20%), which could affect reproducibility of molecular analyses based on this method; 4) DNA yields from never-tilled soils were approximately four times higher than yields from cultivated soils; and 5) DNA obtained with these purification methods was of high molecular weight (> 23 kDa) and pure enough for PCR amplification.

Future research that would build on the findings from this dissertation would include additional replications of these molecular analyses at more frequent time intervals. Additional replications over time would help verify the reproducibility and reliability of these techniques. Any molecular analyses of soils would be greatly enhanced by measuring ^{15}N transformations in the same samples to observe relationships between gross nitrification and AAO population structure. Since the field of molecular microbial ecology continues to advance, additional primers and probes are likely to become available, particularly for AAOs in the gamma-subgroup of Proteobacteria. The LTER treatments at the KBS site offer important opportunities for continued research on the microbial ecology of nitrification.

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Chapter Two

CULTURAL METHODS FOR AMMONIA-OXIDIZING BACTERIA

INTRODUCTION

Nitrification is the microbial oxidation of ammonia to nitrate, and it leads to significant nitrogen (N) losses from agricultural systems when nitrate-N is leached or denitrified (Keeney, 1986). Autotrophic ammonia-oxidizing bacteria (AAOs) carry out the rate-determining step of nitrification in most soils, but little is known about AAO population ecology. In this study, AAO population sizes and structures were evaluated in cultivated and uncultivated soils from the National Science Foundation Long-Term Ecological Research (LTER) site at Kellogg Biological Station, near Kalamazoo, Michigan. The LTER site was established in 1988 to study ecological interactions affecting productivity, nutrient availability, and biotic diversity in cropped, successional, and native ecosystems representative of the Upper Midwest United States (Robertson et al., 1996). AAOs were selected for study in these soils, because they are a key functional group affecting nitrogen cycling in microbial and plant communities, and because nucleic-acid-based methods have recently become available for their analysis (McCaig et al., 1993).

Standard cultural methods for isolating pure cultures of AAOs are difficult and time-consuming due to the long generation times of these bacteria and their tendency to be overgrown by heterotrophs (Watson et al., 1989). As a consequence, few pure AAO cultures are available for study, and these cultures probably do not represent the breadth of AAO diversity in nature. Despite their limitations, laboratory culture methods offer an important entry into the study of AAO populations. The standard cultural method for estimating AAO population size in soils is the most-probable-number (MPN) procedure, in which soil dilutions are added to replicate tubes containing growth medium composed of mineral salts and 0.5 g of $(NH_4)_2SO_4$ per liter (Schmidt and Belser 1982). Tubes are examined for AAO growth after 4 to 8 weeks incubation (or longer), and MPN counts are determined on the basis of the highest dilution at which growth is observed. Some workers have observed significant differences in MPN counts when using higher or lower concentrations of $(NH_4)_2SO_4$ in growth media (Donaldson and Henderson, 1989; Suwa et al., 1994). Our study employed MPN analyses at three different $(NH_4)_2SO_4$ concentrations to see if such differences could be observed, and whether these differences could be related to soil treatment.

In this study, both cultural and nucleic acid-based approaches were used to evaluate AAO population sizes and structures in soils under different management regimes. This paper describes the performance of cultural methods for enumerating and isolating AAOs from LTER soils and for maintaining and storing AAO stock cultures during development of molecular techniques for AAO population analysis. Results of nucleic acid-based analyses of AAO populations in these soils will be reported in a subsequent paper.

MATERIALS AND METHODS

Strains. Six strains of ammonia-oxidizing bacteria previously isolated from soils were used in this study. Strains in this collection included three of the five genera known to occur in soils--*Nitrosomonas*, *Nitrosolobus*, and *Nitrosospira*. *Nitrosomonas europaea* ATCC 25978 and *Nitrosolobus* *multiformis* ATCC 25196 were obtained from the American Type Culture Collection (ATCC). *N. europaea* ATCC 19718, *Nitrosolobus* strain 24-C, *Nitrosospira* strain NpAV, and *Nitrosospira* strain Np39-19 were obtained from Dr. Ed Schmidt of the University of Minnesota. Cultures representing the other two genera--*Nitrosovibrio* and *Nitrosococcus*--were not available for this study.

Liquid culture media. A preliminary study was carried out to select a liquid medium suitable for culture maintenance. Growth of AAO strains was evaluated over a 2-month period in two types of liquid media (ATCC Medium 929 and ATCC Medium 1573). Media were filter-sterilized after pH adjustment, because they contained Mg^{++} and Ca^{++} ions, which could become unavailable for bacterial growth as a result of complexation during autoclaving (Table 2.1).

Each culture was transferred in triplicate to 16x125 mm screw-capped tubes containing 5 ml of the two different media and incubated at 25°C without shaking. Cultures were checked daily for acid production resulting from ammonia oxidation, which was indicated by color change of the phenol red indicator from pink to yellow. A sterile solution of 0.05 M K_2CO_3 was added dropwise to cultures that had turned yellow to bring the pH back to 7.5. Cultures were evaluated for growth consistency among the triplicate tubes and the length of time each culture remained viable after repeated pH adjustment. Transfers to fresh medium were made three times during the 2-month period. Cultures of *N. multiformis* ATCC 25196 and *Nitrosolobus* strain 24-C appeared to decline more rapidly in vigor than the other cultures, as indicated by their tendency to stop producing acid after two weeks. Since growth of *Nitrosolobus* strains was more consistent in Medium 929 than in Medium 1573, the former was chosen for culture maintenance.

TABLE 2.1. Compositions of modified ATCC Medium 929 and ATCC Medium 1573.

Components	Medium 929 (*Nitrosolobus* Medium) per liter distilled water	Medium 1573 (*Nitrosomonas europaea* Medium) per liter distilled water
$(NH_4)_2SO_4$	1.32 g[1]	1.70 g[1]
$MgSO_4$	380 mg	200 mg
$CaCl_2.2H_2O$	20 mg	200 mg
K_2HPO_4	87 mg	15 mg
Fe·EDDHA	1 mg	1 mg
Phenol red (0.5% solution)	0.25 ml	0.25 ml
Trace elements solution	1.0 ml of following mix:	1.0 ml of following mix:
$Na_2MoO_4{\cdot}2H2O$	10 mg	10 mg
$MnCl_2{\cdot}4H_2O$	20 mg	20 mg
$ZnSO_4{\cdot}7H_2O$	10 mg	10 mg
$CoCl_2{\cdot}6H_2O$	0.2 mg	0.2 mg
$CuSO_4{\cdot}5H_2O$	---	2 mg
Distilled water	100 ml	100 ml

Adjust pH to 7.5 with 0.5 M K_2CO_3. Filter sterilize with 0.2-μ filter.

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[1] Corresponds to 20 mM NH_4-N (270 ppm) and 26 mM NH_4-N (350 ppm) for Medium 929 and Medium 1573, respectively.

All transfers were made in a laminar flow hood; extreme care was taken to prevent contamination. All lots of liquid medium were checked for contamination before inoculation by spotting 0.1 ml onto the surface of Plate Count Agar and R2A Agar (Difco, Detroit, MI). The culture liquid was allowed to be absorbed by the agar before plates were inverted for incubation at 32°C for up to one month. Stock AAO cultures were checked every month for heterotrophic contamination by this method.

Solid medium. Growth of pure AAO cultures was tested on a solid medium containing Medium 929 without phenol red and an agar base of 1.5% Noble Agar (Difco, Detroit, MI) and 1% $CaCO_3$ powder. To prepare one liter of medium, 500 ml double-strength agar base and 500 ml double-strength Medium 929 were prepared separately. The agar base was autoclaved in a 2-liter flask containing a heavy stir bar for mixing. When the agar base had been cooled to about 65°C, filter-sterilized Medium 929 (double-strength) was carefully poured into the molten agar while the mixture was being stirred on a hot plate. Approximately 30-40 ml of agar medium were added per petri plate, since inoculated plates would be incubated for extended periods. Plates were prepared immediately prior to inoculation. To inoculate a plate, 50 to 100 µl of culture were spotted onto its surface, and the plate was left overnight at room temperature to allow water from the culture to be absorbed. Plates were wrapped in parafilm and placed inverted into plastic bags for incubation at 25°C in the dark. The bags also contained water-soaked paper towels, which were replaced every 2 months, to provide a moist atmosphere for the plates.

Treatments and soils. LTER treatment plots (1 ha each) are replicated in a randomized block design. Soils from two agricultural treatments (cultivated Treatment 1 and low-input-cultivated Treatment 3) were used to provide cultivated soil samples. The cultivated treatment had been a corn-soybean rotation from 1989 to 1994, with wheat introduced as a third rotation crop in 1995. This treatment was conventionally tilled (annual moldboard plowing, disking, and cultivation), treated with prescribed applications of herbicides and insecticides, and fertilized with ammonium nitrate. The N fertilizer was broadcasted at a rate of 84 kg N per ha to the corn crop and 56 kg N per ha to the wheat crop. The low-input-cultivated treatment had been a corn-soybean-wheat rotation since 1989, with a leguminous winter cover (34 kg per ha hairy vetch broadcasted in August) and reduced chemical inputs. This treatment was given a starter fertilizer application only (ammonium nitrate alongside of row using 28 kg N per ha). Never-tilled soils were obtained from Treatment 8 plots that had not been tilled after clearing of the native deciduous forest in 1959. (Most of the vegetation in these plots now consists of C3 grasses.) Successional soils were obtained from Treatment 7 plots, which were under a disturbance regime intermediate between the cultivated and never-tilled treatments. Successional plots had been in cultivated corn and soybeans for at least 40 years before they were left in 1989 to revegetate with extant flora. Nitrogen-fertilized microplots (5m x 5m) within these successional plots were also sampled to evaluate fertilization vs. disturbance effects. These microplots received 125 kg N per ha (ammonium nitrate) broadcasted in July of each year.

Soils at the LTER site are classified as Typic Hapludalfs (U. S. Soil Classification System, 1992) belonging to the Kalamazoo and Oshtemo soil series (fine, loamy, mixed, mesic). The A horizons in these soils are typically 20 cm deep. Soils were sampled in April 1992, July 1993, and August 1995 to a 10-cm depth with a 2.5-cm soil corer (approximately 14 g fresh soil per core). Soil samples in 1992 and 1995 were composites of 20 soil cores per replicate plot taken near LTER sampling station #1 in each plot. Two replicate plots were sampled per treatment. Prior to subsampling, soils were mixed inside plastic bags by manually kneading and shaking the bags. Gravel and other debris were removed from these soils manually. The 1992 and 1995 samples were thus true replicate samples from two different plots.

Soils in 1993 were subsamples from the large LTER midsummer sampling done for annual data collection (Robertson et al., 1996). In the larger LTER sampling procedure, 20 core samples from each of five sampling stations per replicate plot were composited and mixed by moist sieving (4 mm sieve). (Cultivated Treatment 1 has six replicate plots; never-tilled Treatment 8 has four replicate plots.) The mixed samples from all replicate plots were composited and sieved again prior to removing subsamples for MPN analysis. The 1993 samples were analytical samples from a composited sample from six or four replicate plots.

All soil samples were stored at $4^{o}C$ until they were analyzed in the laboratory. Soil moisture contents were determined from two 10-g subsamples dried at $110^{o}C$ for 48 h. All results reported here are based on soil dry weights.

Most-probable-number (MPN) analyses. In initial MPN tests, April 1992 soil samples were taken from the low-input-cultivated (in wheat) and never-tilled treatments (replicate plots 1 and 2). Three different media, based on the MPN medium described by Schmidt and Belser (1982) were used in these trials: 1) full-strength Schmidt-Belser (SB) Medium; 2) full-strength SB Medium with 0.3% bentonite clay (Sigma Chemical Co., St. Louis, MO); and 3) 1/10-strength SB Medium without clay. The bentonite clay had been added to one set of MPN tubes to evaluate whether the increased particle surface area provided by the clay would enhance ammonia oxidizer growth, as had been previously reported in the literature (Verhagen and Laanbroek, 1991). Tenfold dilutions of soil were added to 16x125 mm screw-capped tubes containing 5 ml of medium (five tubes per dilution). Tubes were incubated in the dark for 8 weeks at 25^{o}C and examined for color change in the bromthymol blue indicator from blue to yellow, which indicated a pH of 6.8.

In July 1993, Medium 929 with varying amounts of $(NH_4)_2SO_4$ was used as the recovery medium for AAOs in soil samples from cultivated (in corn) and never-tilled plots. These samples were tested with 5-tube MPNs using tenfold dilutions and media containing 0.05, 0.5, and 5.0 g of $(NH_4)_2SO_4$ per liter, corresponding to 1/10X, 1X, and 10X standard NH_4-N concentrations, respectively (Table 2.2). In August 1995, samples were analyzed from cultivated (in wheat), never-tilled, and successional plots (unfertilized main plot and fertilized microplots). For these analyses, 96-well microtiter plates were used for MPNs (Rowe et al., 1977) by making twofold dilutions (8 wells per dilution) in 1/10X, 1X, and 20X concentrations of NH_4-N.

After subsamples were taken for moisture determination, 10 g moist soil were blended with 190 ml 100 mM phosphate buffer (pH 7) in a Waring Blendor for 1 min. Coarse particles were allowed to settle for 1 min, then 10 ml of the slurry were transferred to a sterile plastic tray for further dilution in the microtiter plates. Prior to inoculation, all wells of the microtiter plates were filled with 100 µl of the appropriate media. A multichannel pipettor was used to transfer eight 100-µl sample aliquots from the plastic tray to the first row of wells in the microtiter plates. When sample aliquots had been mixed with the medium in the first row of wells, 100 µl of this mixture were transferred to the next set of wells, thus making a 1:2 dilution. A series of 18 twofold dilutions were typically made after the first 1/20 dilution in the blender.

MPN tubes or plates were incubated in the dark at 25^{o}C for 8 weeks. Microtiter plates were double-wrapped in parafilm to prevent moisture loss and packed in plastic bags containing water-soaked pads to maintain a moist atmosphere. MPN cultures were checked for nitrate as an indicator of ammonia

TABLE 2.2. NH_4-N concentrations used in MPN recovery media.

Medium	$(NH_4)_2SO_4$ g per liter	NH_4-N mM	NH_4-N ppm	Comments
1/10X	0.05	0.74	10	Concentration which gave higher MPN recoveries from forest soils (Donaldson and Henderson, 1989).
1X	0.5	7.4	100	Standard concentration for MPNs on soils (Schmidt and Belser, 1982).
10X	5.0	74	1,000	Concentration which inhibited NH_4-sensitive *Nitrosomonas* strains (Suwa et al., 1994).
20X	10.0	148	2,000	Twice as high as the concentration which inhibited NH_4-sensitive *Nitrosomonas* strains (Suwa et al., 1994).

oxidizer growth. Since the diphenylamine test for nitrate described by Schmidt and Belser (1982) did not give reliable results, another nitrate test reagent, Szechrome NB (Polysciences, Inc., Warrington, PA) was used (Jeanette Norton, personal communication.) Szechrome NB reagent was prepared according to the manufacturer's instructions in a 2:3 mixture of concentrated phosphoric and sulfuric acids. To check for nitrate, 50 µl of MPN medium were added to the well of a plate and mixed with 250 µl of the Szechrome reagent. After 15 min, the color of the test mixture was compared to that of nitrate standards ranging from 0.1 ppm to 100 ppm. The reagent formed a blue color in the presence of at least 0.1 ppm nitrate. Microtiter plates containing uninoculated MPN media were held for 8 weeks in the same bags as the control plates, and 50-µl aliquots of these media were used as negative controls.

Enrichments from never-tilled soils. The samples and SB media used to test initial MPNs on never-tilled soils in 1992 were also used for long-term enrichment cultures. Replicate 1-liter flasks were inoculated with 1 g moist soil. Flasks contained 250 ml full-strength SB Medium (with and without 0.3% bentonite clay) or tenfold-diluted SB Medium containing no clay. After being incubated in the dark (without shaking) at room temperature for 6 months, the flask cultures were streaked out onto solid medium. Twelve-week-old MPN tubes from low-input-cultivated and never-tilled soils were also streaked out onto solid medium. Inoculated plates were held in moist bags in the dark at 25^{o}C for one to six months and examined for putative AAO colonies. Some of these colonies were transferred to liquid medium and monitored for subsequent growth.

Long-term storage methods for AAOs. AAO viability after freezer storage (-70^{o}C) and room-temperature storage on 929/$CaCO_3$ plates was evaluated. One-ml aliquots of 3-week-old cultures in Medium 929 were added to 1 m1 20% sterile glycerol in 17 x 55 mm glass vials (8-ml capacity) and equilibrated for 15-30 minutes. These mixtures were placed in a dry ice/ethanol bath, which froze the cultures solid within 2 minutes. Triplicate samples were tested for outgrowth after the following treatments: (1) equilibration in glycerol; (2) equilibration, freezing, and one-day storage at -70°C; (3) equilibration, freezing, and 3-month storage; and (4) equilibration, freezing, and one-year storage. Frozen glycerol stocks were quick-thawed (within 2 minutes) by swirling the vials in a beaker of water at room temperature. One-ml of the glycerol stock was transferred to 5 ml fresh Medium 929 for incubation at 25°C. Outgrowth was evaluated by noting how many of the tubes turned yellow and how long the cultures needed to be incubated before they turned yellow. In some cases, the glycerol stock mixture appeared yellow immediately after thawing. The pH of these mixtures had to be adjusted to 7.5 before incubation, otherwise culture viability could not be determined. This color change was likely artifactual, because uninoculated Medium 929 would occasionally turn yellow after freezing and thawing.

Growth scraped from one-year-old plates of 929/$CaCO_3$ solid medium was transferred to tubes containing fresh Medium 929. Tubes were checked for acid production as an indicator of AAO outgrowth.

RESULTS

AAO growth on solid medium. Colonies (approximately 0.2 mm in diameter) could be observed visually after 4 weeks incubation of 929/$CaCO_3$ plates that had been streaked with stock cultures of *N. europaea*, *N. multiformis*, *Nitrosospira* strain Apple Valley, and *Nitrosospira* strain 39-19. Inoculation of plates by spotting 50-100 ml aliquots of cultures onto plate surfaces produced lawns of confluent growth after 3 to 4 weeks incubation. Clear zones, indicating dissolution of $CaCO_3$ by acid production, could be observed in the agar below and surrounding the lawns. Individual AAO colonies were too small to produce noticeable clearing, although clear zones could be observed under more heavily inoculated areas after several months incubation.

Individual AAO colonies on these plates were large enough after 4 to 8 weeks incubation to provide sufficient cell material for rep-PCR. This required at least five colonies, and attempts to use a single colony for PCR were not successful. Lawns of growth scraped from plate surfaces also yielded sufficient cell material to allow fixation and embedding for transmission electron microscopy (Figures 2.1 through 2.4). All cells exhibited the typical morphological features that have been used to distinguish AAO strains (Watson et al., 1989). These included intracytoplasmic membranes, carboxysomes, and typical cell shapes. Plates of 929/CaCO3 Medium were also inoculated with enrichment and MPN cultures from LTER soils.

Most-Probable Number (MPN) Analyses. MPN counts from low-input-cultivated soils using SB Medium were at least tenfold higher than counts obtained from never-tilled soils for April 1992 samples (Table 2.3). Instead of enhancing growth, addition of bentonite clay reduced MPN counts by at least two orders of magnitude for never-tilled soils. The presence of clay did not appear to significantly affect MPN counts for low-input-cultivated soils. MPN counts in diluted medium were reduced by factors of 100 and 30-50 for never-tilled and low-input-cultivated soils, respectively (Table 2.3).

MPN counts of never-tilled soils were also approximately tenfold lower than counts from cultivated soils in 1994 and 1995 (Tables 2.4 and 2.5). For soils sampled in 1994, very little or no difference could be seen between counts

FIGURE 2.1. Transmission electron microscopy photograph of *Nitrosomonas europaea* ATCC 25978 showing intracytoplasmic membranes as lamellae at the periphery of the cell. (39,000X magnification.)

FIGURE 2.2. Transmission electron microscopy photograph of *Nitrosolobus multiformis* ATCC 25196 showing intracytoplasmic membranes compartmentalizing cell into a lobate structure. (29,000X magnification.)

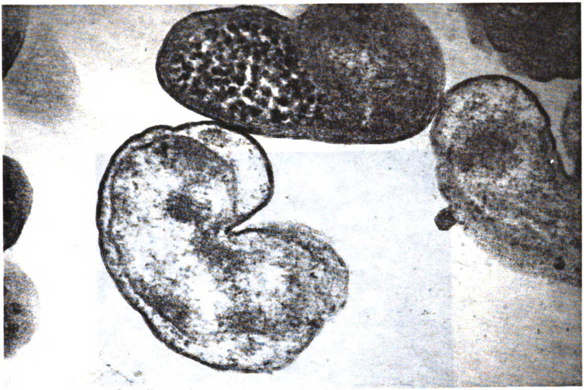


FIGURE 2.3. Transmission electron microscopy photograph of *Nitrosospira* strain Np AV (Schmidt) showing lack of intracytoplasmic membranes. Cell is comma-shaped, due to a single spiral turn. (39,000X magnification.)

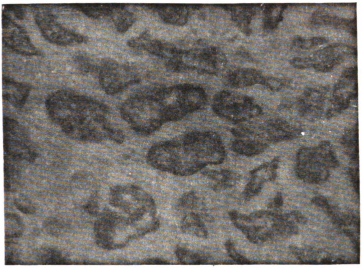


FIGURE 2.4. Transmission electron microscopy photograph of *Nitrosospira* strain R1 grown on Noble agar plates containing mineral medium and $CaCO_3$. (29,000X magnification.)

TABLE 2.3. Comparative MPN counts (x 10^3 AAOs per gram of soil) obtained in Schmidt-Belser medium with and without bentonite clay (one soil sample per replicate plot in 1992).

Soil	1/10X	1X	1X + Clay
Cultivated low-input (wheat)			
Rep 1	2.3	70	1.1
Rep 2	2.3	130	130
Average (n=2)	2.3	100	66
Never-tilled			
Rep 1	< 0.1*	2.3	< 0.1*
Rep 2	< 0.1*	13	< 0.1
Average (n=2)	< 0.1	7.7	< 0.1

* no growth in any tube

TABLE 2.4. Comparative MPN counts (x 10^3 AAOs per gram dry soil) obtained in Medium 929 containing 1/10X, 1X, and 10X standard NH_4-N concentrations (two subsamples per composite sample in 1993).

Soil	1/10X	1X	10X	Ratio of MPN counts (1/10X over 10X)
Cultivated (corn)				
Subsample 1	490	330	79	6.2
Subsample 2	170	79	17	10.0
Average	330	200	48	6.9
Never-tilled				
Subsample 1	7.0	1.7	3.3	2.1
Subsample 2	13	22	33	0.4
Average	10	12	18	0.6

TABLE 2.5. Comparative MPN counts (x 10^3 AAOs per gram of soil) obtained in Medium 929 containing 1/10X, 1X, and 20X standard NH_4-N concentrations (samples from two replicate plots in 1995).

Soil	1/10X	1X	20X	Ratio of MPN counts 1/10X over 20X
Cultivated (wheat)				
Rep 5	120	100	45	2.7
Rep 6	130	85	80	1.6
Average	130	93	63	2.1
Never-tilled				
Rep 3	13	33	0.18	72
Rep 4	12	59	0.19	63
Average	13	46	0.19	68
Unfertilized				
successional	74	97	65	11.4
Rep 1	46	250	40	1.2
Rep 3				
Average	60	170	23	2.6
Unfertilized				
Fertilized				
successional	190	120	58	3.3
Rep 1				
Rep 3	83	410	83	1.0
Average Fertilized	140	270	71	2.0

in 1X and 10X concentrations of NH_4-N. When a higher NH_4-N concentration (20X) was used in 1995, the MPN ratios (1X counts/20X counts) were more than thirtyfold higher for never-tilled soils than ratios for cultivated and fertilized successional soils (Table 2.5).

Enrichments. Six-month enrichments of never-tilled soil in flask cultures were streaked out onto solid medium, and the inoculated plates were examined after six weeks incubation. Numerous, small (< 0.2 mm), rust-colored colonies could be observed on plates that had been inoculated with the enrichments grown in 1/10X SB Medium. Since these colonies were identical in size and morphology, ten colonies were picked to one tube containing Medium 929. The resulting culture was confirmed to be free from heterotrophic contamination and was designated "R1" for "rust-colored." A few similar colonies could be observed on plates inoculated with the 1/10X + Clay enrichment, but plates inoculated with 1X and 1X + Clay enrichments were overgrown with spreading fungal and bacterial colonies.

When MPN tubes from low-input-cultivated and cultivated soils were streaked out onto solid medium, most of the resulting growth was obviously heterotrophic. Approximately 60 small, isolated, putative AAO colonies were picked to tubes containing Medium 929, but none of these produced viable cultures after transfer. Heterotrophic colonies were large (> 3 mm), mucoid, and white, yellow, or orange in color.

Long-term storage of AAOs. Equilibration of cells in glycerol (10% final concentration), freezing and thawing, and frozen storage reduced the viability of all AAO strains, as indicated by the incubation periods needed for cultures to turn yellow (Table 2.6). When equivalent numbers of untreated cells were transferred to fresh medium, all cultures turned yellow within 1 day, except

TABLE 2.6. Evaluation of effect of freezing and frozen storage on viability of AAOs. Numbers in columns are days required for cultures to turn yellow after thawing and transfer to fresh medium.

Treatment	*N. europaea* ATCC 25978	*N. europaea* ATCC 19718	*Nitrosolobus multiformis* ATCC 25196	*Nitrosolobus* strain 24-C	*Nitrosospira* strain Apple Valley	*Nitrosospira* strain 39-19
None	1	1	1	2-3	1	1
Glycerol only	1	2	1-2	3	3	1-2
Glycerol + freezing	1	1-2[a]	3-5	3[a]	2-3	2-5[a]
One-month frozen storage[b]	3[a]	3-5	3[a]	3-5	3[a]	3
One-year frozen storage	14-17	14[a]	45	14-21	17	14-17

[a] No pH change in one out of three tubes.

[b] Duplicate, rather than triplicate, tubes used.

for *Nitrosolobus* strain 24-C, which grew somewhat more slowly than the other strains. Exposure of cells to glycerol without freezing retarded cell outgrowth, because these cultures needed to be incubated 1-2 days longer than untreated cultures before they turned yellow. Freezing without storage also had a growth-retarding effect for most of the AAO strains, as did extended storage at -70°C. *N. europaea* ATCC 25978 appeared to be the most resistant of the six strains to these treatments, while *N. multiformis* ATCC 25196 appeared to be the most sensitive. All of the AAO strains had at least one tube (out of duplicate or triplicate tubes) containing nonviable culture after freezing (Table 2.6). Thus, if freezing is used as a storage method for the AAOs, two or three glycerol stocks may need to be thawed so that at least one viable culture will grow out. The glycerol stocks prepared in this study yielded viable cultures after 2 1/2 years of frozen storage, although for some strains (e.g., *N. multiformis*), two or three vials had to be thawed before a viable culture was obtained.

DISCUSSION

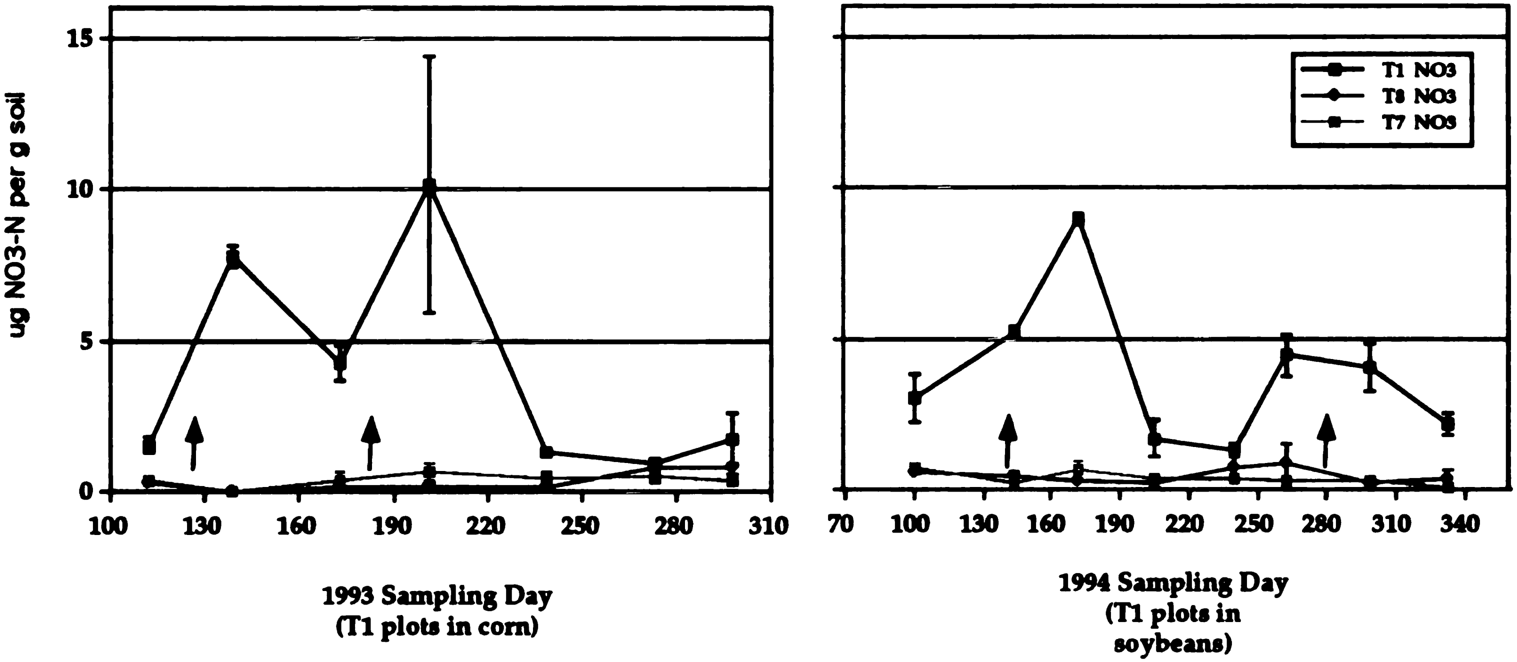
MPN media containing three different concentrations of $(NH_4)_2SO_4$ were used to obtain a more reliable comparison of AAO population sizes than would be obtained with a single NH_4-N concentration. MPN counts of cultivated and uncultivated soils in Medium 929 were generally higher at the 1/10X (10 ppm) NH_4-N concentration than at the 1X (100 ppm) concentration by factors ranging from 2 to 10 (Tables 2.4 and 2.5). When the highest MPNs among the three NH_4-N concentrations were considered for each sample, MPN counts of never-tilled soils were consistently tenfold lower than counts of cultivated soils.

Soil NO_3-N and NH_4-N measurements support the conclusion that *in situ* AAO populations in never-tilled soils were one-tenth as large as populations in cultivated soils. Measurements made throughout the 1993 and 1994 growing seasons showed that never-tilled soils had very low NO_3-N levels, ranging from 0 to 1.5 ppm, with a mean of 0.4 ppm. Cultivated soils during the same period (corn in 1993, soybeans in 1994) had NO_3-N levels that were generally tenfold higher, and these ranged from 0.9 to 14.4 ppm, with a mean of 3.9 ppm (Figure 2.5). NH_4-N levels in never-tilled soils exhibited a fourfold variation, ranging from 4.2 to 18 ppm, with a mean level of 10.2 ppm (Figure 2.6). The mean NH_4-N level was lower in cultivated soils (3.0 ppm), and NH4-N varied more widely, with a sevenfold variation from 1.5 to 10.2 ppm (Figure 2.5). The low NO_3-N levels found concomitantly with higher NH_4-N levels in the never-tilled soils indicate that AAO populations in these soils were not as active in producing NO_3-N as the populations in cultivated soils. This conclusion is compatible with previous studies showing correlations between AAO numbers and nitrifying activity in systems permitting visual enumeration by fluorescent probes (Laanbroek et al., 1993).

The reason for lower populations of AAOs in the presence of higher NH_4-N concentrations in never-tilled soils is unclear. One explanation for lower AAO populations and NO_3-N levels in never-tilled soils could be that the higher amounts of organic matter in these soils tied up the NH_4-N and made it less available to AAOs. This explanation, however, is not supported by the NO_3-N and NH_4-N data from successional soils (Figures 2.5 and 2.6), which also contained higher amounts of organic C (Bruns et al., manuscript in preparation). Another explanation is that some factor(s) in the never-tilled soils inhibited the growth or activity of AAOs (Rice and Pancholy, 1972).

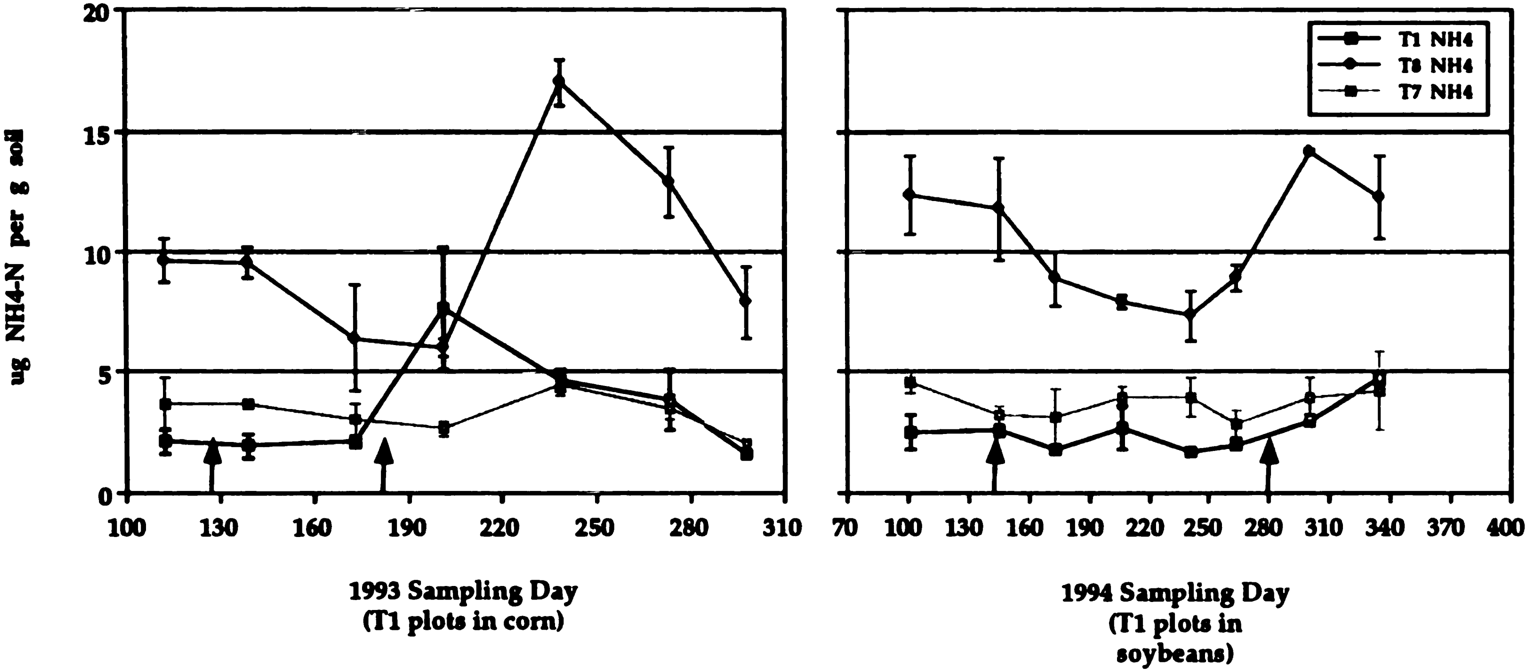
The differences in inorganic N dynamics and MPN counts between cultivated and uncultivated soils may indicate differences in AAO population structures. The MPN results for LTER soils can be related to the observations of Suwa et al. (1994), who obtained estimates of the prevalence of NH_4-

FIGURE 2.5. Mean NO3-N levels in cultivated (T1), never-tilled (T8), and successional (T7) soils in 1993 and 1994. Error bars show standard errors for NO3-N measurements from duplicate plots. First arrows in each graph indicate planting day. Second arrows indicate fertilization day and harvest day, respectively, for 1993 and 1994. (Soybean crop was not fertilized.)



Source: LTER Data Catalog, 1996.

FIGURE 2.6. Mean NH4-N levels in cultivated (T1), never-tilled (T8), and successional soils in 1993 and 1994. Error bars show standard errors for NH4-N measurements from duplicate plots. First arrows in each graph indicate planting day. Second arrows indicate fertilization day and harvest day, respectively, for 1993 and 1994. (Soybean crop was not fertilized.)



Source: LTER Data Catalog, 1996.

sensitive AAOs in activated sludges by comparing ratios of MPN counts obtained at low (20 ppm) vs. high (1000 ppm) NH_4-N concentrations. For sludges with higher N loadings (mean of 1.2 g total Kjeldahl nitrogen (TKN) per liter per day), ratios of MPN counts at low vs. high NH_4-N concentrations ranged from 0.6 to 5.7. For sludges with lower N loadings (mean of 0.2 g TKN per liter per day), the MPN ratios ranged from 3.7 to > 3900. Suwa et al. (1994) observed that isolates from low-N sludges grew in media containing 20 ppm NH_4-N but not in media containing 1000 ppm NH_4-N, while isolates from high-N sludges grew at either NH_4-N concentration. Dominant AAOs in low-N sludges thus appeared to be NH_4-sensitive, while dominant AAOs in high-N sludges tolerated 1000 ppm NH_4-N and higher.

The 1/10X (10 ppm) and 10X (1000 ppm) concentrations of NH_4-N used in our MPN media were comparable to the low and high concentrations used by Suwa et al. (1994). The ratios of MPN counts at 1/10X over 10X did not exceed 10 for any of the soils (Table 2.4), suggesting that the type of NH4-sensitive strains described by Suwa and coworkers did not dominate the AAO populations in these soils. When we used a higher NH_4-N concentration (20X or 2000 ppm) in subsequent MPN experiments, ratios of counts at 1/10X over 20X were much higher for never-tilled soils than for cultivated soils (Table 2.5). Overall, the AAOs in LTER soils were not as sensitive to NH_4-N as the AAOs from low-N sludges in the study by Suwa et al. (1994). The differences in MPN ratios detected with the 20X concentration, however, suggested that AAO populations in never-tilled soils were more sensitive to NH_4-N than AAOs from cultivated soils. These results could help explain why NH_4-N levels remained consistently lower in cultivated than in never-tilled soils. Cultivated soils may have selected forAAO populations that tolerated higher and more variable concentrations of NH4-N, resulting from repeated N-fertilization and release of

N-rich root exudates upon crop harvests. This explanation is supported by the higher variance in inorganic N levels observed for cultivated soils, relative to never-tilled and successional soils (Table 2.7).

Differences in the 1/10X over 20X ratios between cultivated and uncultivated soils also appeared to be reflected in the ratios for fertilized and unfertilized successional soils. Like the cultivated soils, fertilized successional soils exhibited low ratios. One of the two unfertilized successional soils exhibited a higher ratio, although it was not as high as the ratios for uncultivated soils (Table 2.5). This result could be an indication in this successional soil of an increase in AAOs that were more sensitive to NH_4-N. The highest MPN counts in the unfertilized successional soils, however, had been observed at 1X NH_4-N concentration, rather than 1/10 X concentration. When MPN ratios were calculated for these soils from the 1X over 20X NH_4-N counts, the ratios were slightly higher than analagous ratios for the cultivated and fertilized successional soils. These results also suggest that AAOs in cultivated and fertilized successional soils were more tolerant of higher NH_4-N concentrations.

The commonly used NH_4-N concentration for MPN counting of AAOs in soils has been 7.6 mM, or 100 ppm (Schmidt and Belser, 1982). Of the different NH_4-N concentrations used in our study, the 1/10 X concentration (10 ppm) was the most similar to measured soil inorganic N levels (NH_4-N plus NO_3-N) during the 1993 and 1994 growing seasons. Mean inorganic N levels were 6.9, 10.6, and 3.9 ppm for cultivated, never-tilled, and unfertilized successional soils, respectively (Table 2.7). Higher recoveries of AAOs from cultivated and never-tilled soils at the 1/10X NH_4-N concentration can probably be attributed to the fact that this concentration was most similar to soil inorganic N levels. MPN counts for successional soils, on the other hand, were somewhat higher at 100 ppm than at 10 ppm NH4-N in the recovery media (Table 2.5).

TABLE 2.7. Inorganic N measurements (NO_3-N plus NH_4-N) during 1993 and 1994 growing seasons in cultivated, never-tilled, and unfertilized successional soils, with n=30.

Soil treatment	Minimum	Maximum	Mean	Variance	Standard deviation
Cultivated	2.6	24.6	6.9	17.2	4.1
Never-tilled	4.5	18.3	10.6	11.1	3.3
Unfertilized successional	2.2	6.1	3.9	1.2	1.1

The use of the lower NH_4-N concentration in enrichment media made it possible to obtain the novel AAO isolate (Figure 2.4) from uncultivated soil, because this strain appeared to be much more numerous in the enrichments containing 10 ppm NH_4-N than in enrichments with 100 ppm NH_4-N concentrations. If enrichments had been carried out only at the standard NH_4-N concentration, it is unlikely that this AAO would have been isolated. The enrichment containing 10 ppm NH_4-N yielded numerous identical colonies on solid medium that could be picked to one tube to provide enough biomass to generate a viable culture. Sequence analysis of its 16S ribosomal RNA genes indicated that it belongs in a novel clade of *Nitrosospira* that is distinct from previously cultured *Nitrosospira* spp. (Bruns et al., 1995). Solid media have been used by other workers to isolate AAOs. When Koops and coworkers employed their solid medium, they were able to isolate eight new *Nitrosomonas* spp. (Koops et al., 1991). These successes in obtaining novel AAOs by cultural methods demonstrate the need to continue and expand the use of cultural methods in learning more about AAO diversity.

The solid medium used in this study supported the growth of all pure AAO cultures and could be used to store cultures for several months, as long as the plates were kept moist. Storage on solid medium offers an alternative to frozen storage of AAOs, which tend to become less viable the longer they are kept at $-70^{o}C$ (Table 2.6). This medium could also be used to generate larger amounts of biomass (i.e., from lawns of surface growth) for microscopy or DNA extraction. Although the solid medium was useful in maintaining AAO cultures that were already pure, it did not provide an efficient means to purify individual AAO strains from mixed cultures. Isolated colonies of putative AAOs from enrichment cultures could be obtained after 2-3 months incubation, as judged by clear zones around some colonies. However, most of these colonies did not produce viable cultures after they were transferred to liquid medium, probably because of the low amount of biomass in the colony. A number of these liquid cultures were "false positive" for growth, because they turned orange in color but were still negative for nitrite and nitrate after 2-3 months incubation. Other workers (Prosser, personal communication) have made similar observations about AAO growth media and attributed them to chemical changes in the pH indicator.

Cultural methods become much more powerful in characterizing AAO populations when they incorporate molecular analysis techniques. Growth from MPN enrichments, for example, could be amplified using 16S rDNA PCR to identify AAOs that become predominant at different NH_4-N concentrations during incubation. This approach, however, was not used in the molecular analysis portion of this study. Instead, PCR was directly performed on bacterial community DNA extracted from LTER soils. This latter approach provides more information on *in situ* AAO populations, rather than on the AAOs that grow out from these populations. Results of direct PCR analysis will be reported in Bruns et al. (manuscript in preparation).

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Chapter Three

EVALUATION OF *NITROSOMONAS EUROPAEA* GENE PROBE HYBRIDIZATIONS WITH *NITROSOSPIRA* DNA

INTRODUCTION

Autotrophic ammonia-oxidizing bacteria (AAOs) convert ammonia to nitrite in the first, rate-limiting step of nitrification. This step, which is followed by conversion of nitrite to nitrate by nitrite-oxidizing bacteria, is a critical link in nitrogen cycling in diverse ecosystems (Bock et al., 1989). AAOs thus represent a key functional group of bacteria in microbial communities in soils, sediments, waste treatment systems, building facades, and fresh, brackish, and marine waters (Watson et al., 1989; Koops and Moller, 1992). Despite their widespread distribution, AAOs appear to comprise a phylogenetically narrow group of eubacteria (Teske et al., 1994). To date, the only phylogenetic subdivision known to contain AAOs of terrestrial origin is the beta subgroup of Proteobacteria (Woese et al., 1984), although some marine and brackish water strains fall within the gamma subgroup (Woese et al., 1985). The apparently close phylogenetic relationship among terrestrial AAOs is likely to be reflected in the close similarity of functional gene sequences coding for enzymes involved in oxidation of ammonia, the sole source of energy for AAOs (Bock et al., 1989).

The ammonia oxidation pathway of AAOs has been studied almost exclusively in *Nitrosomonas europaea*, a culture that was purified from soil enrichments approximately 40 years ago (Lewis and Pramer, 1958). This isolate appears to be more amenable to laboratory culture than most other AAO strains (Prosser, 1989). A large body of literature exists (Hollocher et al., 1981; Arciero et al., 1991; Hyman and Arp, 1992; Arciero and Hooper, 1993) on the biochemical characterization of two enzymes involved in energy generation in *N. europaea*--ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). These enzymes carry out the following reactions:

$$(1)\ NH_3 + O_2 + 2e^- + 2H^+ \xrightarrow{\text{AMO}} NH_2OH + H_20$$

$$(2)\ NH_2OH + H_2O \xrightarrow[\text{as e- acceptor}]{\text{HAO with c554}} HNO_2 + 4e^- + 4H^+$$

Gene sequences have recently been published for AMO (McTavish et al., 1993a) and HAO (Sayavedra-Soto et al., 1994) in *N. europaea*. These sequences were obtained from PCR products amplified from *N. europaea* genomic DNA and primers based on amino acid sequences of purified *N. europaea* AMO and HAO proteins. AMO oxidizes ammonia to hydroxylamine, and HAO oxidizes hydroxylamine to nitrite. Two genes, *amoA* and *amoB*, code for AMO subunits (McTavish et al., 1993a). The gene *amoA* codes for the subunit containing AMO's active site. The gene *amoB* codes for another polypeptide involved in AMO activity and is located immediately downstream of *amoA*. The gene *hao* codes for the polypeptide unit of HAO (McTavish et al., 1993b; Sayavedra-Soto et al., 1994), which is a multimer of this unit (Arciero and Hooper, 1993). Another protein involved in energy generation in *N. europaea* is cytochrome *c*-554, believed to be the immediate electron acceptor for HAO (Arciero et al., 1991). The gene coding for cytochrome *c*-554, *hcy*, is located 1200 bp downstream of *hao* (Sayavedra-Soto et al., 1994).

We obtained *Escherichia coli* clones containing plasmids with inserts coding for ammonia oxidation enzymes from the laboratory of Dan Arp (Oregon

State University). From these inserts we prepared DNA probes for hybridization with genomic DNA extracted from pure cultures of four terrestrial AAOs within the *Nitrosospira* group. The *Nitrosospira* group is phylogenetically distinct from the *Nitrosomonas* group of AAOs in the beta subdivision of the Proteobacteria and includes the genera *Nitrosolobus*, *Nitrosospira*, and *Nitrosovibrio*. (Head et al., 1993; Hiorns et al., 1995). Our objective was to evaluate the hybridization signals generated by these functional gene probes against genomic DNA of other AAOs that have been isolated from soils. This was a first step in evaluating whether these probes would be reliable indicators of AAO population size if they were used in hybridization tests with DNA from mixed AAO populations in soils. Such probe hybridization tests have the potential to be more rapid in estimating AAO population sizes in environmental samples than the Most-Probable-Number (MPN) method, which involves 4 to 8 weeks of incubation (Schmidt and Belser, 1982; Bruns et al., manuscript in preparation).

Other gene sequences of interest in evaluating diversity among ammonia oxidizers are those coding for enzymes involved in denitrification. Previous reports have shown that *N. europaea* produces soluble nitrite reductase enzyme (Hooper, 1968; Ritchie and Nicholas, 1974) Poth and Focht (1985) also demonstrated N_2O production from NO_2^- in *N. europaea* cultures. The nitrite reductase enzymes found in AAOs may be unique to this group, or they could exhibit similarity to enzymes found in other denitrifying bacteria. To explore these possibilities, we also applied gene probes for nitrite-reductase (Smith and Tiedje, 1992; Ye et al., 1993) and nitrous oxide reductase (Viebrock and Zumft, 1988) from *Pseudomonas* strains in hybridization tests against genomic DNA of AAOs.

MATERIALS AND METHODS

DNA probes. Probes developed from *N. europaea* genes for ammonia oxidation enzymes and *Pseudomonas* spp. genes for denitrifying enzymes were used in hybridization studies (Table 3.1). *Escherichia coli* cells containing plasmids with probe inserts were grown in batch culture with appropriate antibiotics to maintain plasmids. Plasmid DNA was extracted from the cultures and purified in cesium chloride gradients according to standard methods (Sambrook et al., 1991). Probe fragments were isolated by agarose gel electrophoresis after digestion of plasmids with appropriate restriction enzymes. The Gene-Clean kit (Bio-101, Inc., La Jolla, California) was used to purify probe DNA from the gel. The purified fragments were heat-denatured and labelled with [α-^{32}P]dCTP (3000 Ci/mM, Dupont New England Nuclear Research Products, Wilmington, Delaware) by using a random priming kit from Boehringer Mannheim Biochemicals. Unincorporated nucleotides were removed from the labelled probes with a spun column approach (Sambrook et al., 1991). Labelled probe was added to hybridization fluid to obtain a final activity of approximately 10^6 cpm/ml. *Pseudomonas stutzeri* strain JM300 and *Pseudomonas* sp. strain G-179 were used as positive controls for hybridizing with probes for the Cu-type (Ye et al., 1992) and heme-type (Smith and Tiedje, 1993) dissimilatory nitrite reductases, respectively.

Bacterial strains. Six strains of ammonia-oxidizing bacteria previously isolated from soils were used in this study (Table 3.2). Strains in this collection included three of the five genera known to occur in soils--*Nitrosomonas*, *Nitrosolobus*, and *Nitrosospira*. These were obtained from the American Type Culture Collection (ATCC) and Dr. Ed Schmidt of the University of Minnesota. Cultures representing the other two genera--*Nitrosovibrio* and *Nitrosococcus*--were not available for this study.

TABLE 3.1. DNA probes used in this study

Probe designation	Organism (probe source)	Protein encoded by gene with probe sequence	Plasmid	Probe size	Laboratory source and reference
amoA	*Nitrosomonas europaea* ATCC 19718	Ammonia mono-oxygenase (AMO) acetylene-binding polypeptide		0.70 kb *Kpn*I-*Eco*RI	D. Arp (McTavish et al., 1993)
amoB	*Nitrosomonas europaea* ATCC 19718	Polypeptide involved in AMO activity, gene located immediately downstream of *amoA*		0.67 kb *Eco*RI-*Eco*RI	D. Arp (McTavish et al., 1993)
hao	*Nitrosomonas europaea* ATCC 19718	Hydroxylamine oxido-reductase	pNH110	0.72 kb *Kpn*I-*Eco*RI	D. Arp (Sayavedra-Soto et al., 1994)
hcy	*Nitrosomonas europaea* ATCC 19718	Cytochrome c-554, believed to accept electrons from HAO	pNHc55p1	0.73 kb *Eco*RI-*Bam*HI	D. Arp (Sayavedra-Soto et al., 1994)
TN45 probe	*Nitrosomonas europaea* ATCC 25978	Auxiliary polypeptide possibly involved in HAO activity	pTN45	4.5 kb *Eco*RI-*Eco*RI	T.Tokuyama (Tokuyama et al., 1988)
Cu-dNir	*Pseudomonas* sp. strain G-179	Copper-containing nitrite reductase	pRTc1.9	1.8 kb *Cla*I-*Cla*I	J. Tiedje (Ye et al., 1993)
Heme-dNir	*Pseudomonas stutzeri* JM300	Heme-containing nitrite reductase	pBsGTH	2.4 kb *Eco*RI-*Bam*HI	J. Tiedje (Smith and Tiedje, 1992)
nosz	*Pseudomonas stutzeri* ZoBell	Nitrous oxide reductase		1.2 kb *Pst*I-*Pst*I	J. Tiedje (Viebrock and Zumft, 1988)

TABLE 3.2. Strains of autotrophic ammonia-oxidizing bacteria used in this study

Strain	Source	Morphology	Comments
Nitrosomonas europaea ATCC 25978	American Type Culture Collection	Rod	Neotype, isolated from soil (location unknown) and purified with serial dilutions (Lewis and Pramer, 1958)
Nitrosomonas europaea ATCC 19718	Dr. Ed Schmidt, University of Minnesota	Rod	Same strain as ATCC 25978, separate culture deposited in ATCC by Dr. Ed Schmidt
Nitrosolobus multiformis ATCC 25196	American Type Culture Collection	Lobate	Isolated from soil from Surinam, South America (Watson et al., 1989).
Nitrosolobus strain 24-C	Dr. Ed Schmidt, University of Minnesota	ND	Isolated from agricultural soil in Minnesota, cultures tend to lose vigor quickly.
Nitrosospira strain Apple Valley	Dr. Ed Schmidt, University of Minnesota	Spiral, curved rod	Isolated from agricultural soil in Minnesota.
Nitrosospira strain 39-19	Dr. Ed Schmidt, University of Minnesota	ND	Isolated from agricultural soil in Minnesota.

Cultures of three heterotrophic bacteria were also used to provide DNA for positive and negative controls. The type stain of *Arthrobacter globiformis* (ATCC 8010) was included because it is a heterotrophic nitrifier found in soils (Verstraete and Alexander, 1972). It was of interest to see if DNA from a terrestrial, heterotrophic nitrifier (Kurokawa et al., 1985) would cross-hybridize with gene probes for ammonia oxidation enzymes from an autotrophic bacterium.

AAO batch culture scale-up, DNA extraction and REP-PCR. Tube cultures of AAOs in ATCC Medium 929 were grown to a cell concentration of 1-2x10^7 cells per ml (indicated by slight turbidity) by adjusting the pH of the cultures with 0.05M K_2CO_3 every other day over a 2-week period. One-ml aliquots of tube cultures were transferred to 50 ml fresh medium in 250-ml Erlenmeyer flasks, and the pH of these cultures was repeatedly adjusted with 0.1M or 0.3M K_2CO_3 for 2-3 weeks. Five-ml aliquots from the small flask cultures were transferred to 500 mL fresh medium in 2-L Erlenmeyer flasks, and these cultures were grown for another 2-4 weeks. All incubation was at 25°C in the dark without shaking. Cell counts in flask cultures were determined by examining wet mounts of cultures in a Petroff-Hauser counting chamber (Koch, 1981) at 1000x magnification.

Cells from the large flask cultures were harvested by centrifugation in 250-mL bottles at 5000 x g for 30 min. Cell pellets from 2 to 4 bottles were resuspended in Medium 929 without $(NH_4)_2SO_4$ and transferred to 30-ml Oakridge tubes to concentrate bacterial biomass. The miniprep procedure for extracting bacterial genomic DNA of Ausubel et al., 1990 (page 2.4.1) was modified by using tenfold larger volumes of all extraction reagents. This procedure was used to obtain genomic DNAs of all AAO strains and the two *Pseudomonas* strains. Genomic DNA of *A. globiformis*, which was more resistant to lysis, was extracted according to the method of Visuvanathan et al. (1989). REP-PCR (de Bruijn, 1992) was also performed on genomic DNA samples to compare agarose gel rep patterns among AAOs in the culture collection. This procedure, which is based on PCR amplification of conserved and repeated DNA elements in eubacteria, generates characteristic fingerprints for each organism and allows comparison and grouping of identical and related isolates.

Restriction digests, Southern blots, and probe hybridizations. Genomic DNA preparations were digested with *Eco*RI, loaded into lanes on 1% agarose gels, (3 ug DNA per lane) and subjected to electrophoresis (Sambrook et al. 1989). Visual examination of the relative brightness of UV-illuminated DNA in a typical agarose gel (Fig. 3.1) confirmed that all lanes contained equivalent amounts of DNA, except for lanes containing DNA from *Nitrosolobus* sp. strain 24-C. This AAO strain grew more slowly and had lower cell densities than the other AAOs, making it difficult to obtain comparable amounts of genomic DNA. The amount of *Nitrosolobus* sp. strain 24-C DNA available for loading into one lane was half the amount loaded for other bacterial strains. Subsequent densitometric measurements of this strain's hybridization bands were doubled to compensate for the lower amount of target DNA available for probe binding.

Restriction enzyme digestion, electrophoresis, and Southern blotting of restricted DNA onto Hybond N+ membranes (Amersham Life Sciences, New York) were carried out as described by Ausubel et al. (1992). The DNA on the blots was cross-linked by UV light using a Stratalinker (Stratagene, La Jolla, California). Blots were prehybridized for 16 h in heat-sealed bags containing a standard prehybridization solution consisting of 50% formamide, 5X Denhardt's

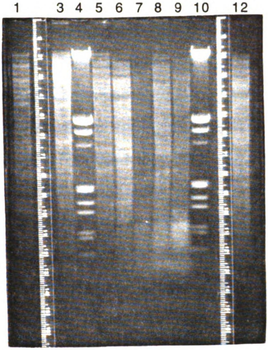

FIGURE 3.1. Ethidium-bromide-stained agarose gel containing *Eco*RI-digested DNA of ammonia oxidizers and control bacteria. (Lane 1), *Pseudomonas stutzeri* JM300 ; (Lane 2), blank; (Lane 3) *A. globiformis* ; (Lane 4), Lambda/HindIII DNA size marker; (Lane 5), *N. europaea*; (Lane 6), *N. multiformis* (Lane 7), *Nitrosolobus* sp. strain 24-C; (Lane 8), *Nitrosospira* sp. strain AV; (Lane 9), *Nitrosospira* sp. strain 39-19; (Lane 10), Lambda/HIII DNA size marker; Lane 11, blank; (Lane 12), *Pseudomonas* sp. strain G-179. All lanes contained approximately 3 ug genomic DNA, except for Lane 7 containing DNA from *Nitrosolobus* sp. strain 24-C, with approximately 1.5 ug.

solution, 5X SSPE, and 200 mg per ml salmon sperm DNA (Sambrook et al., 1989). After the prehybridization solution was removed, blots were immersed in hybridization solution containing ^{32}P-labelled probes for 24 h at 30°C (low stringency) or 42°C (moderate stringency). After hybridization, blots were washed once for 15 min at room temperature with 2X SSC (17.53 g of NaCl and 8.82 g of sodium citrate per liter; pH 7.0)-0.1% sodium dodecyl sulfate (SDS).

Additional washes and hybridizations were performed at different levels of stringency: (1) hybridization at 30°C and washing at 30°C in 2X SSC; (2) hybridization at 30°C and washing at 42°C in 0.1S SSC; (3) hybridization at 42°C and washing at 42°C in 0.1X SSC; and (4) hybridization at 42°C and washing at 55°C in 0.1X SSC. Either X-omat (Kodak) or Dupont NEN film was exposed to the probed blots using one Cronex Lightning Plus KE intensifying screen (DuPont) at -70C for 7-10 h prior to development. To facilitate re-probing, blots were stripped of radioactivity by washing twice for 15 min in distilled water that had been heated to boiling and amended with 1% SDS, followed by rinsing in 0.1X SSC for 15 min at room temperature. Membranes were kept moist in air-tight containers at 4°C between hybridizations to prevent drying.

Densitometric measurements from autoradiograms. Digitized images of autoradiogram films were obtained with the CCD camera of a Gel Print 2000i (BioPhotonics Corp., Ann Arbor, Mich.). Hybridization band intensities were determined using densitometry measurements of the digitized images with Spectrum IP Lab image processing software (Signal Analytics Corp., Vienna, Va.). Band intensities measured for *Nitrosolobus* and *Nitrosospira* strains were compared to those of *N. europaea* to estimate percent sequence similarities between the probes and homologous genes.

RESULTS

Genomic DNA yields from AAO cultures. Cell counts of 4-week-old AAO cultures were estimated to be 4 x 10^7 cells per ml at OD_{600} of 0.01. At this cell concentration the theoretical DNA yield from 1 ml of culture was 360 ng, assuming average cellular DNA content of 9 x 10^{-15} g per cell (Hecht et al., 1975). Typical DNA recoveries from 4-week-old cultures were 100-120 ng DNA per ml culture. DNA recoveries were lower when older cultures (e.g., 6 weeks) were used for extraction, presumably due to production of extracellular polysaccharides that interfered with DNA extraction. Slightly improved DNA recoveries could be achieved with AAO cultures that had just reached slight turbidity (0.5-1 x 10^7 cells per ml).

REP-PCR patterns. REP-PCR patterns were obtained from purified genomic DNA from the six AAO stock strains (Figure 3.2). The REP-PCR patterns for *N. europaea* ATCC 25978 and *N. europaea* ATCC 19718 appeared identical, suggesting that the strains were the same (de Bruijn, 1992). This observation was confirmed by D. Pramer (personal communication), who indicated that the source of the two separate ATCC accessions had been a culture purified in his laboratory from a soil enrichment obtained from D.L. Jensen in Denmark (Lewis and Pramer, 1958).

DNA probe hybridization patterns. Genomic DNA was digested with *Eco*RI prior to Southern blotting and hybridization tests. *N. europaea* DNA exhibited two hybridization bands with the *amoA*and *amoB* probes, three bands with the *hao* probe, and one band with the *hcy* probe (Figures 3.3 through 3.6). Other workers have indicated that *N. europaea* has two copies of the *amo* gene

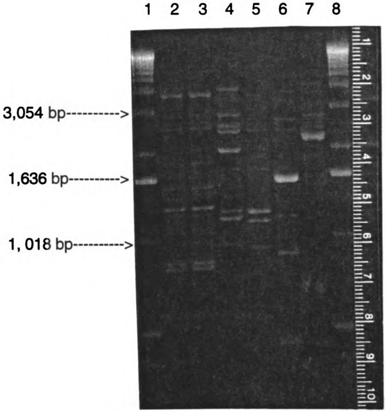

Figure 3.2. REP-PCR patterns of autotrophic ammonia oxidizer DNA observed in agarose gels stained with ethidium bromide and illuminated under UV light. Lane 1, *Nitrosomonas europaea* ATCC 25978; Lane 2 *Nitrosomonas europaea* ATCC 19718; Lane 3, *Nitrosolobus multiformis* ATCC 25196; Lane 4, *Nitrosolobus* 24-C (Schmidt); Lane 5, *Nitrosospira* NpAV (Schmidt); and Lane 6, *Nitrosospira* 39-19 (Schmidt).

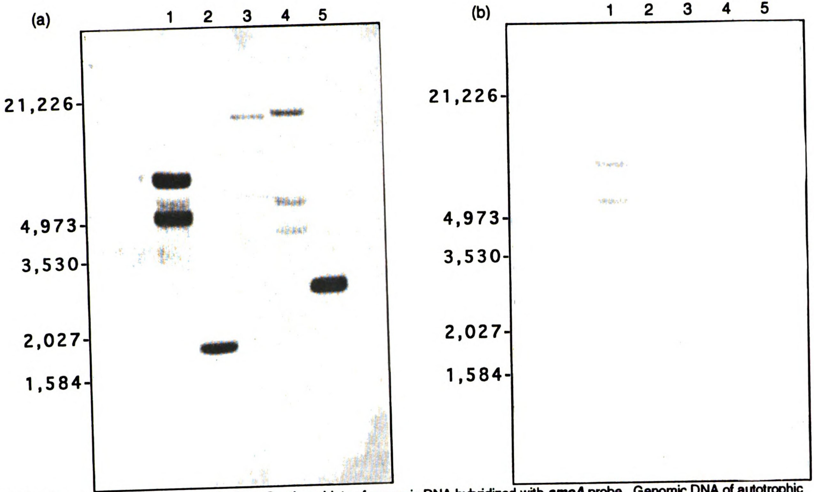

FIGURE 3.3. Autoradiogram photographs from Southern blots of genomic DNA hybridized with ***amoA*** probe. Genomic DNA of autotrophic ammonia oxidizers and control bacteria were digested with *Eco*RI: (Lane 1), *N. europaea* ; (Lane 2), *N. multiformis* ; (Lane 3), *Nitrosolobus* sp. strain 24-C ; (Lane 4), *Nitrosospira* sp. strain AV ; (Lane 5), *Nitrososopira* sp. strain 39-19 . Blots were hybridized with ^{32}P-labeled DNA probe for *amoA* (703 b): (a) Hybridization patterns under low-stringency conditions (hybridization at 30^{O}C, washing at 42^{O}C in 0.1X SSC). (b) Hybridization patterns under higher-stringency conditions (hybridization at 42^{O}C, washing at 42^{O}C in 0.1X SSC).

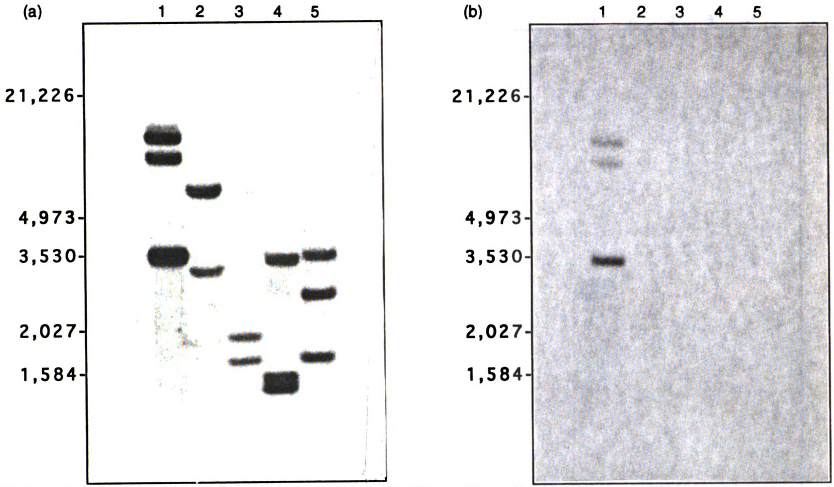

FIGURE 3.4. Autoradiogram photographs from Southern blots of genomic DNA hybridized with ***hao*** probe. Genomic DNA of autotrophic ammonia oxidizers and control bacteria were digested with *Eco*RI: (Lane 1), *N. europaea*; (Lane 2), *N. multiformis*; (Lane 3), *Nitrosolobus* sp. strain 24-C (Lane 4), *Nitrosospira* sp. strain AV; (Lane 5), *Nitrososopira* sp. strain 39-19. Blots were hybridized with ^{32}P-labelled DNA probe for *hao* (723 b): (a) Hybridization patterns under low-stringency conditions (hybridization at 30^{O}C, washing at 42^{O}C in 0.1X SSC). (b) Hybridization patterns under higher-stringency conditions (hybridization at 42^{O}C, washing at 42^{O}C in 0.1X SSC).

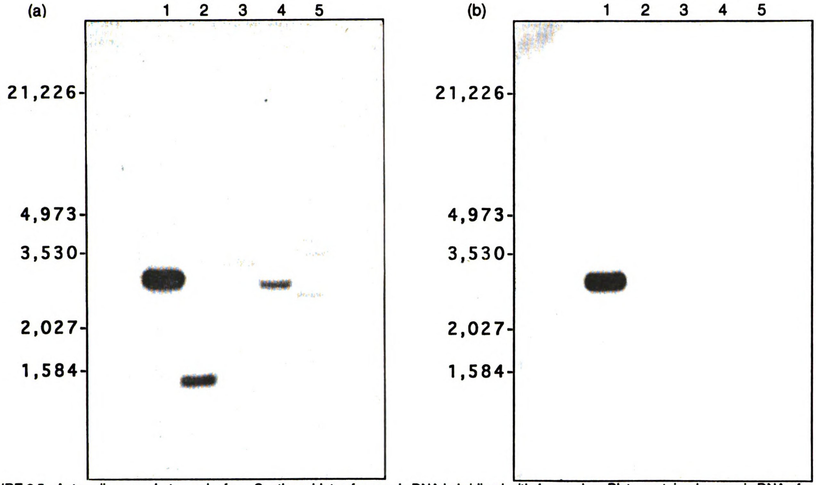

FIGURE 3.5. Autoradiogram photographs from Southern blots of genomic DNA hybridized with ***hcy*** probe. Blots contained genomic DNA of autotrophic ammonia oxidizers and control bacteria, digested with *Eco*RI: (Lane 1), *N. europaea* ; (Lane 2), *N. multiformis* ; (Lane 3), (Lane 4), *Nitrosolobus* sp. strain 24-C; *Nitrosospira* sp. strain AV ; (Lane 5), *Nitrososopira* sp. strain 39-19 . Blots were hybridized with ^{32}P-labeled DNA probe for *hcy* (623 b): (a) Hybridization patterns under low-stringency conditions (hybridization at 30^{O}C, washing at 42^{O}C in 0.1X SSC). (b) Hybridization patterns under higher-stringency conditions (hybridization at 42^{O}C, washing at 42^{O}C in 0.1X SSC).

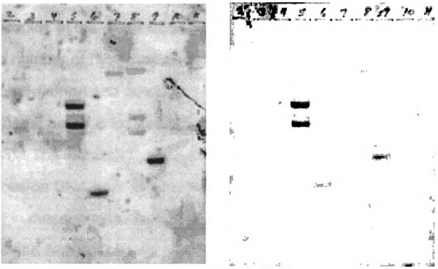

Figure 3.6a. Southern blots of AAO genomic DNA hybridized with *amoA* probe (left) and *amoB* probe (right). Hybridization was carried out at 30°C. Blots were washed at 30°C in 2X SSC buffer. Autoradiogram films were exposed for 6.5 h. Sources of genomic DNA were *N. europaea* (Lane 5); *N. multiformis* (Lane 6); *Nitrosolobus* sp. strain 24-C (Lane 7); *Nitrosospira* sp. strain AV (Lane 8); *Nitrosospira* sp. strain 39-19 (Lane 9).

Figure 3.6b. Southern blots of AAO genomic DNA hybridized with *amoA* probe (left) and *amoB* probe (right). Hybridization was carried out at 42°C. Blots were washed at 42°C in 0.1X SSC buffer. Autoradiogram films were exposed for 9.5 h. Sources of genomic DNA were *N. europaea* (Lane 5); *N. multiformis* (Lane 6); *Nitrosolobus* sp. strain 24-C (Lane 7); *Nitrosospira* sp. strain AV (Lane 8); *Nitrosospira* sp. strain 39-19 (Lane 9).

(McTavish et al., 1993a), three copies of the *hao* gene (McTavish et al., 1993b), and three copies of the *c*-554 gene (Sayavedra-Soto et al., 1994).

The number of hybridization bands and the locations of each band were identical for the *amoA* and *amoB* probes for all AAO strains tested (Figure 3.6). In *N. europaea*, *amoB* is located immediately downstream of *amoA* (McTavish et al., 1993). The identical hybridization patterns obtained with *amoA* and *amoB* probes on DNA from all other AAO strains indicates that these genes are also linked in these strains. Norton et al. (1996) have reported that *N. multiformis* and *Nitrosospira* sp. strain AV each contain three copies of the *amoA* gene.

Comparisons of summed band intensities. To evaluate whether *N. europaea* gene probes would provide reliable estimates of population sizes of other terresterial AAOs, the intensities of all hybridization bands for each strain, under lowest stringency conditions, were summed to obtain an estimate of total probe signal from each AAO (Table 3.3). The total probe signal depends on both gene copy number and percent similarity between the DNA probe and corresponding genes in different strains. Total probe signal of *N. europaea* was designated as 100%, because all probes were derived from *N. europaea* DNA sequences. Total probe signals from the other four AAO strains (Table 3.3) were divided by the total probe signal from *N. europaea* to obtain percentage values (Table 3.4). These percentages reflect the relative signal that could be obtained from one genome of an AAO strain when it is hybridized with the corresponding *N. europaea*-derived probe. In general, *N. multiformis* exhibited the lowest percentages (20 to 48%) of the four AAO strains tested. The *hao* probe gave the highest overall percentages (48 to 68%) among the four strains (Table 3.4).

TABLE 3.3. Summed intensities[1] of all hybridization bands obtained with each probe from genomic DNA of five ammonia oxidizer strains. Numbers in parentheses indicate the numbers of hybridization bands contributing to total intensities. Hybridizations were carried out under low stringency conditions (hybridization at 30°C, washing at 30°C with 2X SSC.)

Ammonia oxidizer strain	*amoA* probe	*amoB* probe	*hao* probe	*hcy* probe
Nitrosomonas europaea [2]	220,500 (2)	365,300 (2)	580,600 (3)	277,300 (1)
Nitrosolobus multiformis	80,500 (1)	74,400 (1)	276,400 (2)	128,400 (1)
Nitrosolobus sp. strain 24-C	166,600 (2)	46,700 (2)	372,200 (3)	75,400 (1)
Nitrosospira sp. strain AV[3]	158,300 (3)	114,200 (3)	394,400 (3)	75,500 (1)
Nitrosospira sp. strain 39-19	98,500 (1)	107,300 (1)	365,500 (3)	165,300 (3)

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[1] Densitometric measurements of bands in digitized images of autoradiogram films that had been exposed to Southern blots hybridized with ^{32}-P-labelled probes (Figures 3.3, 3.4, and 3.5).

[2] *Nitrosomonas europaea* contains 2 copies of the *amo* genes (McTavish et al., 1993a and 1993b), 3 copies of the *hao* gene, and 3 copies of the cytochrome *c*-455 (*hcy*) gene (Sayavedra-Soto et al., 1994).

[3] *Nitrosospira* sp. strain AV contains 3 nearly identical copies of the *amoA* gene (Norton et al., 1996).

TABLE 3.4. Summed band intensities for *N. multiformis*, *Nitrosolobus* sp. strain 24-C, *Nitrosospira* sp. strain AV, and *Nitrosospira* sp. strain 39-19, expressed as percentages of the summed intensities for *N. europaea* (Table 3.3).

Ammonia oxidizer strain	*amoA* probe	*amoB* probe	*hao* probe	*hcy* probe
N. europaea	100%	100%	100%	100%
N. multiformis	36	20	48	46
Nitrosolobus sp. strain 24-C	76	52	64	27
Nitrosospira sp. strain AV	72	31	68	27
Nitrosospira sp. strain 39-19	45	29	63	59

No hybridization was observed between any of the *N. europaea* probes and DNA from *A. globiformis*, *P. stutzeri* strain JM300, or *Pseudomonas* sp. strain G-179 (data not shown).

Hybridization results with other DNA probes. No hybridization was observed between DNA from the four AAO strains and the TN45 probe derived from *N. europaea* (Table 3.1). This probe thus appears to be derived from a DNA sequence unique to *N. europaea* among the strains tested. No hybridization was observed with DNA from any AAO and the probes for the heme dNir or nitrous oxide reductase. Slight cross-hybridization was observed between the probe for the Cu-type dNir and DNA from *Nitrosolobus* sp. strain 24-C and *Nitrosospira* sp. strain AV under moderate stringency conditions (Figure 3.7). No hybridization was seen with DNA from the other AAOs.

DISCUSSION

Results from the first comparative hybridization tests between a suite of*N. europaea* functional gene probes and genomic DNA from four representatives of the *Nitrosospira* group of ammonia-oxidizing bacteria are presented. These results provide important information for understanding how molecular analysis techniques may be applied to the study of heterogeneous AAO populations in soils and other environmental samples. The key findings in this study were that DNA from all four *Nitrosospira* strains hybridized with each *N. europaea* functional probe under low-stringency conditions (Figures 3.3, 3.4, 3.5), except for the TN45 probe. Total hybridization signal at low stringency from each of the four strains ranged from 20% to 76% of total signal from *N.europaea* DNA (Table 3.4). No hybridization was observed for *amo* and *hao* probes under higher-stringency conditions (hybridization and washing at 42°C), except for very faint bands between DNA from *Nitrosospira* strain Np 39-19 and probes for

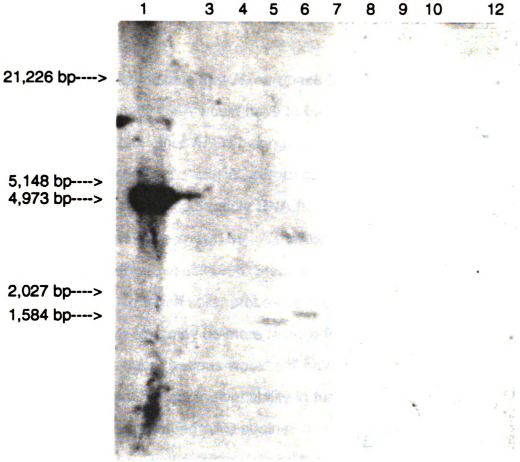

FIGURE 3.7. Autoradiogram photograph of Southern blot hybridized with *nir* probe for a copper-type nitrite reductase gene obtained from *Pseudomonas* sp. strain G-179 (Ye et al., 1992). The blot contained *Eco*RI-digested DNA from the following cultures: *Pseudomonas* sp. strain G-179 (Lane 1); Lane 2 blank; Lambda DNA marker (Lane 3); *Nitrosospira* sp. strain 39-19 (Lane 4); *Nitrosospira* sp. strain AV (Lane 5); *Nitrosolobus* sp. strain 24-C (Lane 6); *N. multiformis* (Lane 7); *N. europaea* (Lane 8); Lambda DNA marker (Lane 9); *A. globiformis* (Lane 10); Lane 11 blank; *Pseudomonas stutzeri* strain JM300 (Lane 12). The positive control, *Pseudomonas* sp. strain G-179, exhibited the expected hybridization band. Note that two AAO strains, *Nitrosospira* sp. strain AV and *Nitrosolobus* sp. strain 24-C, exhibited slight hybridization with the probe.

amoA (Figure 3.3) and *amoB* (Figure 3.6). The *hcy* probe also produced very faint bands with DNA from all four strains under higher-stringency conditions (Figure 3.5).

The results indicate that *N. europaea* DNA probe hybridization with microbial community DNA would have to be carried out under low-stringency conditions to detect other AAOs belonging to the *Nitrosospira* group. Use of low-stringency conditions with *N. europaea* DNA probes would be particularly critical when analyzing community DNA from soils, because cultural and molecular studies have shown that *Nitrosospira* spp. are the predominant AAOs in many soils (Hiorns, et al., 1996; Stephen et al., 1996; Bruns et al., manuscript in preparation). In these soils, probes developed from functional genes derived from *Nitrosospira* spp. may be more reliable for AAO population studies. It is likely that probe hybridizations would still have to be carried out at low stringencies to counteract the variability in functional gene homology (Norton et al., 1996) observed among AAO strains.

The need for low-stringency hybridizations with functional probes raises the problem of potential nonspecific binding of the probes to DNA from similar genes in unrelated organisms. Methane oxidizers, for example, have DNA that may cross-hybridize with probes for ammonia monooxygenase, because this enzyme has substrate specificity similar to that of the particulate methane monooxygenase (McTavish et al., 1993). Since methane-oxidizing bacteria are also found in soils, DNA from these populations may also bind to *amo* probes under low-stringency conditions. For this reason, an *hao* probe is likely to be more specific for AAOs than an *amoA* probe (D. Arp, personal communication). The *hao* probe exhibited the highest total hybridization signal of the four *N. europaea* probes tested in this study (Table 3.4), suggesting that this gene is more conserved among AAOs. Of these four probes, the *hcy* probe would be expected to have the most cross-reactivity with DNA from other other bacteria, because the sequence for the *c*-554 gene shares homology to genes coding for other heme cytochromes (Sayavedra-Soto et al., 1994). Under the higher-stringency conditions used in our study, the *hcy* probe exhibited greater cross-reactivity with DNA from all four *Nitrosospira* strains than the other three probes (Figure 3.5).

Functional gene probe hybridizations with community DNA cannot be considered to give absolute estimates of AAO population sizes in mixed communities. A key problem in using probe hybridizations to estimate AAO population size stems from our lack of knowledge about the extent of cross-hybridization between DNA probes that have been developed from cultured AAOs and the genomic DNA of uncultured AAOs. As a group, the AAOs are extremely difficult to culture and purify, and only 10 to 20 different cultures of AAOs are available in international culture collections. In addition, the physiological and biochemical characteristics of *N. europaea* have been assumed to be generalizable to other AAOs (Prosser, 1989). Physiological diversity among AAOs may be much greater than previously assumed. The lack of any cross-hybridization between the TN45 probe sequence from *N. europaea* and DNA from the *Nitrosospira* strains could allude to biochemical attributes in the *Nitrosomonas* group that are absent in the *Nitrosospira* group. Similarly, the cross-hybridization between *Pseudomonas* dNir probes and DNA from two out of four *Nitrosospira* strains (Figure 3.7) may be an indication of broader physiological diversity within this group. Continued efforts to modify enrichment conditions and improve cultural procedures may result in the isolation from soils of AAO bacteria which belong to phylogenetic divisions other than the beta subgroup of Proteobacteria. The ammonia oxidation enzymes of such AAOs would likely have undergone greater divergence and would exhibit less homology to *N. europaea* DNA probes than the terrestrial strains used in this study.

Hybridization tests in this study were performed only on genomic DNA from pure AAO cultures so that cross-reactivity between *N. europaea* gene probes and DNA from *Nitrosospira* representatives could be compared directly. Since no attempt was made to apply these probes to community DNA extracted from soils, this study did not address the possibility that AAO numbers in soils are too low to produce detectable hybridization signals. The lowest detection limit for high-activity radioactive probes with single-copy targets of 1 kb is 10^4 organisms per gram of soil (Holben et al., 1988; Harris, 1994). Since MPN counts of AAOs in soils may be 10^4 or fewer per gram (Schmidt and Belser, 1982), some AAO populations would be too small to be detected with functional probes. Thus, it would be more feasible to use the probe hybridization approach with community DNA extracted from soils expected to have high AAO populations (e.g., soils that have been amended with ammonia-based fertilizers or animal manures; high-nitrogen-loaded sludges). Probe hybridizations may be most useful in situations where comparisons of relative population sizes, rather than absolute counts, can be used to track spatial, temporal, or treatment variations.

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Chapter Four

USE OF 16S rDNA ANALYSIS TO COMPARE DIVERSITY OF AUTOTROPHIC AMMONIA OXIDIZER POPULATIONS IN SOILS

INTRODUCTION

Autotrophic ammonia-oxidizing bacteria (AAOs) are a key functional group within soil microbial communities, because they carry out the rate-determining step in nitrification. Terrestrial AAOs represent a narrow phylogenetic group within the β-Proteobacteria (Head et al., 1993; Teske et al., 1994), and they are known to be difficult to enumerate and isolate (Schmidt and Belser, 1982). Their distinctive functional and phylogenetic characteristics increase the likelihood that oligonucleotide probes and primers developed from their genomic DNA sequences will be specific and useful for analyzing DNA from AAO populations in complex communities. Genes coding for small subunit ribosomal RNA (16S rDNA) have been used most extensively in phylogenetic analysis of bacteria (Woese et al., 1984; Maidak et al., 1994). The 16S rRNA genes probably have greater potential than functional genes for providing the means to evaluate AAO population sizes and structures within complex communities.

Genes for enzymes in the autotrophic ammonia oxidation pathway (McTavish et al., 1993; Sayavedra-Soto et al., 1994; Klotz and Norton, 1995) may not provide sufficient target within community DNA to bind functional probes, because AAO populations typically comprise less than 0.01% of the total microbial community in soils (Schmidt and Belser, 1982). Functional gene copy numbers and nucleotide sequences appear to vary enough within this narrow group of β-Proteobacteria so that functional probe hybridizations may only be able to provide relative estimates of AAO population sizes (Norton et al., 1996; Bruns et al., manuscript submitted). Stephen et al. (1996a) recently compiled a database containing 110 partial 16S rDNA sequences for AAOs in the β-subgroup of Proteobacteria. Six distinct sequence clusters, based on differences in variable regions of these sequences, were identified (Stephen et al., 1996a). Marker sequences identified for each cluster appeared to be useful for evaluating the composition of mixed AAO populations (Stephen et al., 1996b).

Our study describes the use of PCR-based 16S rDNA analysis to compare AAO populations in soils with different disturbance and fertilization regimes. In contrast to workers who have used universal 16S rDNA primers with PCR to characterize whole bacterial communities in simpler systems (Giovannoni et al., 1990; Schmidt et al., 1991) we used more specific primers (McCaig et al., 1994; Kowalchuk et al., 1996) targetted for AAOs and their close relatives within the larger soil microbial community. We also analyzed PCR amplification products with oligonucleotide probes developed from the database of Stephen et al. (1996a). Two approaches were used: 1) cloning of PCR amplification products followed by probe hybridizations of clone blots; and 2) separation of PCR products by denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993), followed by probe hybridizations of DGGE blots (Kowalchuk et al., 1996; Stephen et al., 1996b). We observed reproducible differences in 16S rDNA sequences generated from the soil community DNA samples and attempted to relate these observations to known differences in soil treatments (i.e., disturbance and fertilization histories). These soils were shown to have different AAO population sizes and different nitrification capacities (Bruns et al., manuscript in preparation). We propose that the differences in rDNA sequences obtained from soil communities reflect actual differences between *in situ* AAO populations within these communities.

MATERIALS AND METHODS

Long-Term Ecological Research site. Soils containing the AAO populations to be analyzed were sampled from the Long-Term Ecological Research (LTER) site at Kellogg Biological Station (KBS) in southwest Michigan U.S.A. (Robertson et al., 1996). This site was established in 1988 to study ecological interactions affecting productivity, nutrient availability, and biotic diversity in cropped, successional, and native ecosystems representative of the Upper Midwest United States. Chemical, biological, and physical measurements and agronomic parameters are taken regularly at this site during each cropping season. Much of these data are accessible through the KBS LTER home page on the World Wide Web (http://kbs.msu.edu/lter/). Some KBS LTER data for relevant treatments, experimental plots, and sampling periods are cited in this paper (see below). These provide supplemental information for ecological interpretations of results from microbiological and molecular analyses.

Soils and treatments. Soils from cultivated and never-tilled plots at the LTER site were sampled in July 1993, july 1994, and August 1995. Soils from successional plots were sampled in August 1995 only. Analysis of 16S rDNA from soil bacterial community DNA was performed on 1994 and 1995 soil samples obtained from two 1-ha replicate plots per treatment.

The cultivated plots (LTER Treatment 1) had been in a corn-soybean rotation from 1989 to 1994, with wheat introduced as a third rotation crop in 1995. This treatment was conventionally tilled (annual moldboard plowing, disking, and cultivation), treated with prescribed applications of herbicides and insecticides, and fertilized with ammonium nitrate. The N fertilizer was broadcasted at a rate of 84 kg N per ha to the corn crop and 56 kg N per ha to the wheat crop. Crops in this treatment were corn, soybeans, and wheat in 1993, 1994, and 1995, respectively. The never-tilled plots (LTER Treatment 8) had not been tilled after clearing of the native deciduous forest in 1959. (Most of the vegetation in these plots now consists of C3 grasses.) Successional plots (LTER Treatment 7) had been in corn and soybeans for at least 40 years before they were left in 1989 to revegetate with extant flora. The successional plots were therefore under a disturbance regime intermediate between the cultivated and never-tilled treatments. Nitrogen-fertilized microplots (5m x 5m) were also sampled to evaluate fertilization vs. disturbance effects. These microplots (designated here as Treatment 7F) received 125 kg N per ha broadcasted in July of each year.

Soils at the LTER site are classified as Typic Hapludalfs (U.S. Soil Classification System, 1992) belonging to the Kalamazoo and Oshtemo soil series (fine, loamy, mixed, mesic). Soils were sampled to a 10-cm depth with a 2.5-cm corer (approximately 14 g fresh soil per core). The A horizons in these soils are typically 20 cm deep. Soil samples in 1994 were composited from five sampling stations in each of the two replicate plots per treatment. Samples in 1995 were composites of 20 soil cores taken near LTER sampling station #1 in each plot (Robertson et al., 1996). All soil samples were stored at $4^{o}C$ until they were analyzed in the laboratory. Prior to subsampling, soils were mixed inside plastic bags by manually kneading and shaking the bags. Gravel and other debris were remoeved from these soils manually. Soil moisture contents were determined from two 10-g subsamples dried at $110^{o}C$ for 48 h. All results reported here are based on soil dry weights.

Descriptions of each treatment and other biological and chemical characteristics of soils from these treatments are summarized in Table 4.1. Nitrate and ammonium measurements are made monthly during ice-free conditions as part of routine KBS LTER site data collection (Robertson et al., 1996). The nitrate and ammonium contents reported in Table 4.1 were obtained from the KBS LTER Site Data Catalog on the World Wide Web (http://kbs.msu.edu/lter). These were determined on composited samples from each replicate plot between April and November in 1994. Soil samples were sieved (4 mm) and subsampled in triplicate. Subsamples (10 g fresh weight) were extracted in 100 ml of 1 M KCl by shaking for one min, standing overnight, and reshaking prior to filtering with a type A/E 1-um pore size glass fiber filter. Filtrates were analyzed on an Alpkem segmented flow analyzer (KBS LTER Site Data Catalog, Soil Inorganic Nitrogen, 1996).

Organic C, microbial biomass C, and direct microscopic counts reported in Table 4.1 were determined from composited samples taken in July, 1994 from each replicate plot. Carbon analysis was performed on oven-dried, ground samples in a Carlo Erba NA 1500 series 2 nitrogen/carbon analyzer (Fisons Instruments, Beverly, Mass.). Microbial biomass C contents were measured by the chloroform-fumigation-incubation method (Horwath and Paul, 1995). Direct microscopic counts were made by staining soil smears with DTAF [5-(4,6-dichlorotriazin-2-yl)amino fluorescein (Sigma Chemical Co., St. Louis, Mo.)] and obtaining random digitized images of the smears under epifluorescence microscopy (D. Harris, personal communication) with a charge-coupled device camera (Princeton Instruments, Trenton, N. J.). Images were transferred to a Power Macintosh 7100/66 for counting as previously described (Zhou et al., 1996). Most-probable number (MPN) counts of AAOs were determined for 1995 samples by blending soils in a Waring Blendor with 100

TABLE 4.1. Management histories, biochemical measurements, and biological characteristics of soils sampled from cultivated, successional, and never-tilled treatments at the LTER site, Kellogg Biological Station, Michigan, U.S.A (includes 1993, 1994, and 1995 data).

Parameter	Treatment 1 (cultivated)	Treatment 8 (never-tilled)	Treatment 7 (successional)	Treatment 7F (fertilized successional)
Management history[1]	Cultivated for at least 40 years prior to 1989; under high-input, conventional-till corn/soybean/wheat rotation since 1989; 84 kg N per ha applied during corn years; 56 kg N per ha on wheat	Native deciduous woodland cleared in 1959; left under native successional vegetation, with annual fall-mowing since 1989	Under cultivation about 40 years prior to 1989; under unmanaged native successional vegetation since 1989	Same history as Treatment 7 except for N fertilization; annual application of 125 kg N per ha
Mean NO_3-N (µg NO_3-N per g soil) April-October, 1993[2]	4.0 (±3.7)	0.3 (±0.3)	0.4 (±0.3)	ND
Mean NH_4-N (µg NH_4-N per g soil) April-October, 1993[2]	3.4 (±2.2)	9.9 (±3.9)	3.3 (±0.8)	ND
Microbial biomass C (µg per g soil)[3]	189	320	299	ND
Direct bacterial counts per g soil (July 1994 samples)	2.9 (±0.7) x 10^9	5.1 (±1.0) x 10^9	ND	ND
MPN counts of AAOs per g soil (August 1995)[4]	1.3 (±0.1) x 10^5	1.3 (±0.1) x 10^4	6.0 (±1.4) x 10^4	1.4 (±0.6) x 10^5
Bacterial heterotroph : AAO ratio	21,000 : 1	364,000 : 1	ND	ND

ND = Not determined

[1] Robertson et al., 1996; KBS LTER Agronomic Protocol, 1996, KBS LTER home page on World Wide Web, http://kbs.msu.edu/lter/..

[2] Measurements taken monthly from April through November, 1993. Means are from measurements for two replicate plots, with n=14. Standard deviations for the means are given in parentheses (KBS LTER Site Data Catalog, Soil Inorganic Nitrogen, 1996, KBS LTER home page.)

[3] Chloroform fumigation-incubation method (Horwath and Paul, 1995); mean of 4 samples, with one sample measured per replicate plot in 1993 and 1995 (D. Harris, personal communication).

[4] Bruns et al., manuscript in preparation.

mM phosphate buffer (pH 7.0) and making twofold dilutions in 96-well microtiter plates (Bruns et al., 1996). The wells in the microtiter plates contained ATCC Medium 929 modified by reducing the $(NH_4)_2SO_4$ concentration tenfold to 10 ppm (Bruns et al., manuscript in preparation). Inoculated plates were sealed with parafilm, wrapped in plastic bags, and incubated at 25°C in the dark for two months before testing aliquots from the wells for nitrate and nitrite (Bruns et al., manuscript in preparation).

DNA extraction from soils. Soil samples were stored at 4°C until they were analyzed in the laboratory. Prior to subsampling, soils were mixed inside plastic bags by manually kneading and shaking the bags. Gravel and other debris were removed from the soils manually. Soil moisture contents were determined from two 10-g subsamples dried at 110°C for 48 h. All results reported here are based on soil dry weights. Soil community DNA was extracted from 5-g subsamples (fresh weight) by the method of Zhou et al. (1996). DNA was separated from soil humic substances by subjecting the entire crude extract to electrophoresis in 0.8% low-melting-point agarose (Gibco BRL, Gaithersburg, Md.). The DNA bands were excised from the gels, and the agarose was dissolved with agarase (Boehringer Mannheim Corp., Indianapolis, Ind.). The DNA mixture was concentrated and washed twice with distilled water in Centricon-100 ultracentrifugal filters (Amicon, Inc., Beverly, Mass.). DNA concentrations and purities were determined at 260, 280, and 230 nm with a Hewlett Packard 8452A spectrophotometer (Hewlett Packard Co., Sunnyvale, Calif.).

Polymerase chain reaction with 16S rDNA primers. Purified DNA was amplified using the 16S rDNA primers, described by McCaig et al. (1994). These have a higher specificity than universal eubacterial primers for AAOs in the β-subgroup of Proteobacteria. The sequence of the forward primer, β-AMOf, was 5'-TGGGGRATAACGCAYCGAAAG-3', which corresponded to positions 141-161 of the *Escherichia coli* rDNA molecule. The sequence of the reverse primer, β-AMOr, was 5'-AGACTCCGATCCGGACTACG-3', corresponding to *E. coli* positions 1301-1320 (McCaig et al., 1994). The PCR was carried out in 50-µl reaction volumes with a Perkin-Elmer GeneAmp PCR System 9600 (Perkin -Elmer, Foster City, Calif.), using a hot-start procedure to reduce nonspecific amplification (Chou et al., 1992). Each reaction mixture contained 10 ng template DNA, 4 pmol of each primer, and 1 Unit of Taq polymerase (Perkin Elmer, Foster City, Calif.) in a final concentration of 2.5 mM $MgCl_2$, 0.12 mM dNTPs in PCR reaction buffer. Positive controls contained 10 ng of *Nitrosomonas europaea* genomic DNA as template. Negative controls contained dilutions of processed agarose gel slices that were cut from areas in the purification gels containing no DNA. PCR reaction conditions were as follows: initial denaturation at $94^{o}C$ for 2 min; 35 cycles of $94^{o}C$ for 1 min/$55^{o}C$ for 1 min/$72^{o}C$ for 1 min; final extension at $72^{o}C$ for 7 min. Four to five duplicate reaction mixtures were amplified at a time, and the PCR products were pooled to reduce bias resulting from PCR drift (Moyer et al., 1994).

Cloning of PCR products. Pooled mixtures of PCR products were concentrated in a Microcon-100 unit (Amicon, Inc., Beverly, Mass.) and added to agarose gels for electrophoresis to isolate PCR product bands. Gel slices containing the PCR products (approximately 1100 bp) were excised and treated with QIAEX II reagents (Qiagen, Inc, Chatsworth, Calif.) to purify the DNA from the gel. Purified DNA was washed again and concentrated in a Microcon-100 unit. PCR products were ligated into the pGEM-T vector using T4 ligase (Promega, Inc., Madison, Wisc.). Epicurean coli XL1-Blue supercompetent cells (Stratagene, Inc., La Jolla, Calif.) were prepared and transformed with the ligation mixtures according to the manufacturer's directions. The entire transformation mixtures were plated out onto Luria-Broth (LB) agar plates containing 20 mg ampicillin, 80 mg methicillin, and 10 mg kanamycin per liter of agar. (Methicillin was added to reduce incidence of false-positive satellite colonies.) Immediately prior to inoculation, plates had been freshly prepared and spread with 50 µl IPTG solution (0.2 M isopropyl-1-thio-β-*D*-galactoside in water) and 50 µl Xgal solution (20 mg 5-bromo-4-chloro-3-indolyl-β-*D*-galactoside per ml of dimethylformamide) to permit chromogenic detection of white transformant colonies from blue nontranformant colonies. Plates were incubated at 37°C for 24 h, then held at 4°C for 48 h to allow nontransformant colonies to develop deeper blue color. Well-isolated, white colonies were picked to fresh LB plates containing 100 µg ampicillin per ml to produce a 16S rDNA clone library for each soil sample. Plasmid DNA preparations were obtained from these clones using the Wizard Minipreps kit (Promega Corp., Madison, Wisc.).

T-tracking and sequencing of PCR inserts. Previous observations during development of the database by Stephen et al. (1996a) had shown that the primers of McCaig et al. (1994) could generate nonpecific PCR amplification products from non-AAO DNA (e.g., DNA from *Comamonas testosteroni* and some other related β-Proteobacteria). T-tracking was used to distinguish AAO rDNA inserts from non-AAO inserts. (With the T-tracking method, PCR inserts are partially sequenced with a single terminating dideoxynucleotide (dATP) to generate single-lane band patterns in sequencing gels.) The T-track band patterns were generated from the 16S rDNA region corresponding to positions 300-500 of *E. coli* rDNA. T-tracks from AAO rDNA had characteristic motifs that were used to screen out clones containing non-AAO rDNA (Stephen et al., 1996a). In this study, T-tracks for 45 clones were obtained from plasmid DNA samples with the 536r 16S rDNA sequencing primer, ^{35}S-dATP, and a Sequenase kit (Amersham Corp., Arlington Heights, Ill.).

Plasmid DNA samples, which generated different T-tracks having the characteristic AAO motif, were sequenced manually across the 16S rDNA region corresponding to *E. coli* positions 200-530. Sequences obtained from these plasmids were aligned using the GDE program (Maidak et al., 1994) with other partial 16S rDNA AAO sequences in the database of Stephen et al. (1996). Phylogenetic analysis (Jukes and Cantor, 1969) was performed using PAUP (Swofford, 1993) and Phylip (Felsenstein, 1993) to generate a phylogenetic tree based on nearest-neighbor distances (Saitou and Nei, 1987). GenBank accession numbers for these sequences are U56606 to U56633. Three plasmid DNA samples that generated T-tracks with non-AAO motifs were also sequenced to confirm their positions in the phylogenetic trees.

Colony blots and probe hybridizations. Clones were transferred as 3- to 4-mm patches onto LB-ampicillin plates and incubated overnight at 37°C. Growth from these plates was transferred to replicate 10 cm x 10 cm plates (100 clones per plate) and incubated at 37°C for 18-20 h. Colony transfers were made from each plate by applying a Hybond N+ membrane (Amersham Corp., Arlington Heights, Ill.) to the surface of a plate and leaving it for one min. The membrane was then lifted from the plate and placed colony-side-up on a blotting paper soaked with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 min to lyse the cells. The membrane was neutralized (1.5 M NaCl, 1 M Tris, pH 7.4) and rinsed in 2X saline-sodium citrate (SSC) buffer before air-drying. The DNA from the clones was cross-linked to the membrane by UV irradiation in a Stratalinker (Stratagene, Inc., La Jolla, Calif.). Clones containing plasmids with PCR inserts with known sequences were included in the clone libraries during membrane preparation to serve as positive and negative hybridization controls.

The sequence database of Stephen et al. (1996)a was used to identify oligonucleotides that could be used as probes (Table 4.2) to differentiate various subgroups of AAO sequences in the database. Probes were end-labeled with g^{32}P-ATP (DuPont NEN Biotechnology Division, Wilmington, Del.) with T4 polynucleotide kinase (Stratagene, Inc., La Jolla, Calif.). The ^{32}P-labelled probes were added to hybridization solution to obtain activities of approximately 1 mCi per ml.

Membranes containing DNA from clone libraries were prehybridized in QuikHyb solution (Stratagene, Inc., La Jolla, Calif.) for 1 to 2 h in glass hybridization tubes in a Techne Hybridization oven (Techne, Inc., Princeton, N.J.). Membranes were hybridized with ^{32}P-labelled probes at 42°C for 6 to 18 h. Membranes were washed at 42°C in 2X SSC containing 0.1% sodium dodecyl sulfate (SDS) and placed in film cassettes for exposure to autoradiogram film (Kodak, Inc., Rochester, N.Y.).

Denaturing gradient gel electrophoresis (DGGE). Purified DNA extracts (20 ng per reaction) were used in PCR amplification reactions with the primers CTO178F-GC and CTO637r (*E. coli* numbering), which were developed specifically for DGGE analysis of AAO populations by Kowalchuk et al. (1996). This primer pair generates a 497-bp fragment containing 459 bp of rDNA sequence and a 38-bp GC clamp. PCR reactions were carried out using the Expand High Fidelity PCR System (Boehringer-Mannheim Corp., Mannheim, Germany) in 50-µl volumes overlaid with an equal volume of paraffin oil. PCR conditions in an OmniGene Thermal Cycler were as follows: Initial denaturation at 94°C for 1 min; 35 cycles of denaturation (92°C for 30 sec)/annealing (57°C for 1 min)/extension (68°C for 45 sec with an incremental increase of 1 sec per

TABLE 4.2. Oligonucleotide probe sequences identified from the database of 110 partial rDNA sequences from β-subgroup autotrophic ammonia-oxidizing bacteria (Stephen et al., 1996a).

Probe label	Target group	16S rDNA sequence and E. coli positions (5' to 3')	Anti-sense probe sequence	Non-target strains which can cross-hybridize
Ammo_Cl_2/3/4/6	All β-subgroup AAOs (*Nitrosospira* and *Nitrosomonas* spp. represented by cultured isolates)	GATGTCTGATTAGCTAGT (233-253)	ACTAGCTAATCAGACATC	*Pseudomonas solanacearum; P. syzigii; P. picketti; Kingella orale; Alcaligenes eutropha*
All_Spira	All *Nitrosospira*	CTTTCAGCCGGAACGAAA (435-452)	TTTCGTTCCGGC GAAG	None identified
Spira_Cl_2	Uncultured *Nitrosospira* (molecular isolates that are frequently retrieved from some terrestrial samples)	CACGGTTAATAACTGTGA (444-461)	TCACAGTTATTAACCGTG	No sequences in RDP should hybridize
Spira_Cl_3	Cultured *Nitrosospira* (sequences similar to but distinct from *Nitrosospira* sequencesin RDP	AGGTATTAGCCGTGACCG (450-468)	AGGTATTAGCCGTGACCG	*Nitrosovibrio tenuis* is only RDP strain that will cross-hybridize
Spira_Cl_4	Novel *Nitrosospira* previously uncultured strains now represented by pure cultures (see text).	GAACGAAAAGGTTACGGT (456-473)	ACCGTAACCTTTTCGTTC	No sequences in RDP should hybridize
Monas_Cl_6b	Cultured *Nitrosomonas* (all sequences in RDP)	TTAGTCGGAAAGAAAGAG (438-455)	CTCTTTCTTTCCGACTAA	None identified
Monas_Cl_6a	Uncultured *Nitrosomonas* (molecular isolates that are frequently retrieved from some terrestrial samples)	CTTTCAGTCGAGAAGAAA (435-452)	TTTCTTCTCGACTGAAAG	*Nitrosomonas marina* is only RDP strain that will cross-hybridize
β_Deep	Clones with deep-branching sequence inserts, no known cultural representatives	GATTACTGCCATGATG	CTAATGACGGTACTAC[1]	Unknown

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[1] Not an antisense probe.

cycle); and final extension at 68^{o}C for 5 min (Stephen et al., 1996a). DGGE was carried out according to the methods of Muyzer et al., (1993) in 20x20 cm, 1.5-mm-thick, 0.8% polyacrylamide gradient gels (0.5X TAE; 37:1 acrylamide/bisacrylamide) with a D-Gene system (Bio-Rad Laboratories, Hercules, Calif.). (1X TAE contains 0.04 M Tris base, 0.02 M sodium acetate, and 1 mM EDTA, pH 7.4). Gels were poured with a CBS gradientmaker (Del Mar, USA) and a Bio-Rad Econo-pump (Bio-Rad Laboratories,) at a speed of 5 ml per min (40-ml gradient volume) to obtain a gradient of typically 38 to 50% denaturant. DGGE gels were run in 0.5X TAE at 60^{o}C for either 6 1/2 h at 200 V or 16 h at 85 V. Gels were stained with ethidium bromide to visualize DNA bands under UV transillumination. Southern blots were prepared from the gels, and these were hybridized with selected ^{32}P-labelled probes (Table 4.2).

RESULTS

DNA yields from soils. DNA yields from cultivated soils were significantly lower ($p < 0.05$) than yields from never-tilled soils in 1993 and 1994 (Table 4.3). No significant differences were observed between DNA yields from cultivated, never-tilled, and successional soils in 1995. DNA quality, as measured by optical density (O. D.) ratios of DNA preparations at 260 nm/280 nm, was slightly higher for 1994 samples (1.8 to 1.9) than for 1995 samples (1.5 to 1.7). The O. D.$_{260/230}$ ratios for the same samples were also higher in 1994 (2.3 to 4.4) than in 1995 (1.3 to 1.5). Since the latter ratio is a measure of humic substance contamination in nucleic acid samples, the 1995 DNA samples were less pure than the 1994 samples. Both sets of samples, however, appeared to yield equivalent PCR product band intensities when amplified at 10 ng DNA per 50-µl reaction.

TABLE 4.3. DNA yields (µg DNA per g soil dry weight) from cultivated, never-tilled, and successional soils. Numbers in parentheses indicate ranges of experimental values for each treatment.

Treatment	1993[1] (corn)	1994[2] (soybean)	1995[2] (wheat)
Cultivated (T1)	2.6 (±1.4)	2.9 (±0.1))	12.82 (±3.8)
Never -tilled (T8)	15.4 (±3.3)	13.0 (±2.8))	15.7 (±3.7)
Successional (T7)	ND	ND	13.6 (±1.4)
Fertilized successional (T7F)	ND	ND	19.6 (±3.7)

[1] Mean of three analytical replicate subsamples from 1 composited sample for each treatment.

[2] Mean of 2 replicate samples from each treatment.

Sequence diversity in clone libraries. Twenty-six clones obtained from community DNA of the never-tilled soils sampled in 1994 were screened by T-tracking. Half of these (13 clones) exhibited characteristic AAO motifs. Nearly all T-tracked clones (18 out of 19) from community DNA of cultivated soils exhibited AAO motifs (Figure 4.1). The AAO motif was characterized by the sequence TCTTT at *E. coli* positions 432-436, shown by the bracketed region in Figure 4.1. T-tracks that did not have this motif (tracks 2, 18, 21, 22, 24, 25, 26, 28, 29, 31, 34, and 35) were considered to represent nonspecific PCR amplification products (cloned DNA that did not arise from AAO rDNA). From the percentage of T-tracks with AAO motifs in this sample set of clones, we concluded that clone libraries from community DNA of never-tilled soils contained proportionally more clones with nonpecific amplification products than libraries from cultivated soil community DNA. These T-tracks also provided information on comparative diversity within this sample of cloned rDNA sequences. One out of 13 clones containing the AAO motif in the never-tilled sample set had a redundant T-track pattern (similar to that of another clone in the set). Six out of 18 (one in three) clones with AAO motifs had redundant patterns. Although the sample set of clones from never-tilled soil community DNA contained proportionally fewer AO rDNA inserts, fourfold more clones had unique T-track patterns than did clones in the cultivated soil sample set.

Phylogenetic analysis was performed on 24 cloned sequences that had been aligned to other AAO sequences in the database of Stephen et al., 1996a. A phylogenetic tree was constructed (Figure 4.2) containing 13 sequences from cultivated soil community libraries and 11 from never-tilled soil community libraries. All sequences from cultivated soil community libraries fell within one clade (Cluster 3 *Nitrosospira*) in the phylogenetic tree (Figure 4.2). The most dissimilar AAO sequences among these clones differed at 3% of the base

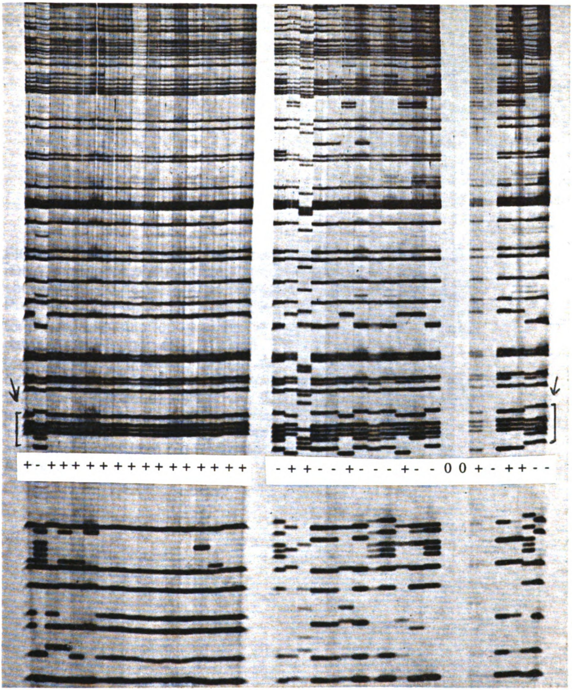

FIGURE 4.1. T-track banding patterns observed with single-dideoxynucleotide termination sequencing reactions. DNA samples sequenced were partial 16S rDNA gene inserts obtained from PCR amplification of soil community DNA with the b-AMO primers of McCaig et al., (1994). The left-hand set of 17 banding patterns (T-tracks 1-17) were sequences amplified from cultivated soil community DNA. All T-track patterns, except one, in this set, contained the characteristic AAO motif (see indicator arrows) composed of a singlet band, a singlet gap, and a triplet of bands. The right-hand set of 18 band patterns (T-tracks 18-35, not including the two inner lanes with no DNA) were sequences amplified from never-tilled soil community DNA. Seven of the 18 T-track patterns contained the AAO motif, while 11 patterns appeared to be derived from non-AAO DNA. (Lanes with the AAO motif are marked "+"; lanes without this motif are marked "-".)

FIGURE 4.2. Phylogenetic tree showing distances between 16S rDNA sequences obtained from cultivated soil samples (KZOO-H sequences)and never-tilled soil samples (KZOO-D sequences). Phylogenetic analysis included other partial 16S rDNA sequences for β-subgroup autotrophic ammonia oxidizers from the sequence database of Stephen et al. (1996a). Clusters indicated are phylogenetically distinct groups of sequences identified in this database. Sources of other sequences in the tree were: neutral Craibstone (Scotland) soils (PH7); acid Craibstone soils (PH4.5 and 4.5); marine waters and sediments (Env and JUNE); low-nitrogen-loaded activated sludge (AL212 from Y. Suwa); and enrichments from pig waste slurries (JWU sequences from A. Princic).

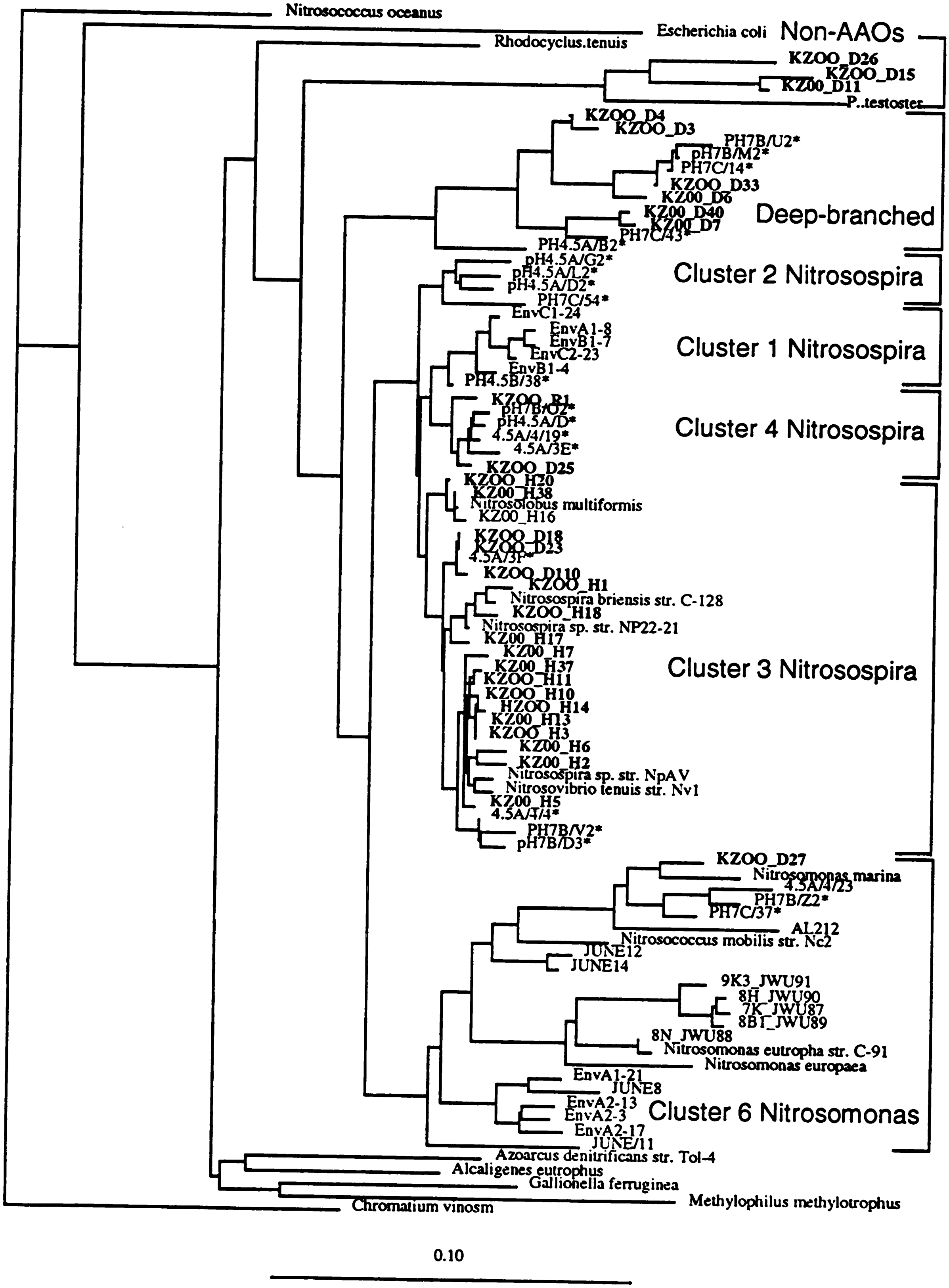
Nitrosococcus oceanus
Escherichia coli
Non-AAOs
Rhodocyclus.tenuis
KZOO_D26
KZOO_D15
KZ00_D11
P..testoster
KZOO_D4
KZOO_D3
PH7B/U2*
pH7B/M2*
PH7C/14*
KZOO_D33
KZ00_D6
KZ00_D40
KZ00_D7
PH7C/43*
Deep-branched
PH4.5A/B2*
pH4.5A/G2*
pH4.5A/L2*
pH4.5A/D2*
PH7C/54*
Cluster 2 Nitrosospira
EnvC1-24
EnvA1-8
EnvB1-7
EnvC2-23
EnvB1-4
PH4.5B/38*
Cluster 1 Nitrosospira
KZOO_R1
pH7B/O2*
pH4.5A/D*
4.5A/4/19*
4.5A/3E*
KZOO_D25
Cluster 4 Nitrosospira
KZOO_H20
KZ00_H38
Nitrosolobus multiformis
KZ00_H16
KZOO_D18
KZOO_D23
4.5A/3F*
KZOO_D110
KZOO_H1
Nitrosospira briensis str. C-128
KZOO_H18
Nitrosospira sp. str. NP22-21
KZ00_H17
KZ00_H7
KZ00_H37
KZOO_H11
KZOO_H10
HZOO_H14
KZ00_H13
KZOO_H3
KZ00_H6
KZ00_H2
Nitrosospira sp. str. NpAV
Nitrosovibrio tenuis str. Nv1
KZ00_H5
4.5A/4/4*
PH7B/V2*
pH7B/D3*
Cluster 3 Nitrosospira
KZOO_D27
Nitrosomonas marina
4.5A/4/23
PH7B/Z2*
PH7C/37*
AL212
Nitrosococcus mobilis str. Nc2
JUNE12
JUNE14
9K3_JWU91
8H_JWU90
7K_JWU87
8B1_JWU89
8N_JWU88
Nitrosomonas eutropha str. C-91
Nitrosomonas europaea
EnvA1-21
JUNE8
EnvA2-13
EnvA2-3
Cluster 6 Nitrosomonas
EnvA2-17
JUNE/11
Azoarcus denitrificans str. Tol-4
Alcaligenes eutrophus
Galliohella ferruginea
Chromatium vinosm
Methylophilus methylotrophus
0.10

FIGURE 4.2

positions (out of a total 318 bases). Of the 11 sequences from libraries of never-tilled soil community DNA, three fell within *Nitrosospira* Cluster 3, two within *Nitrosospira* Cluster 5, one within the *Nitrosomonas* clade (Cluster 6), and six within a deep-branching clade having intermediate phylogenetic distance between the *Nitrosomonas* and *Nitrosospira* groups. The three Cluster 3 *Nitrosospira* sequences were identical, so these were counted as one distinct sequence to give a total of 10 different sequences from never-tilled soil community libraries (Table 4.4). The most dissimilar AAO sequences derived from never-tilled soil community DNA differed at 14% of the 318 base positions. Based on this comparison, AAO sequences from never-tilled soil community libraries exhibited fourfold greater diversity than AAO sequences from cultivated soil community libraries.

Probe hybridizations of clone-blots. Three oligonucleotide probe sequences (Table 4.2), developed from the database of Stephen et al. (1996a), were used in hybridization tests with clone-blots to screen larger numbers of clones for non-AAO PCR products. Based on information in this database, the Ammo_Cl_2/3/4/6 probe could hybridize to all terrestrial AAO sequences, except for sequences falling within the deep-branched clade. The β-Deep and All_Spira probes could hybridize to sequences in the deep-branched clade and all-*Nitrosospira* group, respectively. The percentages of clones with DNA hybridizing to the Ammo_Cl_2/3/4/6 probe were higher in libraries from cultivated soil community DNA than in never-tilled soil community libraries (Table 4.5). Percentages of clones cross-hybridizing with the β-Deep probe were higher in never-tilled soil community libraries than in cultivated soil community libraries.

Probe hybridizations with clone libraries derived from soils sampled in 1995 were performed to determine whether similar hybridization percentages

TABLE 4.4. Numbers of different partial 16S rDNA sequences falling within subgroups in β-Proteobacteria (1994 samples).

Treatment	Total number of sequences	Typical *Nitrosospira* clade	Novel *Nitrosospira* clade	*Nitrosomonas* clade	Deep-branched clade
Cultivated	13	13	0	0	0
Never-tilled	10	1	2	1	6

could be obtained in a subsequent year. In addition, clone libraries were obtained from soils sampled from successional plots that had a disturbance history intermediate between those of never-tilled and cultivated plots. The degree of nonspecific amplification from community DNA of 1995 soils (84 to 99%) was much higher than that from community DNA of 1994 soils (60 to 80% in Table 4.5). The number of clones hybridizing to the Ammo_Cl_2/3/4/6 probe were higher in libraries from cultivated and fertilized successional plots than for never-tilled and successional plots (Table 4.6). Numbers of clones hybridizing to the β-Deep probe were highest in libraries from never-cultivated soils.

Denaturing gradient gel electrophoresis (DGGE). PCR products were obtained from all soil samples using DGGE primers with greater specificity for AAOs (Kowalchuk et al., 1996). The DGGE patterns for replicate samples within each treatment were remarkably similar, except for soils from fertilized successional plots (Figure 4.3). Very similar DGGE patterns were also observed in samples from the same plots taken in 1994 and 1995. Bands 1 and 2 in lanes containing DNA from cultivated soils were more intense than corresponding bands at these positions in lanes containing DNA from never-tilled soils. These same bands appeared stronger in lanes containing DNA from successional soil samples. Their intensities, however, were not as great as they were in lanes with DNA from cultivated soil samples. The third band observed in all lanes containing DNA from cultivated soil samples was not seen in lanes containing DNA from never-tilled soil samples. Conversely, the third band in lanes containing DNA from never-tilled soil samples was not observed in any of the lanes containing DNA from cultivated soil samples.

DNA in DGGE gels was transferred to a hybridization membrane and probed with the All_Spira probe, which hybridizes with all *Nitrosospira* sequences in the database of Stephen et al. (1996a). Equivalent hybridization

TABLE 4.5. Percentages of probe-positive clones in libraries obtained from soil community DNA (1994 samples).

Treatment	Replicate plot	Total clones tested	Percentage negative for all probes	Percentage positive for typical and novel AAOs	Percentage positive for deep-branched clade probe
Cultivated	1	81	36%	64%	0%
	2	200	44%	56%	0%
Never-tilled	1	346	78%	10%	12%
	2	326	79%	4%	17%

TABLE 4.6. Numbers of probe-positive clones in libraries obtained from soil community DNA (1995 samples).

Treatment	Replicate plot	Total clones tested	Number hybridizing with all-AAO probe	Number hybridizing with deep-branched probe
Cultivated	1	500	73	6
	2	494	4	0
Never-tilled	1	482	3	15
	2	480	3	46
Successional	1	369	2	2
	2	457	7	2
Fertilized successional	1	396	32	9
	2	486	10	3

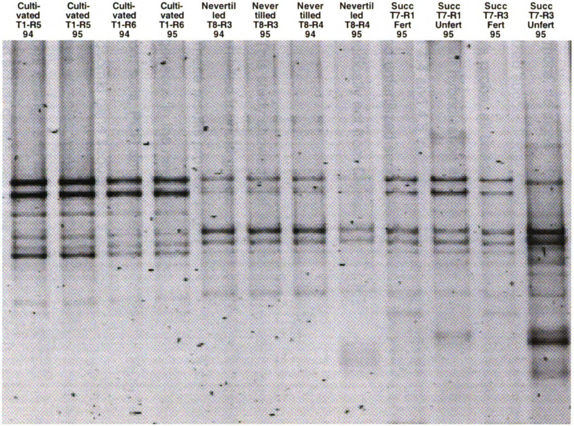

FIGURE 4.3. DGGE band patterns of PCR-amplification products from community DNA observed in ethidium-bromide-stained denaturing gradient gels under UV illumination. DNA bands represent 16S rDNA fragments (497 bp) from mixtures obtained by PCR amplification of soil community DNA with CTO_PCR primers of Kowalchuk et al. (1996). Samples were loaded into the gel as follows (replicate plots and sampling years as indicated): Lane 1, cultivated rep 5, 1994; Lane 2, cultivated rep 5, 1995; Lane 3, cultivated rep 6, 1994; Lane 4, cultivated rep 6, 1995; Lane 5, never-tilled rep 3, 1994; Lane 6, never-tilled rep 3, 1995; Lane 7, never-tilled rep 4, 1994; Lane 8, never-tilled rep 4, 1995; Lane 9, fertilized microplot in successional rep 1; Lane 10, unfertilized successional rep 1; Lane 11, fertilized microplot in successional rep 3; Lane 12, unfertilized successional rep 3. Successional treatments were all sampled in 1995.

signals were observed from banding patterns from all samples. When the blots were probed with the Spira_Cl_3 probe (for Cluster 3 *Nitrosospira*), strong hybridization signals were obtained in lanes containing DNA from cultivated and fertilized successional soil (Figure 4.4). No signal was observed between the Spira_Cl_3 probe and DNA from both replicate plots of never-tilled soils. Furthermore, no signal was observed in the lane containing DNA from one unfertilized successional replicate plot. When blots were probed with the All_Spira probe, all bands from all samples had exhibited approximately equivalent hybridization intensities (Figure 4.5). Hybridization patterns in Figures 4.4 and 4.5 indicated that bands from community DNA of never-tilled soils and unfertilized successional soils were derived from *Nitrosospira* DNA but specifically not from Cluster 3 *Nitrosospira*.

DISCUSSION

This study showed that AAO ribosomal gene sequences from never-tilled soils exhibited fourfold greater dissimilarity than AAO sequences from cultivated soils. These results provide strong evidence that AAO populations were more heterogeneous in the never-tilled soils than in the cultivated soils. Extraction of DNA from soils, PCR amplification, or cloning can cause biased sampling of *in situ* ribosomal gene sequences (Giovannoni et al., 1996). However, the fact that these samples were analyzed with the same procedures under identical conditions strengthens the argument that observed sequence differences reflected actual differences in the *in situ* AAO populations. A reasonable explanation for these differences is that AAO diversity is reduced in cultivated soils by a selection process resulting from regular additions of N fertilizers. Lower AAO diversity in cultivated soils could also be caused by a reduction in niche heterogeneity due to regular plowing disturbance.

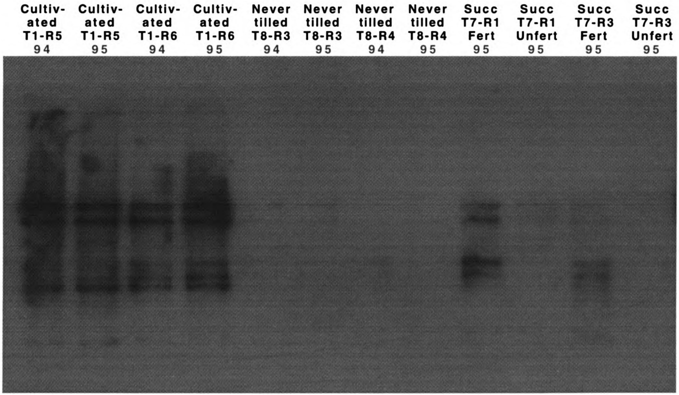

FIGURE 4.4. Autoradiogram of DGGE blot probed with 32P-labelled Spira_Cl_3 probe. Blot shows DNA from gel in Figure 4.2. Lanes show probe hybridizations of band fragments amplified from community DNA extracted from the following soils (with replicate plots and sampling years): Lane 1, cultivated rep 5, 1994; Lane 2, cultlivated rep 5, 1995; Lane 3, cultivated rep 6, 1994; Lane 4, cultivated rep 6, 1995; Lane 5, never-tilled rep 3, 1994; Lane 6, never-tilled rep 3, 1995; Lane 7, never-tilled rep 4, 1994; Lane 8, never-tilled rep 4, 1995; Lane 9, fertilized microplot in successional rep 1; Lane 10, unfertilized successional rep 1; Lane 11, fertilized microplot in successional rep 3; Lane 12, unfertilized successional rep 3. Successional treatments were all sampled in 1995.

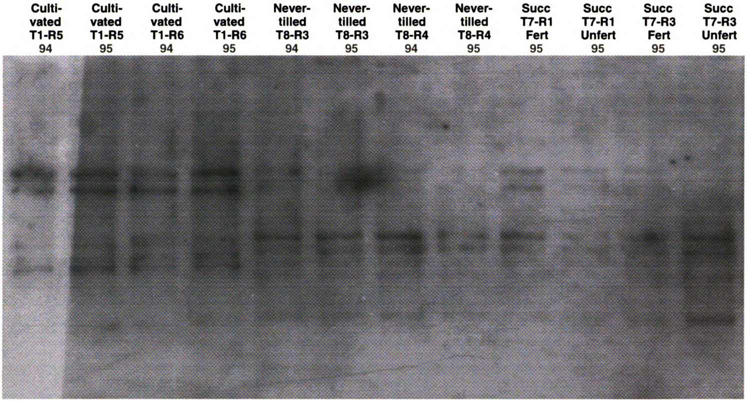

FIGURE 4.5. Autoradiogram of DGGE blot probed with ^{32}P-labelled All_Spira probe. Blot shows DNA from gel in Figure 4.3. Lanes show probe hybridizations with fragments amplified from community DNA extracted from the following soils: Lane 1, cultivated rep 5, 1994; Lane 2, cultivated rep 5, 1995; Lane 3, cultivated rep 6, 1994; Lane 4, cultivated rep 6, 1995; Lane 5, never-tilled rep 3, 1994; Lane 6, never-tilled rep 3, 1995; Lane 7, never-tilled rep 4, 1994; Lane 8, never-tilled rep 4, 1995; Lane 9, fertilized microplot in successional rep 1; Lane 10, unfertilized successional rep 1; Lane 11, fertilized microplot in successional rep 3; Lane 12, unfertilized successional rep 3. Successional treatments were all sampled in 1995.

Another important finding was that *Nitrosospira* Cluster 3 sequences predominated in DGGE blots from cultivated and fertilized successional soils, whereas they were not detected in blots from never-tilled and successional soils (Figures 4.3 and 4.4). *Nitrosospira* Cluster 3 is one among seven phylogenetically distinct β-AAO clusters identified by Stephen et al. (1996a and 1996b) from a sequence database derived from physically and geographically diverse samples. The *Nitrosospira* and *Nitrosomonas* groups in this database comprise four and three clusters, respectively. The *Nitrosospira* group consists of Cluster 1 (sequences from marine samples), Cluster 2 (sequences frequently retrieved from soils but without any cultured representatives), Cluster 3 (sequences of *Nitrosospira* spp. commonly cultured from soils), and Cluster 4 (novel sequences from previously uncultured *Nitrosospira* spp. with pure cultures recently isolated; Stephen et al., 1996a). *Nitrosospira* Cluster 3 includes cultures and sequences that were commonly obtained from agricultural soils in Scotland and the Netherlands (Stephen et al., 1996a). The presence of Cluster 3 sequences in fertilized soils and the lack of these sequences in never-tilled and successional soils suggest that this cluster is associated with soils that have been recently amended with N fertilizers.

Nitrosospira sequences other than Cluster 3 sequences predominated in DGGE mixtures from never-tilled and successional soils, because DGGE blots from these samples hybridized only with the All_Spira probe (Figure 4.4). Since it was not likely that soils contained Cluster 1 *Nitrosospira* (marine strains), these DGGE mixtures probably contained Cluster 2 or 4 *Nitrosospira*. These clusters appear to include sequences from less readily culturable *Nitrosospira* spp. No representatives of Cluster 2 sequences have been isolated in culture, whereas a few Cluster 4 isolates have recently been obtained (J. I. Prosser, personal communication). Bruns et al. (1996) isolated a representative of Cluster 4 *Nitrosospira* from a never-tilled sample after a six-month enrichment in mineral medium containing 10 ppm NH_4-N. This NH_4-N concentration, which is tenfold lower than concentrations typically used for most-probable-number (MPN) enumerations of AAOs (Schmidt and Belser, 1982), was an important factor in the successful isolation of *Nitrosospira* strain R1, a novel representative of Cluster 4 *Nitrosospira* (Bruns et al., manuscript in preparation). Other workers have used lower NH_4-N concentrations in MPN media to increase AAO recovery from forest soils (Donaldson and Henderson, 1989). This may indicate that the most commonly used methods to culture AAOs have been inappropriate for recovering certain AAO groups. Predominance of Cluster 4 sequences from never-tilled and successional soils may shed light on previous studies, in which no or low numbers of nitrifiers were recovered from grassland, forest, and climax ecosystem soils that exhibited nitrification after the vegetation was removed (Rice and Pancholy, etc.). These observations led some researchers to suggest that certain plants inhibit nitrifier populations (Rice and Pancholy, 1978). An alternative explanation, based on our sequence observations, is that the predominant nitrifiers in these grassland, forest, and climax soils were not culturable in recovery media used in these studies.

Another difference observed in our study was that sequences from a deeply branched cluster of unidentified β-proteobacteria appeared to be more common in clone libraries from never-tilled soils than in libraries from cultivated soils. This difference in frequencies of deep-branched sequences was observed in both replicates of each treatment in 1994 and 1995 (Tables 4.5 and 4.6). The deep-branched sequences appeared to be derived from bacteria which are related to AAOs. These bacteria cannot be designated as AAOs until cultured representatives have been shown to oxidize ammonia autotrophically.

Deep-branched sequences have also been retrieved from Scottish soils, sediments, and freshwater samples using the β-AMO primers of McCaig et al. (1994). The CTO_PCR primers used for DGGE (Kowalchuk et al., 1996) were more specific for AAOs than the β-AMO primers (McCaig et al., 1994), and they did not amplify DNA from plasmids containing inserted sequences from the deep-branched cluster (unpublished observations). Thus, the DGGE blots in our study were not expected to contain any deep-branched sequences. The information on this cluster was available only from the clone library analysis and not from DGGE results. The higher frequency of deep-branched sequences in clone libraries appears to be associated with less disturbed soils, but the phylogenetic and functional significance of this association is unknown. Further assessments for the presence of these sequences in other environmental samples and successful isolation of deep-branched representatives are needed to learn more about the function and significance of these bacteria in soils. If their association with undisturbed soils is found to be robust, these deep-branched β-Proteobacterial sequences might potentially serve as disturbance indicators in soil restoration programs.

A final important finding was the close similarity between DGGE band patterns for PCR mixtures from replicate plots of the same treatments from both years (Figure 4.2). All replicate plots had an area of 1 ha and were located among a total of 46 plots in a randomized block design over a 48-ha site (Robertson et al., 1996). Variability of soil properties at this site (including net nitrification) was observed to be high and spatially dependent over distances of 1 to 40 m (Robertson et al., 1993). Approximate distances between sampled replicate plots in our study were 900 m, 500 m, and 100 m, for cultivated, successional, and never-tilled treatments, respectively. Nearly identical DGGE patterns were obtained with each sample that had been composited from 25 cores taken from different sites in each plot. At least two explanations can be given for DGGE pattern reproducibility. First, reproducibility could result from AAO populations in these soils being truly robust and strongly dependent on disturbance history. Alternatively, reproducibility could be due to nucleic acid selectivity of PCR and DNA extraction procedures. If such selectivity did occur during our procedures, it appeared to be treatment-dependent, because the DGGE blots produced distinctly different probe hybridization results. The consistent differences between treatments can be used to interpret how environmental factors have influenced AAOs in these soils, even if possible technical bias precludes use of the results to describe actual *in situ* populations.

In contrast to the DGGE patterns, cloning and probe hybridization results were not as reproducible from 1994 to 1995. This was due to the high incidence of nonspecific (non-AAO) PCR amplification products in the 1995 clone libraries, which was probably due to two technical reasons. First, the reverse primers used in the initial PCR reactions on soil DNA extacts were slightly different in the two years. In 1995, we used the original β-AMOr primer of McCaig et al. (1994). The previous year we used a modified reverse primer from a 16S rDNA sequence starting three bases upstream from the original primer. This modification had been made to reduce nonspecific PCR amplification, specifically from DNA of *Comamonas testosteroni.* The use of the original reverse primer in 1995 may explain the greater incidence of nonspecific PCR amplification products observed. A second possible reason for higher nonspecific amplification was that the 1995 soil DNA extracts were less pure than 1994 extracts, because they had lower $O.D._{260/280}$ and $O.D._{260/230}$ ratios. DNA quality in 1995 may thus have affected the specificity of the *Taq* DNA polymerase in the PCR reactions. Nevertheless, the 1994 and 1995 clone libraries were similar in that both exhibited higher frequencies of deep-branched sequences in libraries from never-tilled soils.

These findings from 16S rDNA analysis are substantiated by results we have obtained using cultural methods to estimate AAO population sizes in these soils (Bruns et al., 1996). Most-probable-number (MPN) counts of AAOs were consistently lower by a factor of ten in never-tilled soils (10^4 AAOs per g) than in cultivated soils (10^5 AAOs per g). To characterize AAO population structure, we obtained simultaneous MPN counts for AAOs at various NH_4-N concentrations, an approach first used by Suwa et al. (1994). AAOs in never-tilled soils had 60-fold higher recovery in MPN medium containing 10 ppm NH_4-N than in medium containing 2000 ppm NH_4-N. Recovery of AAOs in cultivated soils was only twofold higher at 10 ppm NH_4-N than at 2000 ppm NH_4-N (Bruns et al., 1996). Greater recovery of AAOs at the lower NH_4-N concentration suggests that a higher proportion of AAOs in never-tilled soils do not grow out in NH_4-N concentrations above 10 ppm (Suwa et al., 1994). This is consistent with the apparent predominance in never-tilled soils of 16S rDNA sequences representing less easily cultured AAOs (Figure 4.3).

Consistently observed differences in the properties of cultivated and never-tilled soils (Table 4.1) suggest that these soils provided distinctly different habitats for the development of AAO populations. An important environmental factor affecting AAO numbers and activity is their supply of inorganic N in the form of NH_4-N (Robertson,1982). Inputs of inorganic N were higher for cultivated soils than for never-tilled and successional soils. Cultivated soils at the LTER site received 84 kg fertilizer N per ha when the plots were cropped in corn and 56 kg N per ha when the plots were in wheat (Robertson et al., 1996). The method of fertilizer application used at LTER (broadcasting of ammonium nitrate) would result in higher concentrations of NH_4-N being localized at the surface of the soil, due to rapid binding of NH_4^+ to soil colloids. Subsequent incorporation of crop residues during moldboard plowing, as well as soil mixing by disking and cultivating, would produce a more spatially homogeneous distribution of inorganic N. The lower diversity of AAO 16S rDNA sequences from cultivated soils suggests that fertilization and disturbance have increased selection for more homogeneous populations of AAOs. AAO populations in never-tilled and successional soils may thus be more diversified as a result of spatial heterogeneity in substrate availability.

Another factor that affects the supply of inorganic N to AAO populations is NH_4-N uptake by heterotrophic microorganisms. Never-tilled soils have significantly higher biomass C and bacteria per g of soil than cultivated soils (Table 4.1). Thus, AAOs comprise a much smaller proportion of the soil bacterial community in never-tilled soils, where they are outnumbered by bacterial heterotrophs 364,000:1 (Table 4.1). AAOs are subject to less competition for NH_4-N by bacterial heterotrophs in cultivated soils, where they are outnumbered 21,000:1. Lower fungal biomass in cultivated soils (D. Harris, personal communication) would also result in less fungal competition for NH_4-N. Higher inorganic N inputs and less heterotrophic competition could thus increase the supply of NH_4-N to AAOs in cultivated soils. These factors could select for AAOs that favor comparatively higher NH_4-N concentrations.

The cultural and molecular evidence that AAO populations were different in these soils is supported by soil data indicating differences in nitrification activity (Tables 4.1 and 4.7). Never-tilled soils in 1994 were characterized by a higher mean concentration of NH_4-N (10.5 μg per g soil) than cultivated soils, which had a mean concentration of 2.6 μg NH_4-N per g soil (Table 4.7). Never-tilled soils had a mean NO_3-N concentration (0.5 μg per g soil) that was significantly lower than the mean concentration for cultivated soil (3.9 μg NO_3-N

TABLE 4.7. Indicators of the effects autotrophic ammonia oxidizers may have on nitrogen cycle parameters, measured in two replicate plots of cultivated, successional, and never-tilled treatments (1994 LTER data).[1]

Measurement	Cultivated soil	Successional soil	Never-tilled soil
Mean NO_3-N concentration (μg per g soil)	3.9 (±2.49)	0.4 (±0.21)	0.5 (±0.24)
Mean NH_4-N concentration (μg per g soil)	2.6 (±0.98)	3.8 (±0.60)	10.5 (±2.49)
Net nitrification in field incubations (μg/g/day)[2]	0.17 (±0.10)	0.03 (±0.5)	0.19 (±0.15)
Net nitrification in lab incubations (μg/g/day)[2]	0.35	0.18	0.29
Net percentage of NH_4-N converted to NO_3-N during field incubations[3]	68%	30%	21%

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[1] Measurements taken monthly from May through November, 1994. Means are from measurements for two replicate plots, with n=14. Standard deviations for the means are given in parentheses (KBS LTER Site Data Catalog, KBS LTER home page on World Wide Web, http:/kbs.msu.edu/lter/.

[2] Field and lab samples were incubated for 21 days. For field incubations, n=10. For lab incubations, n=2 (D. Harris, personal communication).

[3] Percentage based on the net increase in NO_3-N after 21 days divided by the sum of the initial NH_4-N pool plus the amount of inorganic N mineralized during incubation. Percentages were obtained from inorganic N measurements before and after incubation (KBS LTER Site Data Catalog, KBS LTER home page on World Wide Web).

per g soil). Available data on net nitrification rates during field and laboratory incubations of cultivated and never-tilled soils, however, did not show significant differences (Table 4.7). Differences were observed, however, in the percentages of total NH_4-N pools (initial NH_4-N plus NH_4-N mineralized during incubation) that were converted to NO_3-N during the 21-day incubation period (Table 4.7). Nitrifier populations in cultivated soils converted a larger percentage (68%) of the NH_4-N pool to NO_3-N, whereas populations in never-tilled and successional soils converted 21% and 30% of the NH_4-N to NO_3-N, respectively. Nitrifier populations in the cultivated soils thus appeared to have greater capacity to oxidize available NH_4-N. This observation was consistent with a predominance in cultivated soils of *Nitrosospira* spp. that were more readily cultured in laboratory media. It was also consistent with the fact that AAOs in cultivated soils gave higher MPN counts than AAOs in never-tilled soils at 100 and 2000 ppm NH_4-N.

MPN counts of AAO populations in never-tilled soils remained tenfold lower than counts in cultivated soils, despite the fact that NH_4-N levels in never-tilled soils remained at around 10 ppm throughout two growing seasons (Bruns et al., manuscript in preparation). One explanation for the apparent lack of NH_4-N consumption by these AAO populations is that the NH_4-N was not physically available in these soils. Never-tilled soils contained at least 1.5 times more organic matter than cultivated soils (Table 4.1). Biochemical interactions between NH_4^+ and organic matter (Nommik and Vahtras, 1982) in the never-tilled soils could have made the NH_4-N unavailable for nitrification by AAOs (or immobilization by heterotrophs.) Lack of disturbance in never-tilled soils could also contribute to lower overall nitrification because of spatial discontinuities between sites of bound NH_4-N and locations of nitrifiers in soils.

Another explanation for low AAO population size in the presence of NH_4-N could be that nitrifier populations in never-tilled soils had lower metabolic capacities to oxidize available NH_4-N than nitrifiers in cultivated soils. Belser and Schmidt (1980) and Suwa et al. (1994) showed that different genera and strains of AAOs exhibited varying K_m and V_{max} values for ammonia oxidation. Thus, AAO populations in never-tilled soils could be dominated by strains with relatively low K_m and V_{max} values for ammonia oxidation. AAOs with higher Km values, on the other hand, would be more likely to be selected in cultivated soils that receive fertilizer N.

Finally, another cause for low AAO population size in the presence of NH_4-N could be inhibition of AAOs by allelopathic chemicals from plants (e.g., tannins). This explanation was put forth by Rice and Pancholy (1972) in studies on nitrification in soils from grasslands and climax ecosystems. However, other studies have not detected phytochemical inhibition of nitrification (Barford and Lajtha, 1992), so that this explanation remains controversial.

In conclusion, this study presents strong evidence that AAO populations in fertilized, disturbed soils were less diverse than AAOs in unfertilized, undisturbed soils. Our results also indicated that N-fertilization resulted in distinct differences in the kinds of *Nitrosospira* sequences retrieved from soil communities. In addition, we present preliminary evidence that the molecular analysis results in this study were temporally and spatially reproducible over an area of 1 km^2. Further testing of greater numbers of replicate soils sampled more frequently over longer time periods can confirm the reproducibility and reliability of these techniques in analyzing *in situ* AAO populations. Microbial ecologists have long suspected that different habitats harbor different microbial communities. This hypothesis about microbial diversity, however, could not be tested due to the inadequacies of cultural techniques. Our study shows that molecular techniques now available can be applied to the study of *in situ* soil microbial diversity. This should facilitate integration of soil microbial diversity into the development and testing of general microbial ecology concepts.

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APPENDIX A

DNA RECOVERY FROM SOILS OF DIVERSE COMPOSITION

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DNA Recovery from Soils of Diverse Composition

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A simple, rapid method for bacterial lysis and direct extraction of DNA from soils with minimal shearing was developed to address the risk of chimera formation from small template DNA during subsequent PCR. The method was based on lysis with a high-salt extraction buffer (1.5 M NaCl) and extended heating (2 to 3 h) of the soil suspension in the presence of sodium dodecyl sulfate (SDS), hexadecyltrimethylammonium bromide, and proteinase K. The extraction method required 6 h and was tested on eight soils differing in organic carbon, clay content, and pH, including ones from which DNA extraction is difficult. The DNA fragment size in crude extracts from all soils was >23 kb. Preliminary trials indicated that DNA recovery from two soils seeded with gram-negative bacteria was 92 to 99%. When the method was tested on all eight unseeded soils, microscopic examination of indigenous bacteria in soil pellets before and after extraction showed variable cell lysis efficiency (26 to 92%). Crude DNA yields from the eight soils ranged from 2.5 to 26.9 μg of DNA g^{-1}, and these were positively correlated with the organic carbon content in the soil ($r = 0.73$). DNA yields from gram-positive bacteria from pure cultures were two to six times higher when the high-salt–SDS–heat method was combined with mortar-and-pestle grinding and freeze-thawing, and most DNA recovered was of high molecular weight. Four methods for purifying crude DNA were also evaluated for percent recovery, fragment size, speed, enzyme restriction, PCR amplification, and DNA-DNA hybridization. In general, all methods produced DNA pure enough for PCR amplification. Since soil type and microbial community characteristics will influence DNA recovery, this study provides guidance for choosing appropriate extraction and purification methods on the basis of experimental goals.

Isolation of bacterial nucleic acids from natural environments has become a useful tool to detect bacteria that cannot be cultured (11, 27), to determine the fates of selected bacteria or recombinant genes under natural conditions (10, 19), and to reveal genotypic diversity and its change in microbial ecosystems (22). Many workers have attempted to increase DNA yields from soils by using severe physical treatments such as mechanical bead beating and sonication to lyse indigenous microbial cells. Such treatments can shear DNA to sizes of 5 to 10 kb or less (11, 14), and in at least one study, the average fragment size was 100 to 500 bp (17). Such DNA may not be suitable for community analysis based on *Taq* DNA PCR, because of the risk of forming chimeric products with smaller template DNA (12). Because microbial cells may remain tightly bound to soil colloids, soils high in clay or organic matter pose particular challenges to obtaining high yields of high-molecular-weight DNA. Most DNA extraction methods have been tested on a limited number of soil types, so that their general applicability is unknown for comparative ecological studies.

Extraction of DNA from soils always results in coextraction of humic substances which interfere with DNA detection and measurement. This contamination can inhibit *Taq* DNA polymerase in PCR (18, 25), interfere with restriction enzyme digestion (15), and reduce transformation efficiency (21) and DNA hybridization specificity (19). Since humic substances are difficult to remove, DNA purification is a critical step following direct extraction to obtain DNA of sufficient purity.

Objectives of this study were to evaluate and improve DNA extraction and purification methods for speed and simplicity, DNA yields, DNA fragment size, and applicability to a broader variety of soils. We tested these methods on eight physically and chemically distinct soils, including soils from which DNA is difficult to extract and purify. We emphasized PCR amplification in evaluating DNA purity because *Taq* polymerase is sensitive to humic contamination and because PCR amplification is a major use of extracted soil DNA.

MATERIALS AND METHODS

Soils. Eight soils were used to evaluate the efficiency of DNA extraction and purification procedures. Six of these soils had been selected from a global soil collection (9) to represent a range of soil properties (Table 1). The other two soils, Native Kellogg (NK) and Cultivated Kellogg (CK), were obtained from the National Science Foundation Long-Term Ecological Research site at Kellogg Biological Station near Kalamazoo, Mich. NK and CK soils were from the same soil series (Kalamazoo sandy loam, typic hapludalf), but CK soil has been cultivated for the last 40 years while NK soil has been undisturbed. All soils came from regions having predominantly luvisolic soils, as described under the Food and Agriculture Organization Soil Classification System (7). The six soils from the global collection had been sampled between 5 and 30 cm in depth in 1993. NK and CK soils were sampled between 0 and 15 cm in 1993 and 1994. All soils were kept on ice or stored at 4°C until they were tested in the laboratory.

Soil moisture contents were determined by drying at 110°C for 48 h. Particle size analyses were performed by a modified hydrometer method, in which the clay content was determined after 8 h (4). Carbon and nitrogen contents were determined on oven-dried, ground samples in a Carlo Erba NA 1500 series 2 nitrogen/carbon analyzer (Fisons Instruments, Beverly, Mass.). Soil pH was determined in a slurry (5 parts distilled water, 1 part soil). Soil color was evaluated by visual examination in outdoor sunlight with Munsell color plates.

Bacterial strains and soil inoculation. *Pseudomonas* sp. strain B13 (5) was used as the seed organism. Cells were grown to late exponential phase on M9 medium supplemented with trace minerals and 5 mM 3-chlorobenzoate and resuspended in 2 ml of extraction buffer (see below) before being inoculated into the soils. This cell suspension was mixed with sterilized soils, which were obtained by autoclaving twice at 121°C for 60 min. Seeded soils were kept at room temperature for 30 min prior to DNA extraction.

Effect of CTAB and PVPP on humic contamination of crude extracts. Hexadecylmethylammonium bromide (CTAB) and polyvinylpolypyrrolidone (PVPP) have been used in previous studies to complex and remove contaminants from

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DNA (1, 10). Seeded NK soils were used to evaluate the effect of CTAB and PVPP in the extraction buffer on humic contamination in crude extracts. Soil (5 g) was mixed with extraction buffer (see below) containing (i) no CTAB, no PVPP; (ii) 1% CTAB, no PVPP; or (iii) no CTAB, 2 g of PVPP. Soil suspensions were then processed by the extraction method described below. Spectrophotometric A_{260}/A_{280} and A_{260}/A_{230} ratios were determined to evaluate levels of protein and humic acid impurities, respectively, in the crude extracts (14, 20).

SDS-based DNA extraction method. Since CTAB performed better in reducing humic contamination, it was used in the buffer for sodium dodecyl sulfate (SDS)-based DNA extraction. Soil samples of 5 g were mixed with 13.5 ml of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) and 100 μl of proteinase K (10 mg/ml) in Oakridge tubes by horizontal shaking at 225 rpm for 30 min at 37°C. After the shaking treatment, 1.5 ml of 20% SDS was added, and the samples were incubated in a 65°C water bath for 2 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6,000 × *g* for 10 min at room temperature and transferred into 50-ml centrifuge tubes. The soil pellets were extracted two more times by adding 4.5 ml of the extraction buffer and 0.5 ml of 20% SDS, vortexing for 10 s, incubating at 65°C for 10 min, and centrifuging as before. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 × *g* for 20 min at room temperature, washed with cold 70% ethanol, and resuspended in sterile deionized water, to give a final volume of 500 μl.

Cell lysis and direct microscopic counts. Cell lysis efficiency was estimated for six of the eight soils by direct microscopic counts of soil smears (3) obtained before and after the DNA extraction treatment. Before DNA extraction, 5 to 15 g of soil was blended in a Waring blender for 1 min in 150 to 190 ml of sterile, filtered (0.2 μm), deionized water. The coarse particles in the blended slurry were allowed to settle for 1 min, and then a 10-ml subsample was removed from the upper portion of the slurry and transferred to a 15-ml sterile tube. The subsample was vortexed for 10 s, and 4- or 6-μl aliquots were removed for smears. Each aliquot was spread evenly in a 7-mm circle on a coated slide (Cel-Line Associates, Newfield, N.J.) and quickly dried at 40°C. After DNA extraction, smears were prepared from the remaining soil pellet and the pooled supernatants from each sample. A subsample of soil pellet was removed to determine the moisture content, and the remaining pellet was blended with appropriate amounts of water to prepare smears. Before DNA was precipitated and quantified from each supernatant, a 100-μl subsample of the supernatant was diluted 1:20 in water to make smears.

Dried smears were flooded with 10 μl of a fluorescent staining solution containing 2 mg of DTAF [5-(4,6-dichlorotriazin-2-yl)amino fluorescein; Sigma Chemical Co., St. Louis, Mo.)] per 10 ml of buffer (0.85% NaCl, 50 mM Na_2HPO_4 [pH 9.0]) (16). Flooded slides were held for 30 min in a covered container to prevent drying. The slides were rinsed by immersion in fresh buffer three times for 20 min each, rinsed with water after the final immersion, and air dried. Slides were stored in the dark at 4°C for no longer than 48 h before microscopic analysis.

The slides were examined with a 63× objective on a Leitz Orthoplan 2 epifluorescence microscope, a Lep HBO 50 mercury lamp, and a Leitz I3 filter block (BP 450-490 excitation filter, RKP 510 beam splitter, and LP 515 suppression filter). A charge-coupled device camera (Princeton Instruments, Trenton, N.J.) was used to obtain digitized images of smears (26) by photographing fields selected at random along two central transects (8). Images were transferred to a Power Macintosh 7100/66 via an ST135 detector/controller and GPIB interface card (National Instruments, Austin, Tex.) for display by IP Lab Spectrum image analysis software (Signal Analytics Corp., Vienna, Va.). Bacterial cells were counted by visual examination of images on the computer monitor. All counts were obtained by one investigator.

DNA extraction from gram-positive bacteria. The gram-positive bacteria used for comparing cell lysis methods were grown on tryptic soy agar at 37°C overnight. Three methods were used to evaluate DNA extraction from these bacteria in terms of DNA yield and fragment size: (i) grinding, freezing-thawing, and SDS; (ii) freezing-thawing and SDS; and (iii) SDS. Cell pellets from pure cultures (15 ml; 10^{10} cells ml^{-1}) were ground with a mortar and pestle in the presence of sterile sand and liquid nitrogen before addition of extraction buffer and SDS. The preparations were then frozen at −70°C and thawed by microwave heating until they boiled briefly, a total of three times; then the DNA was extracted by following a protocol similar to the one described above. The DNA yield was determined by spectrophotometry.

Purification of crude DNA extracts. Four methods for purifying small portions of crude extracts were evaluated. One-tenth to one-fifth of the crude DNA extract from 5 g of soil was processed in four ways, and the final volumes of the eluates were adjusted to their original volumes. The four methods were as follows: (i) single minicolumn (extract was passed through one Wizard minicolumn containing 1 ml of Wizard PCR Preps purification resin [Promega, Madison, Wis.]); (ii) double minicolumn (eluate from the first minicolumn was purified further with fresh resin and passage through another minicolumn); (iii) gel plus minicolumn (extract was purified by agarose gel electrophoresis followed by passage of the excised and melted gel band through a Wizard minicolumn); and

TABLE 1. Sampling locations and properties of soils used in DNA extraction procedures

Soil sampling location	Soil texture	Climate/native vegetation	FAO[a] soil grouping	Amt (%) of:			Moisture content %	Moist color (hue, value/chroma)	pH	% C	% N
				Sand	Silt	Clay					
Waitville, Saskatchewan (WV)	Loam	Temperate/boreal forest	Albic luvisol	45	39	16	28	7.5 YR 2/0	6.1	5.85	0.51
Bittern, Saskatchewan (BT)	Sandy loam	Temperate/boreal forest	Albic luvisol	59	37	7	14	10 YR 4/3	5.7	0.86	0.05
Lake Beloye, Russia (RU)	Sandy loam	Temperate/boreal forest	Albic luvisol	54	41	5	29	10 YR 3/1	4.8	4.73	0.12
Native Kellogg, Mich. (NK)	Sandy loam	Temperate/deciduous hardwood	Orthic luvisol	52	43	5	14	10 YR 3/1	5.4	1.50	0.12
Cultivated Kellogg, Mich. (CK)	Sandy loam	Temperate/deciduous hardwood	Orthic luvisol	70	23	7	12	10 YR 3/2	6.7	0.95	0.08
Lagos Penuelas, Chile (LP)	Sandy loam	Mediterranean/sclerophyllous	Chromic luvisol	67	21	12	10	10 YR 3/2	6.1	0.97	0.09
Venice Hills, Calif. (VH)	Loam	Mediterranean/sclerophyllous	Chromic luvisol	42	41	17	11	7.5 YR 4/3	6.4	0.85	0.08
Merredin, Australia (ME)	Sandy clay loam	Mediterranean/sclerophyllous	Xanthic ferralsol	55	14	31	9	5 YR 4/3	9.1	0.59	0.04

[a] FAO, Food and Agriculture Organization.

TABLE 2. Comparison of DNA yield and purity of the crude DNA from seeded NK soil subjected to different treatments[a]

Treatment	DNA yields (μg/g [dry wt] of soil)[b]	A_{260}/A_{280} ratio[b]	A_{260}/A_{230} ratio[b]
No PVPP, no CTAB	17.1 ± 0.9	1.17 ± 0.02	0.72 ± 0.03
CTAB, no PVPP	17.5 ± 1.2	1.35 ± 0.04	0.91 ± 0.03
PVPP, no CTAB	10.9 ± 1.5	1.23 ± 0.05	0.88 ± 0.03
Pure culture		1.89	1.57

[a] NK soil was sampled in 1993 and stored at 4°C for 6 months.

[b] DNA yields (mean values, $n = 3$, ± 1 standard deviation) were determined by fluorometry. The ratios were calculated from spectrophotometric measurements.

(iv) gel plus centrifugal concentrator. (The crude extract was subjected to gel electrophoresis, and the DNA band was excised, melted, and treated with Gelase [Epicentre Technologies, Madison, Wis.] by following the rapid protocol of the manufacturer. Nucleic acids were then washed and concentrated in a Centricon-50 [Amicon Corp., Beverly, Mass.].)

Since the minicolumn capacity was limited to 1 ml of resin, only a fraction (1/5 to 1/10) of the crude extract from 5 g of soil could be purified at a time. A larger-scale gel-plus-column purification procedure was used to purify the entire crude extracts from 5-g samples of the eight test soils. The procedure employed a 20-ml-capacity column with a different resin (Wizard Minipreps Plasmid Purification resin [Promega]) because the resin used in the minicolumns contained a 230-nm-absorbing substance that interfered with spectrophotometric measurement of DNA. The DNA was eluted from the resin twice with 500 μl of hot (70°C) Tris-EDTA buffer to facilitate release of high-molecular-weight DNA.

DNA quantification. After small-scale purifications, DNA was quantified by fluorometry with a TK 100 fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.), by following the extended assay protocol provided by the manufacturer. The fluorometer was calibrated with herring sperm DNA (Boehringer Mannheim, Indianapolis, Ind.). The DNA yields were estimated on the basis of at least three replicate determinations.

DNA was quantified after large-scale purification by determining fluorescence intensities of extracts in agarose gel bands in scanned Polaroid photographs. Crude and final DNA extracts were subjected to electrophoresis in Tris-acetate-EDTA (TAE) buffer containing 0.5 μg of ethidium bromide per ml in 0.7% agarose gels containing DNA standards of 5 to 60 ng of lambda phage DNA (Boehringer Mannheim). Gel photographs were scanned with a Hewlett-Packard ScanJet IIc scanner, generating digitized images that were analyzed with IP Lab Spectrum software. A standard curve of DNA concentration (10 to 50 ng of DNA) versus integrated pixel intensity ($r^2 = 0.98$) was prepared for each gel and used to calculate the final DNA concentrations in the DNA extracts.

PCR, restriction enzyme digestion, and Southern blotting. Primers and PCR conditions for amplifying 16S rRNA and *clcD* genes were described previously (28, 29). Restriction enzyme digests were performed with approximately 0.2 μg of DNA and 4 U of an endonuclease (*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, or *Xho*I) in 20 μl of the appropriate buffer as provided by the manufacturer. After incubation for 12 to 16 h, the DNA fragments were resolved in a 1% agarose gel. To evaluate DNA hybridization, 0.5 μg of the purified DNA from the seeded soils was digested with 10 U of *Bam*HI, separated in an 0.8% agarose gel, and transferred to a GeneScreen Plus membrane (Dupont, Boston, Mass.). Prehybridization, hybridization, and washings were carried out as described previously (29). A PCR-amplified 615-bp fragment of the *clcD* gene was used as the probe.

RESULTS

Soil properties. The physical and chemical properties of the eight soils used in the DNA extraction study were quite different (Table 1). Soils were classed as loams, sandy loams, or sandy clay loams, with clay contents ranging from 5 to 31%. The WV soil had the highest organic C content, which was reflected in its dark color (chroma = 0), and the highest N content. The ME soil had the lowest organic C and N contents, the reddest color, and the lowest moisture content. The pH of the soils ranged from 4.8 to 9.1.

Effect of CTAB or PVPP on DNA extraction from NK soil. When seeded NK soil was treated with different extraction buffers, no difference in DNA yield was observed among treatments with or without CTAB. Significant differences in DNA yield did occur among treatments with or without PVPP (Table 2). The crude DNA solutions from PVPP or CTAB treatments were lighter in color and had higher A_{260}/A_{230} and A_{260}/A_{280} ratios than did the solutions from treatments without PVPP or CTAB (Table 2). Both CTAB and PVPP can effectively remove humic materials, but unlike PVPP, CTAB resulted in no DNA loss. CTAB and PVPP did not completely remove humic compounds, since the A_{260}/A_{230} and A_{260}/A_{280} ratios for crude DNA from soils were significantly lower than the ratios for DNA solutions from pure cultures (Table 2).

DNA extraction efficiency was determined by comparing the total crude DNA obtained from a known cell density of sterile seeded soil samples with the DNA extracted from the pure culture. Crude DNA recoveries from seeded NK and CK soils were 92 to 99%.

No significant differences in DNA yields were observed when the crude DNA was allowed to precipitate in isopropanol for 1 h or overnight, either at room temperature or at −20°C. Repeated extractions of the soil pellets were beneficial, since small amounts of DNA were still recovered after the second and even the third wash, depending on the soil (data not shown). In the optimized method used subsequently, soil pellets were extracted three times. Most of the soil DNA fragments were larger than 23 kb and similar in size to DNA isolated from pure cultures (Fig. 1). These results suggest that the extraction protocol did not cause severe shearing of DNA.

Evaluation of DNA extraction and cell lysis on more challenging soils. Crude DNA was extracted from eight unseeded soils by the SDS-based method with CTAB, and mean yields ranged from 2.5 to 26.9 μg of DNA per g (dry weight) of soil (Table 3). The WV soil had the highest DNA yield, and the ME soil had the lowest yield. Significant correlation was observed between crude DNA yield and soil organic C content ($r = 0.73$, $P = 0.01$).

Lysis efficiencies of the DNA extraction procedure for six soils were estimated by microscopic examination of soil smears before extraction and of soil pellets and pooled supernatants after extraction. No cells were found in any of the 1:20 dilutions of the pooled supernatants, but cells were found in the residual pellets (Table 4). Postextraction counts of WV, RU, LP, and BT soils were significantly lower than preextraction counts, indicating high lysis efficiencies (67 to 92%). Two soils, VH and ME, appeared to show poor lysis by this method. Significant correlation was observed between cell lysis effi-

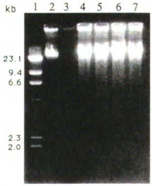

FIG. 1. Agarose gel electrophoresis of total DNA extracted from NK soil by different treatments. Lanes: 1, *Hin*dIII-cut bacteriophage lambda molecular size marker (1 μg); 2, pure-culture DNA from *Pseudomonas* sp. strain B13; 3, sterile soil; 4, sterile soil plus B13; 5, nonsterile soil DNA extracted without PVPP and CTAB; 6, nonsterile soil DNA extracted with PVPP; 7, nonsterile soil DNA extracted with CTAB.

TABLE 3. Crude DNA yields from eight soils and percentages of crude DNA recovered in the large-scale purification method involving gel electrophoresis and passage through one Megacolumn with 10 ml of Wizard Minipreps plasmid purification resin

Soil	Crude DNA yield[a] (μg/g [dry wt] of soil)	Final DNA yield[a] (μg/g [dry wt] of soil)	Crude yield CV (%)[b]	Final yield CV (%)[b]	% of crude DNA recovered
WV	26.9 ± 6.5	20.1 ± 5.0	24	25	75
BT	12.5 ± 5.1	3.4 ± 0.9	41	28	27
RU	13.7 ± 2.3	8.4 ± 2.8	17	34	61
NK	21.6 ± 5.1	12.0 ± 1.3	24	11	56
CK	4.9 ± 1.1	3.9 ± 1.2	22	31	80
LP	5.7 ± 1.1	3.1 ± 1.4	20	44	54
VH	3.0 ± 1.1	2.3 ± 0.5	35	22	77
ME	2.5 ± 0.6	2.0 ± 0.6	22	31	80

[a] DNA yields were estimated by gel staining. Yields are mean values (± 1 standard deviation) with $n = 5$, except for NK and CK soils, for which $n = 6$.

[b] CV, coefficient of variation.

ciency and clay content of the soils ($r = -0.67$; $P = 0.01$). Lysis was also evaluated by comparing the maximum and minimum expected DNA yields based on literature estimates of cellular DNA content for soil bacteria (2, 23). DNA yields for soils WV, RU, NK, CK, and ME were within the ranges of expected DNA yields, but observed values for BT, LP, and VH soils were below the minimum expected DNA yields (Table 4). By this method of evaluation, the ME soil gave the expected amount of DNA.

Comparison of different methods for lysing gram-positive bacteria. Since the SDS-based method may not have lysed some gram-positive bacteria, we evaluated two more physically severe cell lysis methods on pellets from pure cultures of gram-positive bacteria. The DNA yield was two to six times higher for most of the bacteria examined by the grinding-freezing-thawing-SDS method than by the freezing-thawing-SDS and SDS methods (Table 5). While a very small portion of the DNA was sheared by the grinding-freezing-thawing-SDS method, most of the DNA had a high molecular weight and a similar size to the DNA from the freezing-thawing-SDS and SDS methods.

Comparison of DNA purification methods with crude DNA from NK soil. To evaluate DNA purity for enzyme digestion, PCR amplification, and DNA hybridization, four purification methods were compared by using portions of the crude extracts from seeded and unseeded NK soil. Because of the small capacity of the minicolumn used in these methods, only 1/10 of the crude DNA extract from 5 g of soil was purified at a time. All four purification methods resulted in complete removal of the brown color from crude DNA solutions. DNA recovery varied with different purification methods. Higher recovery was obtained with gel-plus-concentrator purification than with column methods (Table 6). However, recovery by gel electrophoresis was more variable and depended on the size distribution of DNA fragments in crude extracts. In addition, loss of DNA was greater for the first minicolumn purification (~20%) than for the second minicolumn purification (~5 to 6%) (Table 6). This suggests that humic materials in crude extracts might interfere with DNA binding to the resin.

Restriction endonuclease digestion was possible only with purified DNA. While all enzymes cut the DNA purified by the gel-plus-minicolumn method, DNA resulting from single-minicolumn purification was only partially digested by most of the enzymes (Table 6). DNA quality could be improved by a second minicolumn purification, since the eluted DNA was digested by most of the enzymes. While all of the purified DNA samples were completely digested by *Bam*HI, most methods resulted in DNA that was only partially digested by *Hin*dIII (Table 6).

Amplification of the 16S rRNA genes was successful when DNAs purified by all tested methods were used as templates (Fig. 2). No PCR products were observed with DNA from

TABLE 4. Direct counts and DNA yields for individual samples of eight soils

Soil	Preextraction count/g (dry wt) of soil[a]	Postextraction count/g (dry wt) of soil	% Lysis efficiency[b]	Expected DNA yield[c] (μg/g [dry wt] of soil)	Crude DNA yield[d] (μg/g [dry wt] of soil)
WV	$(9.7 \pm 0.6) \times 10^9$	$(2.0 \pm 0.2)^e \times 10^9$	79	15–50	33.5
BT	$(7.3 \pm 0.4) \times 10^9$	$(5.7 \pm 2.1)^e \times 10^8$	92	11–39	8.7
RU	$(6.9 \pm 1.1) \times 10^9$	$(1.4 \pm 0.1)^e \times 10^9$	80	9–40	15.5
NK	$(5.1 \pm 1.0) \times 10^9$	ND[f]	ND	7–31	19.6
CK	$(2.9 \pm 0.7) \times 10^9$	ND	ND	4–18	4.9
LP	$(4.6 \pm 0.5) \times 10^9$	$(1.5 \pm 0.2)^e \times 10^9$	67	7–26	4.6
VH	$(3.5 \pm 0.3) \times 10^9$	$(2.6 \pm 0.4) \times 10^9$	26	5–19	2.0
ME	$(1.3 \pm 0.1) \times 10^9$	$(4.2 \pm 0.6) \times 10^9$	—[g]	2–7	2.3

[a] Mean count ± standard deviation (two smears per sample).

[b] Determined from one sample of each soil and calculated from mean counts: [100 − (postextraction count/preextraction count)] × 100.

[c] Range of expected DNA yield obtained by multiplying lower and upper limits of preextraction counts (mean counts ± 1 standard deviation) by low and high literature values reported for cellular DNA content of soil bacteria (1.6 fg cell^{-1}, as reported by Bakken and Olsen [2], and 5 fg cell^{-1}, as suggested by Torsvik and Goksoyr [23]).

[d] DNA yield from the supernatant associated with pellet used for the postextraction direct count. The DNA yield was estimated by gel staining.

[e] Significantly different from the preextraction count at the 5% level.

[f] ND, not determined.

[g] Not determined, since postextraction counts were higher than preextraction counts. Higher counts in ME soil following DNA extraction could have been due to cell masking by soil particles in preextraction ME smears, because they contained more soil (1.1 mg cm^{-2}) than did preextraction smears from other soils (0.14 to 0.7 mg cm^{-2}). Bloem et al. (3) have recently recommended a maximum soil density for direct counts on loam soil of 0.8 mg cm^{-2} to minimize cell masking by soil particles.

TABLE 5. Comparison of DNA yield from gram-positive bacteria[a] by different lysis methods

Treatment	DNA yield (μg/μl) from:							
	A. globiformis	*B. subtilis*	*C. renale*	*M. luteus*	*R. erythropolis*	Ea39	Ben-28	*S. lividans*
Grinding-freezing-thawing-SDS	0.47	0.33	0.40	0.22	0.23	0.19	0.26	0.37
Freezing-thawing-SDS	0.13	0.19	0.46	0.12	ND[b]	ND	ND	0.08
SDS	0.11	0.16	0.43	0.12	0.10	0.07	0.05	0.06

[a] The gram-positive bacteria are *Arthrobacter globiformis*, *Bacillus subtilis*, *Corynebacterium renale*, *Micrococcus luteus*, *Rhodococcus erythropolis*, *Rhodococcus* sp. strain Ea39, *Rhodococcus* sp. strain Ben-28, and *Streptomyces lividans*. The DNA yields were estimated by spectrophotometry.

[b] ND, not determined.

crude extracts. No amplification products were detected when target DNA sequences were not present in the reaction mixtures. A second set of primers (for the *clcD* genes) was used to further test for PCR amplification in parallel seeded soils. The DNA templates purified by all methods produced the expected product (data not shown).

DNA extracted from seeded NK soil was used to determine whether the DNA purified by each of the four methods was pure enough for Southern hybridization. Crude and purified DNA extracts and DNA obtained from pure cultures of the same strain were hybridized with the *clcD* gene probe. Signal intensities were very similar between pure-culture DNA extracts and purified soil DNA from each of the four methods (Fig. 3). Weak hybridization was observed for the crude DNA. The DNA purified by all tested methods was pure enough for Southern hybridization.

Evaluation of purification methods on crude DNA extracts from more challenging soils. Small-scale purification methods were also evaluated with crude DNA extracts from the six global soils. Only the double-minicolumn and gel-plus-minicolumn methods resulted in complete removal of the dark color from all six crude DNA solutions. The gel-plus-concentrator method did not completely remove the dark color from the WV and RU extracts, probably because of the high organic C contents of these soils. Crude extracts from WV, RU, and NK soils all contained greater amounts of high-molecular-weight humic acids, as observed during electrophoretic separation of DNA from humic contaminants. After electrophoresis, excised DNA bands could have contained such contaminants, which cannot be washed through concentrator filters with lower-molecular-weight cutoffs.

PCR amplification of the 16S rRNA genes was successful with DNA purified by the gel-plus-minicolumn and gel-plus-concentrator methods. DNA purified by the double-minicolumn method was amplified in only four of the six soils (WV and RU soils produced no PCR amplification), indicating that this method yielded DNA that was less pure. The DNA fragments from all soils were larger than 20 kb.

TABLE 6. DNA recovery for different purification methods and enzyme digestion of the DNA samples purified from unseeded NK soil

Purification method	Recovery[a] (%)	Digestion[b] by:					
		*Bam*HI	*Dra*I	*Eco*RI	*Eco*RV	*Hin*dIII	*Xho*I
Crude extracts	100	−	−	−	−	−	−
Column	80.6 ± 2.1	+	±	±	±	±	±
Column + column	74.5 ± 3.8	+	+	+	+	±	+
Gel + centricon	91.4 ± 7.5	+	+	±	+	±	+
Gel + column	84.1 ± 7.1	+	+	+	+	+	+

[a] Percentage of DNA recovered as measured by UV absorption compared with DNA in crude extract ± 1 standard deviation.

[b] +, complete digestion; ±, incomplete digestion; −, no digestion.

A larger-scale gel-plus-column method was also evaluated on these soils. The A_{260}/A_{280} and A_{260}/A_{230} ratios of DNA purified by the large-scale method were 1.6 to 1.8 and ≥1.9, respectively, indicating that the DNA was of good quality. For most of the extracts, 0.1-μl aliquots resulted in better PCR amplification than did 1-μl aliquots (Fig. 4). The WV soil extract still appeared to contain substances that interfered with the reaction, because no amplification was observed with 1-μl aliquots. DNA fragments purified by the large-scale procedure were all larger than 20 kb (data not shown). However, the large-scale purification method gave poorer DNA recoveries (27 to 80%; Table 3) than the small-scale methods did. Recoveries were improved slightly (e.g., from 53 to 75% for NK soils and from 68 to 85% for CK soils) when DNA Cleanup resin or Maxipreps Plasmid Purification resin (Promega Corp.) was used instead of the Minipreps resin. The resin choice may have

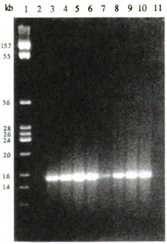

FIG. 2. Agarose gel electrophoresis of 16S rDNA amplification products from DNA of unseeded NK soil samples. Lanes: 1, *Hin*dIII-, *Eco*RI-, and *Bam*HI-cut bacteriophage lambda molecular size marker (1 μg); 2, crude DNA extracts; 3 and 4, undiluted (40.5-ng) and 10^{-1}-diluted (4.1-ng) DNA extracts purified by gel plus centrifugal concentrator; 5 and 6, undiluted (35-ng) and 10^{-1}-diluted (3.5-ng) DNA extracts purified by gel plus minicolumn; 7 and 8, undiluted (30-ng) and 10^{-1}-diluted (3.0-ng) DNA extracts purified by single minicolumn; 9 and 10, undiluted (28-ng) and 10^{-1}- diluted (2.8-ng) DNA extracts purified by two successive minicolumns; 11, reaction mixture only, without DNA template.

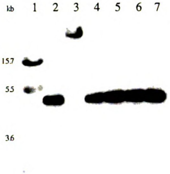

FIG. 3. Autoradiogram of hybridization signals with the *clcD* gene after Southern transfer of DNA from seeded sterile NK soil. Lanes: 1, *Hin*dIII-cut bacteriophage lambda molecular size marker (1 μg); 2, pure-culture DNA; 3, crude DNA extracts; 4, DNA extracts purified by gel electrophoresis plus centrifugal concentrator; 5, DNA extracts purified by gel electrophoresis plus column; 6, DNA extracts purified by single column; 7, DNA extracts purified by double column.

to be optimized when recovery of large amounts of DNA is important.

DISCUSSION

We divided the problem of DNA recovery from soil into two component methods, i.e., (i) cell lysis and extraction of crude DNA and (ii) purification of crude DNA, since there are advantages in combining different lysis and purification methods for different cases. The component methods were first developed on a standard soil (NK) and then tested on a set of more challenging soils. Three evaluation approaches were used to determine whether the SDS-based extraction method recovered most of the bacterial DNA: (i) DNA recovery efficiency from seeded bacteria in a standard soil; (ii) lysis efficiency of indigenous bacteria in more challenging soils; and (iii) comparison of crude DNA yields with expected DNA yields.

The SDS-based extraction method resulted in 92 to 99% recovery of the DNA from bacteria added to soil. These efficiencies were comparable to or higher than those obtained by other laboratories (6, 21). This first approach to evaluating DNA recovery, however, can overestimate extraction efficiency, because indigenous bacteria may be more difficult to lyse than seeded bacteria. When we determined net losses in indigenous cell counts after extraction, lysis efficiencies varied from 26 to 92% among five test soils. The variation in cell lysis apparently reflects differences in soil characteristics and bacterial community composition (i.e., soils exhibiting low cell lysis may have contained higher proportions of gram-positive cells). Crude DNA yields from the SDS-based extraction method agreed reasonably well with expected yields based on direct microscopic counts. We compared each experimental yield with a range of expected yields, rather than a single value, because of the uncertainty regarding the choice of an average cellular DNA content for soil bacteria.

SDS has been the most widely used cell lysis treatment for DNA extraction from pure cultures, soils, and sediments. Trevors et al. (24) found that the SDS-based cell lysis protocol provided the highest DNA yields in comparison with freezing-thawing and Sarkosyl-based lysis protocols. More et al. (13) showed that the percentage of indigenous cells remaining after SDS treatment of a sediment (13%) was lower than the percentage of cells left after 10 min of bead milling (26%). Our results indicated that SDS-based cell lysis, in combination with high-salt treatment and heating, was effective for most of the soils but appeared to be influenced by clay content and was not effective for at least some gram-positive bacteria. Thus, for soils exhibiting poor cell lysis or studies depending on extensive sampling of gram-positive DNA, other lysis treatments or combinations of treatments could be considered. Combining SDS with bead mill homogenization resulted in higher cell lysis efficiency for *Bacillus* endospores (13). However, bead mill homogenization and other physical methods such as sonication generally cause severe DNA shearing (11, 14). Our results showed that the combination of grinding, freezing-thawing, and SDS resulted in much higher DNA yields from most of the gram-positive bacteria but without severe shearing.

Although DNA purified by all methods could be amplified and hybridized, some variation in DNA purity was observed with respect to restriction enzyme digestion (Table 6). The gel-plus-minicolumn method appeared to result in the purest DNA, because the DNA was completely digested by all enzymes examined; the method also provided good recovery with the standard soil. However, the larger-scale gel-plus-column method gave variable DNA recovery from crude extracts from other soils. Single- or double-minicolumn purifications appeared to give DNA which was incompletely digested and less suitable for PCR amplification, as well as having lower recovery efficiency. Single- or double-minicolumn methods, however, were very rapid and less expensive.

The gel-plus-column method gave very pure DNA, while the gel-plus-concentrator method gave the highest recovery. The latter method, however, may not remove all humic contaminants from crude extracts of soils with low chromas (0 or 1), because these soils appear to contain higher proportions of high-molecular-weight humic acids. If gel-plus-concentrator

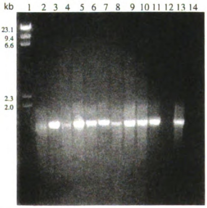

FIG. 4. Agarose gel electrophoresis of 16S rDNA amplification products of DNA samples from six soils purified by the large-scale procedure. Lanes: 1, *Hin*dIII, *Eco*RI, and *Bam*HI-cut bacteriophage lambda molecular size marker (1 μg); 2 and 3, undiluted (15-ng) and 10^{-1}-diluted (1.5-ng) BT extracts; 4 and 5, undiluted (20-ng) and 10^{-1}-diluted (2.0-ng) LP extracts; 6 and 7, undiluted (8.5-ng) and 10^{-1}-diluted (0.85-ng) ME extracts; 8 and 9, undiluted (36-ng) and 10^{-1}-diluted (3.6-ng) RU extracts; 10 and 11, undiluted (8-ng) and 10^{-1}-diluted (0.8-ng) VH extracts; 12 and 13, undiluted (40-ng) and 10^{-1}-diluted (4-ng) WV extracts; 14, reaction mixture only, without DNA template.

purification is used on these soils, concentrator units with the highest-molecular-weight cutoffs should be used, because some humic acids have molecular weights of 100,000 or greater. Larger-scale gel-plus-column purification can provide larger amounts of DNA but is more expensive, requiring additional agarose and DNA-binding resin.

In summary, the DNA extraction and purification methods evaluated here are simple, rapid, and efficient for most soils and purposes. DNA could be extracted from eight soil samples in 6 h by the SDS-based method. Because of the gentle nature of the extraction treatment, the DNA fragment size in crude extracts was >23 kb. DNA purification required 2 to 4 h for the single- or double-column method, 8 to 10 h for the gel-plus-column method, and 12 to 14 h for the gel-plus-concentrator method. If DNA purity is of the greatest concern, we recommend gel-plus-column methods. It is also important to recognize that no single method of cell lysis or purification will be appropriate for all soils and experimental goals. The basic methods suggested should be appropriate for the more common cases, but different combinations and modifications of lysis and purification protocols will probably be needed for some conditions.

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