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Activity and Composition of the Denitrifying Bacterial Community Respond Differently to Long-Term Fertilization

Karin Enwall,[1]* Laurent Philippot,[2] and Sara Hallin[1]

Swedish University of Agricultural Sciences, Department of Microbiology, Box 7025, SE-750 07 Uppsala, Sweden,[1] *and UMR INRA Microbiologie et Géochimie des Sols, 17 rue Sully BP 86510, 21065 Dijon Cedex, France*[2]

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The objective of this study was to explore the long-term effects of different organic and inorganic fertilizers on activity and composition of the denitrifying and total bacterial communities in arable soil. Soil from the following six treatments was analyzed in an experimental field site established in 1956: cattle manure, sewage sludge, $Ca(NO_3)_2$, $(NH_4)_2SO_4$, and unfertilized and unfertilized bare fallow. All plots but the fallow were planted with corn. The activity was measured in terms of potential denitrification rate and basal soil respiration. The *nosZ* and *narG* genes were used as functional markers of the denitrifying community, and the composition was analyzed using denaturing gradient gel electrophoresis of *nosZ* and restriction fragment length polymorphism of *narG*, together with cloning and sequencing. A fingerprint of the total bacterial community was assessed by ribosomal intergenic spacer region analysis (RISA). The potential denitrification rates were higher in plots treated with organic fertilizer than in those with only mineral fertilizer. The basal soil respiration rates were positively correlated to soil carbon content, and the highest rates were found in the plots with the addition of sewage sludge. Fingerprints of the *nosZ* and *narG* genes, as well as the RISA, showed significant differences in the corresponding communities in the plots treated with $(NH_4)_2SO_4$ and sewage sludge, which exhibited the lowest pH. In contrast, similar patterns were observed among the other four treatments, unfertilized plots with and without crops and the plots treated with $Ca(NO_3)_2$ or with manure. This study shows that the addition of different fertilizers affects both the activity and the composition of the denitrifying communities in arable soil on a long-term basis. However, the treatments in which the denitrifying and bacterial community composition differed the most did not correspond to treatments with the most different activities, showing that potential activity was uncoupled to community composition.

Denitrification is an alternative pathway for microorganisms to respire under oxygen-limited conditions, using nitrogen oxides as electron acceptors. During this process, the soluble nitrogen compounds nitrate (NO_3^-) and nitrite (NO_2^-) are reduced in a stepwise manner to nitric oxide (NO), nitrous oxide (N_2O), and nitrogen gas (N_2) (41). Besides denitrification, nitrate can also be reduced to nitrite in another dissimilatory pathway, but in this case, the nitrite produced is instead transformed into ammonium (17). The denitrifying bacteria are known to belong to more than 50 genera (41), and the nitrate-reducing bacteria comprise an even larger group of phylogenetically unrelated microorganisms (24). Denitrification is a significant contributor to emissions of N_2O, which is involved in destruction of the stratospheric ozone layer and in global warming (2, 8). Since denitrification can result in losses of added nitrogen fertilizers from agricultural soils, the process is of interest for both environmental and economic reasons.

While it has been shown in numerous studies that nitrogen fertilization promotes denitrification (3, 19), the impact of fertilizers on the composition of the denitrifying community in arable soil has been studied to a lesser extent. In a field experiment, Wolsing and Prieme (40) observed small variations in the denitrifying community which may have been caused by fertilizer type but not by fertilizer amount. The effects of nitrogen fertilization on both denitrifying bacteria and ammonia-oxidizing bacteria were studied in an incubation experiment by Avrahami et al. (1). They showed that the addition of ammonium in high concentrations increases the N_2O release rate and at the same time induces a shift in the soil-denitrifying community, but not in the ammonia-oxidizing community. Even in long-term field experiments, differences in community composition of ammonia oxidizers due to nitrogen fertilization have rarely been detected, although both the abundance and activity of this functional community can be affected (4, 16, 26, 39). On the other hand, changes in the total bacterial community structure in soil amended with manure or ammonium nitrate have been described previously (21).

Our objective was to explore the long-term effects of different organic and inorganic nitrogen fertilizers on both the composition and activity of soil microbial communities. In addition to total bacteria, special attention was devoted to the functional guilds involved in denitrification due to their importance in nitrogen cycling. Soil was sampled from plots fertilized with cattle manure, sewage sludge, $Ca(NO_3)_2$, and $(NH_4)_2SO_4$ at a field site established in 1956 with unfertilized plots with and without crops as control soils. The *nosZ* and *narG* genes, encoding the nitrous oxide and the nitrate reductases, were used as functional markers to analyze denitrifying community composition using denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP), with subsequent cloning and sequencing. The composition of the total bacterial community was assessed by ribosomal intergenic spacer region analysis (RISA). In addition, the activity of total

* Corresponding author. Mailing address: Swedish University of Agricultural Sciences, Department of Microbiology, Box 7025, SE-750 07 Uppsala, Sweden. Phone: 46 18 67 33 27. Fax: 46 18 67 33 93. E-mail: Karin.Enwall@mikrob.slu.se.

TABLE 1. Soil pH, total organic carbon, and nitrogen for the different treatments in the Ultuna Long-Term Soil Organic Matter Experiment[a]

Treatment designation	Fertilizer regimen	Mean ± SD		
		pH[1]	% of dry soil	
			Total organic C	Total N
A	Unfertilized (bare fallow)	5.47 ± 0.16	1.01 ± 0.03	0.09 ± 0
B	Unfertilized	5.63 ± 0.05	1.22 ± 0.10	0.11 ± 0.01
C	Calcium nitrate $Ca(NO_3)_2$	6.26 ± 0.04	1.43 ± 0.03	0.13 ± 0
D	Ammonium sulfate $(NH_4)_2SO_4$	3.97 ± 0.14	1.29 ± 0.01	0.20 ± 0.01
J	Solid cattle manure	6.02 ± 0.09	2.20 ± 0.05	0.20 ± 0.01
O	Sewage sludge	4.68 ± 0.03	2.76 ± 0.08	0.27 ± 0.01

[a] When the experimental site was established in 1956, the soil pH was 6.5 and the total organic C and total N were 1.5% and 0.17% of the dry solids, respectively.

and denitrifying communities was evaluated by measuring the basal respiration rates and the potential denitrification activity.

MATERIALS AND METHODS

Field site characteristics, soil sampling, and chemical analysis. The field site, the Ultuna Long-Term Soil Organic Matter Experiment, is located at Ultuna, Uppsala, in Sweden (14). This field experiment was established in 1956 to study how different organic and inorganic fertilizers and soil amendments influence the nitrogen and carbon content in the soil and whether pH, soil structure, and crop yield are affected. The soil at the site is a clay loam classified as a Eutric Cambisol according to the system of the Food and Agriculture Organization. The experimental site consists of four blocks, each comprising different treatments in plots of 2 by 2 m separated by wooden frames. One of the blocks does not have randomly distributed plots and was omitted from the study. The organic fertilizers are applied during the fall every second year in an amount corresponding to 8 metric tons of ash-free organic matter ha^{-1}, and mineral fertilizers are applied yearly in the spring at a rate of 80 kg N ha^{-1}. All treatments are fertilized with 22 kg phosphorus and 35 to 38 kg potassium ha^{-1} $year^{-1}$. As controls, two unfertilized plots with and without crops are included in each block. During the years 1956 to 1999, spring-sown cereals dominated and occasionally fodder rape and fodder beet were cultivated (14), but since 2000, the plots have been planted with corn (*Zea maize*). Soil was sampled from six different treatments from three replicate plots. The different treatments are described in Table 1. Composite samples of 10 soil cores (2-cm diameter, 20-cm depth) were taken from each plot in between rows in 2002 after harvest. The soil was sieved (4-mm mesh) and stored at −20°C. The 18 soil samples were analyzed separately throughout this study.

Total soil nitrogen was measured as Kjeldahl nitrogen (ISO 10694). The contents of organic carbon were determined through dry combustion (ISO 13878), and the pH was defined in a solution of 0.01 M calcium chloride (ISO 10390). The chemical analyses were performed by AgriLab (Uppsala, Sweden).

Potential denitrification activity and basal soil respiration. Potential denitrification activity was measured in triplicate for each triplicate field plot according to the method of Pell et al. (22). Thawed soil samples (25 g) were placed in 250-ml flasks and kept at room temperature overnight. On the following day, 25 ml of substrate with 1 mM glucose and 1 mM KNO_3 was added and denitrifying conditions were achieved by evacuating and filling flasks with nitrogen gas five times. Acetylene was added to reach 0.1 atm partial pressure. The soil was incubated at 25°C on a rotary shaker for 3 h, and gas samples were collected every half hour. Nitrous oxide in the gas samples was analyzed on a gas chromatograph (model CP 9000; Chrompack, Rotterdam, The Netherlands) equipped with a ^{63}Ni electron capture detector. The initial denitrification rate was calculated from nonlinear regression of the N_2O produced during incubation (22).

Basal soil respiration was determined in triplicate for each triplicate plot according to the method of Stenberg et al. (34). Soil samples (20 g) were adjusted to a 60% water holding capacity, transferred to 250-ml respirometric jars, and placed in a respirometer (Respicond III; Nordgren Innovation AB, Umeå, Sweden). The soil was incubated at 22°C for 7 days, and the CO_2 produced was absorbed in 0.2 M potassium hydroxide solution (10 ml). The subsequent decrease in conductivity of the solution, measured every 30 min during incubation, was used to calculate the respiration rate.

DNA extractions and PCR amplification of *nosZ*, *narG*, and the ribosomal intergenic spacer region. For each triplicate from the six treatments, three DNA extractions were made using a FastDNA Spin Kit for Soil (Qbiogene) according to the manufacturer's instructions. The three extracts from the same replicate were then pooled before further analysis. Fragments of the *nosZ* gene were amplified with the primers nosZ-F (5′-CGY TGT TCM TCG ACA GCC AG-3′) (15) and nosZ1622R (5′-CGS ACC TTS TTG CCS TYG CG-3′) or nosZ1622R-GC (35). The last-named of these primers was used prior to DGGE. Amplification was performed in a total volume of 25 μl with 2.5 μl 10× PCR buffer, 200 μM (each) deoxyribonucleoside triphosphate, 1.25 U *Taq* polymerase (GE HealthCare, United Kingdom), 1 μM (each) primer, 800 ng μl^{-1} bovine serum albumin, and 20 ng of soil DNA. Touchdown PCR was performed in a minicycler (MJ Research) with 2 min of denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58 to 53°C, and 60 s at 72°C. The first 10 cycles were decreased by 0.5°C per cycle, and the last 25 were kept at 53°C. The program was completed after 10 min at 72°C. For the cloning, three 25-μl PCR mixtures were made for each triplicate experiment of the six samples, while for the DGGE, four 50-μl reaction mixtures were made.

Fragments of the *narG* gene were amplified with the primers narG1960f (5′-TAY GTS GGS CAR GAR AA-3′) and narG2650r (5′-TTY TCR TAC CAB GTB GC-3′) designed by Philippot et al. (25). Hot-start PCR amplification was performed in a total volume of 50 μl with 5 μl of 10× PCR buffer, 200 μM (each) deoxyribonucleoside triphosphate, 1 U of *Taq* polymerase (Qbiogene, France), 40 ng of soil DNA, 6 μM (each) primer, and AmpliWax bead (Applied Biosystems, CA). A Touchdown PCR was performed in a Peltier thermal cycler (MJ Research) with 5 min of denaturation at 95°C, followed by 38 cycles of 30 s at 94°C, 30 s at 59 to 55°C, and 45 s at 72°C. The first eight cycles were decreased by 0.5°C per cycle, and the last 30 were kept at 55°C. The program was completed after 10 min at 72°C. For *narG*, three 50-μl PCRs were performed for each triplicate sample and the same purified products were used for both the RFLP analysis and the cloning.

The PCRs for amplification of the ribosomal intergenic spacer region were performed using the primers 38_r (5′-CCG GGT TTC CCC ATT CGG-3′) and 72_f (5′-TGC GGC TGG ATC TCC TT-3′) (11). Amplification was performed in a total volume of 25 μl with 2.5 μl 10× PCR buffer, 200 μM (each) deoxyribonucleoside triphosphate, 0.5 U *Taq* polymerase (Qbiogene, France), 0.5 μM (each) primer, and 25 ng of soil DNA. The PCR was run using a Peltier thermal cycler (MJ Research) starting with 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°, and completed after 15 min at 72°C.

All PCR products were analyzed on 1% agarose gels. For *nosZ*, no bands other than those expected were visible and the PCR products could be resolved by DGGE without purification, but for *narG*, multiple bands were obtained and a purification step was required prior to RFLP analysis. However, before cloning of *narG* and *nosZ*, the PCRs were purified using the MiniElute gel extraction kit (QIAGEN, France) to avoid insertions of nonvisible amplicons.

DGGE of *nosZ*. PCR products of *nosZ* gene fragments with GC clamps were concentrated through freeze-drying (3×10^{-1} mbar, from −40°C to −50°C) (Edwards Modulyo freeze dryer; BOC Edwards, Crawley, United Kingdom). The concentrated PCR products were applied on an agarose gel and quantified using a low-DNA-mass ladder (Invitrogen, CA) prior to DGGE. The DGGE was performed according to the method of Throbäck et al. (35) using a DCode system from Bio-Rad Laboratories, Inc. Approximately 200 ng of PCR amplicons was loaded onto a 7% (vol/vol) acrylamide–bis-acrylamide (37.5:1) gel with a denaturing gradient of 40 to 70%. After electrophoresis for 17 h at 130 V and 60°C, the gel was stained with SYBR Gold (Molecular Probes, Canada) for 30

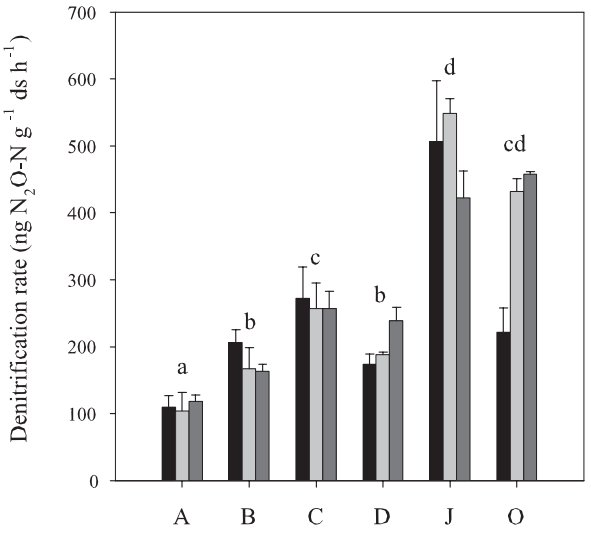


FIG. 1. Potential denitrification rates in soil from the different treatments (A through D, J, and O; mean ± standard deviation, n = 3). The same letters above the bars indicate treatments without significant differences ($P < 0.05$). ds, dry solids.

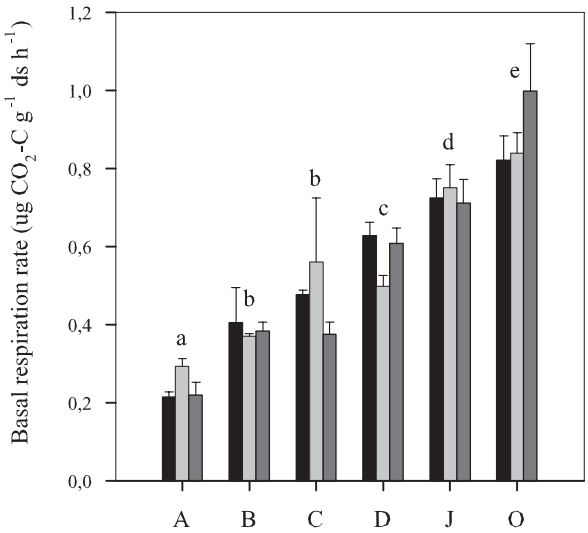


FIG. 2. Basal respiration rates in soil from the different treatments (A through D, J, and O; mean ± standard deviation, n = 3). The same letters above the bars indicate treatments without significant differences ($P < 0.05$). ds, dry solids.

min. Images were documented with the Gel Doc 2000 system and analyzed with Quantity One software (Bio-Rad Laboratories, Inc.).

RFLP of *narG*. The purified PCR products were quantified on an agarose gel using Smart Ladder SF (Eurogentec, Belgium), and equal amounts of the PCR products for each sample (approximately 500 ng) were digested at 37°C with AluI for 2 h. The digested PCR products were separated on a 6% acrylamide–bis-acrylamide (29:1) gel for 11.5 h at 5 mA according to the method of Philippot et al. (25). The acrylamide gel was then stained with SYBR Green II (Molecular Probes, Canada) and scanned with a Storm 960 phosphorimager (Molecular Dynamics).

RISA. The PCR products from the ribosomal intergenic region were quantified on an agarose gel using Smart Ladder SF (Eurogentec, Belgium). Approximately 1 μg of amplicons from each sample was loaded upon a 6% acrylamide–bis-acrylamide (29:1) gel and run for 15 h at 9 mA. The gel was stained with SYBR Green II (Molecular Probes, Canada) and scanned with a Storm 960 phosphorimager (Molecular Dynamics).

Cloning and sequencing. After confirmation that fingerprint patterns of *nosZ* (DGGE) and *narG* (RFLP) were similar from the three field plots within each treatment, purified PCR products from the triplicate plots were pooled before cloning. However, for the cloning of *narG*, sample B3 was omitted because it had two bands that were more intense, which would bias the clone library for this treatment. The pooled PCR products were cloned using the pGEM-T Easy Vector system (Promega, WI) according to the manufacturer's instructions. Forty-eight clones from each of the six *nosZ* and six *narG* clone libraries were screened by transferring small aliquots of cells to PCRs containing the vector primers T7 and SP6. Clones with correct insert sizes were digested with AluI overnight at 37°C and separated by gel electrophoresis on 3% agarose gels. The clones were grouped into RFLP pattern types as determined by similarity in RFLP patterns. Plasmids from the most common clone families were isolated using the QIAprep Spin miniprep kit protocol (QIAGEN). The inserts were sequenced on one strand by Macrogen Inc. (Korea) with an ABI3730 XL automatic DNA sequencer by using the vector primer T7. The sequenced clones from the different RFLP pattern types were digested in silico to ensure the correct division in RFLP types.

Statistical analysis. Potential denitrification rates and basal soil respiration rates were compared using the Mann-Whitney U test. The fingerprint patterns on the gels from DGGE of *nosZ*, RFLP analysis of *narG*, and the RISA were compared using the Quantity One 1-D analysis software (Bio-Rad Laboratories Inc.). Presence-absence matrices were used to determine differences between the patterns, and hierarchical cluster analysis was performed using Dice indices and unweighted-pair group method using average linkages (UPGMA) algorithms. The *nosZ* and *narG* amino acid sequences derived were aligned together with other sequences from environmental clones and pure cultures using the CLUSTALW software (http://www.ebi.ac.uk/clustalw/). Neighbor-joining trees (31) were constructed with the software TREECON (38) using the Kimura (13) distance matrix and bootstrap analysis with 1,000 replicates. The NosZ tree was constructed with sequences from 18 pure cultures and 18 soil clones (18, 27, 28, 30, 35) and the 47 sequences from this study. The NarG tree contains sequences from 18 pure cultures, 16 soil clones (7, 9, 18, 25), and 46 sequences from this study.

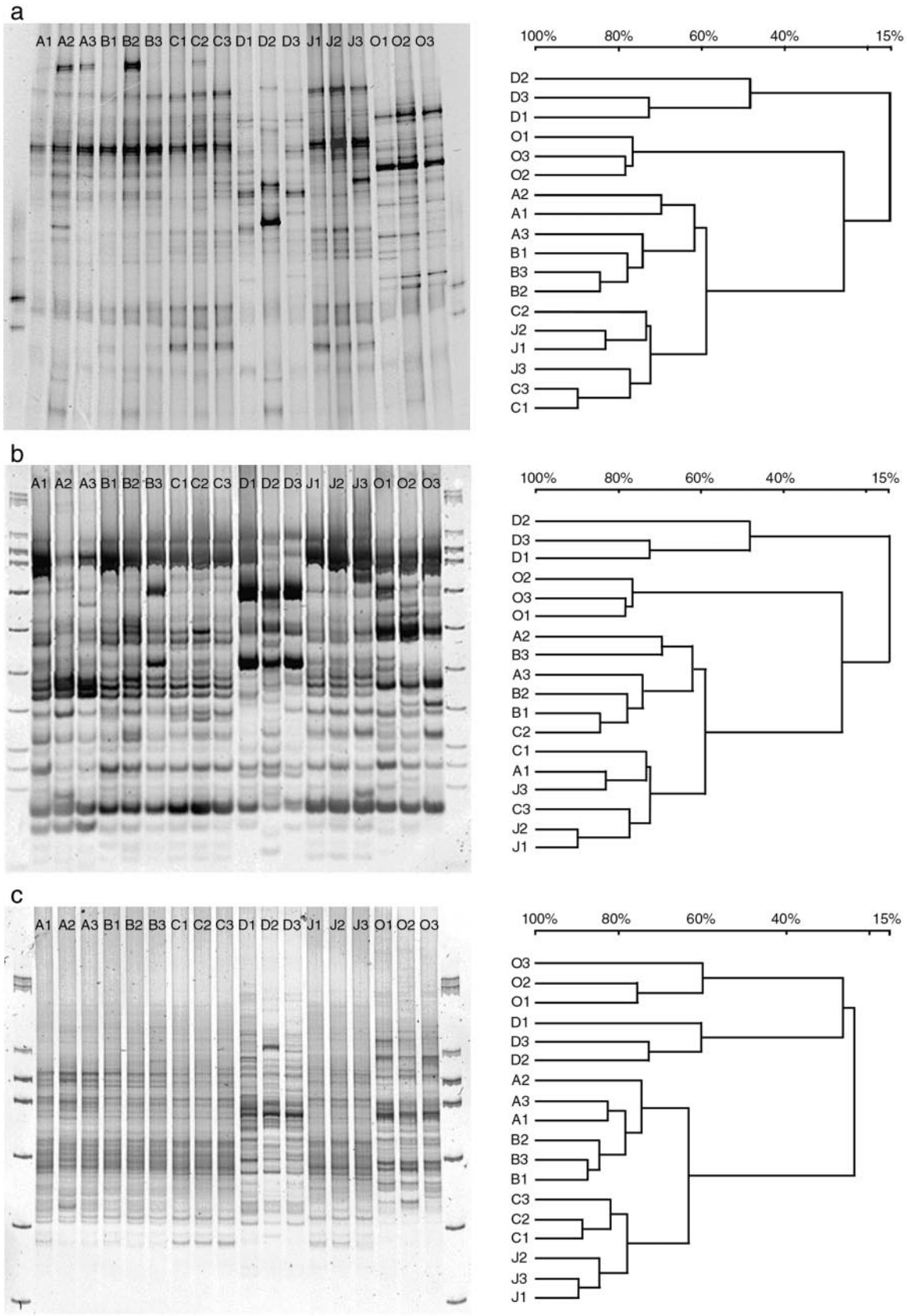
Nucleotide sequence accession numbers. The *nosZ* and *narG* sequences have been deposited in GenBank under accession no. AY955103 through AY955149 and AY955150 through AY955195, respectively.

RESULTS

Denitrification activity and basal soil respiration. Significant ($P < 0.05$) differences in potential denitrification activity were observed between soil samples from most of the different treatments (Fig. 1). The highest denitrification rates were found in the plots amended with organic fertilizers (J and O), although it was only statistically significant for the plots fertilized with cattle manure (J) due to a lower denitrification rate in one of the field replicates fertilized with sewage sludge (O). The measurements for this plot were redone twice, but both times with the same results. The unfertilized plots without crops (A) had the lowest rates. There were no significant differences in the activity between the treatment without nitrogen fertilizer (B) and that with $(NH_4)_2SO_4$ (D) and between the two organically fertilized treatments (J and O).

Significant differences ($P < 0.05$) in basal respiration rates were observed between all treatments, except between the unfertilized field plots with crops (B) and those fertilized with $Ca(NO_3)_2$ (C) (Fig. 2). The basal soil respiration rates were higher for the plots with amendments of manure (J) and sewage sludge (O) than for the plots with mineral fertilizers (C and D) and those without fertilizer (A and B). Similar to the denitrification rates, the lowest respiration rates were found in the unfertilized plots without crops (A).

Fingerprints of soil communities. All the *nosZ*, *narG*, and RISA fingerprints displayed similar patterns among the triplicate field plots within a treatment, and this was confirmed by



the statistical analysis (Fig. 3). In addition, repetition of the *nosZ* PCR and of the DGGE resulted in similar gels (data not shown). Different pattern types by DGGE analysis of *nosZ* genes were distinguished from the six treatments, although the patterns from the unfertilized plots (A and B), the plots fertilized with cattle manure (J), and the plots with nitrogen fertilizer in the form of $Ca(NO_3)_2$ (C) exhibited similarities (Fig. 3a). In agreement, the dendrogram showed that these treatments clustered together. In contrast, the plots fertilized with nitrogen as $(NH_4)_2SO_4$ (D) and sewage sludge (O), which exhibited the most different patterns visible by eye on the gels formed two different clusters. Similar results were obtained for *narG* (Fig. 3b). For the RISA, fingerprints D and O were also clearly different from the rest but more similar to each other than seen for the *nosZ* and *narG* fingerprints (Fig. 3c).

Analysis of *nosZ* and *narG* clone libraries. Six clone libraries, one for each treatment, were constructed for *nosZ* and *narG*, respectively. From each library, 48 clones were randomly picked, which resulted in 39 to 45 clones with fragments of the correct insert size in each library. Restriction analysis of the 254 *nosZ* and 250 *narG* clones resulted in 104 and 120 different pattern types, respectively. For *nosZ*, there were 27 RFLP pattern types containing two clones or more, and for *narG*, there were 28 (data not shown). Two *nosZ* pattern types (no. 2 and 3) were found in all clone libraries except that from the soil fertilized with $(NH_4)_2SO_4$ (D), while RFLP type 4 was detected in all except that from the soil with sewage sludge amendment (O). There was one *narG* RFLP pattern type (no. 1) dominating the clone libraries from all treatments except the one with $(NH_4)_2SO_4$ fertilization (D), where it was not detected at all.

Clones of *nosZ* and *narG* from the most common RFLP pattern types were further analyzed by sequencing, primarily to confirm the identity of the amplified fragments that were used for fingerprint analysis. The 47 *nosZ* and 46 *narG* sequences showed similarities to other *nosZ* or *narG* sequences using GenBank's BLAST search. The NosZ and NarG phylograms were constructed from pure cultures and clones obtained from soil, together with the NosZ (ZRAM) and NarG (GRAM) clones from the study (Fig. 4 and 5).

Most of the NosZ clones from this study were found in clusters together with other soil clones and NosZ from the bacterial strains *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, *Brucella suis*, and *Rhodopseudomonas palustris*. All sequences from the specific RFLP pattern types (9, 14, 16, 19) in the clone library from soil fertilized with $(NH_4)_2SO_4$ (D) clustered together in the phylogram (Fig. 4). Analysis of the NarG tree revealed that sequences were scattered over the tree without any relation to the treatments (Fig. 5). The majority of them were closely related to each other and NarG from *Brucella suis*. One-third of the clones clustered with NarG from the actinomycetes group.

DISCUSSION

Soil microbial diversity is known to be immense, and Girvan et al. (10) suggested that agricultural soils are stable environments with soil type as the overriding factor in community determinations. The 50-year-old Ultuna experimental site represented an excellent model system to reveal the impact of different long-term fertilization regimes on denitrifying and total bacterial communities. Both the structure and function of these communities were analyzed in relation to the application of organic or inorganic fertilizers in a field experiment with replicate treatment plots in the field. The average potential denitrification and the basal respiration rates measured in this work were similar to those observed by Stenberg et al. (34) for different Swedish soils, some of which are located close to our sampling site. The denitrification rates measured also overlap with those obtained by Mulvaney et al. (19), who used ^{15}N to estimate denitrification activity in a soil amended with different inorganic nitrogen fertilizers.

It was verified that the PCR products used for the fingerprint analyses of denitrifying communities were related to the *nosZ* and *narG* genes by generating, screening, and sequencing 12 clone libraries, one for each gene and treatment. Phylogenetic analysis of the sequences obtained showed that most of the NosZ clones clustered with NosZ from α-*Proteobacteria* and that clones from the dominant NarG RFLP pattern type (no. 1) were related to NarG from the *Actinobacteria*. The higher diversity observed for the *narG* gene could be explained by the fact that the narG primers used in this study are able to amplify both gram-positive and gram-negative bacteria while the nosZ primers amplify only gram-negative bacteria, since the genes encoding the nitrous oxide reductase have not been characterized in gram-positive bacteria. Moreover, NarG not only catalyzes the first step of the denitrifying pathway but can also be present in other functional groups capable of dissimilatory nitrate reduction, such as bacteria reducing nitrate into ammonium. However, denitrification is considered to be the dominant dissimilatory nitrate-reducing process in soil (36, 37).

The fingerprint analyses revealed that the composition of the denitrifying and total bacterial communities in the ammonium sulfate (D) and sewage sludge (O) treatments were clearly different from the other treatments. These were also the soil plots with the lowest pH (Table 1). We hypothesize that the long-term fertilization effect was, at least partially, attributable to an indirect effect by soil acidification, which has resulted in a selection of bacteria adapted to low pH. The results reported by Parkin et al. (20) on denitrification activity

FIG. 3. Fingerprints of soil bacterial communities from the different treatments (A through D, J, and O) accompanied by the corresponding UPGMA dendrograms constructed from presence-absence matrices. The scale bar shows percent similarities. (a) DGGE of PCR-amplified *nosZ* gene fragments. The bands in the unmarked lanes to the left and right of the labeled lanes, starting from the top, are partial *nosZ* genes from *Pseudomonas stutzeri* (ATCC 14405) and *Pseudomonas denitrificans* (Pd 1222), respectively. (b) AluI RFLPs of the PCR-amplified *narG* fragments. The unmarked lanes to the left and right of the labeled lanes show the molecular size markers (VIII; Boehringer Mannheim). (c) RISA patterns of the PCR-amplified intergenic ribosomal spacer region. The unmarked lanes to the left and right of the labeled lanes show the molecular size markers (VIII; Boehringer Mannheim).

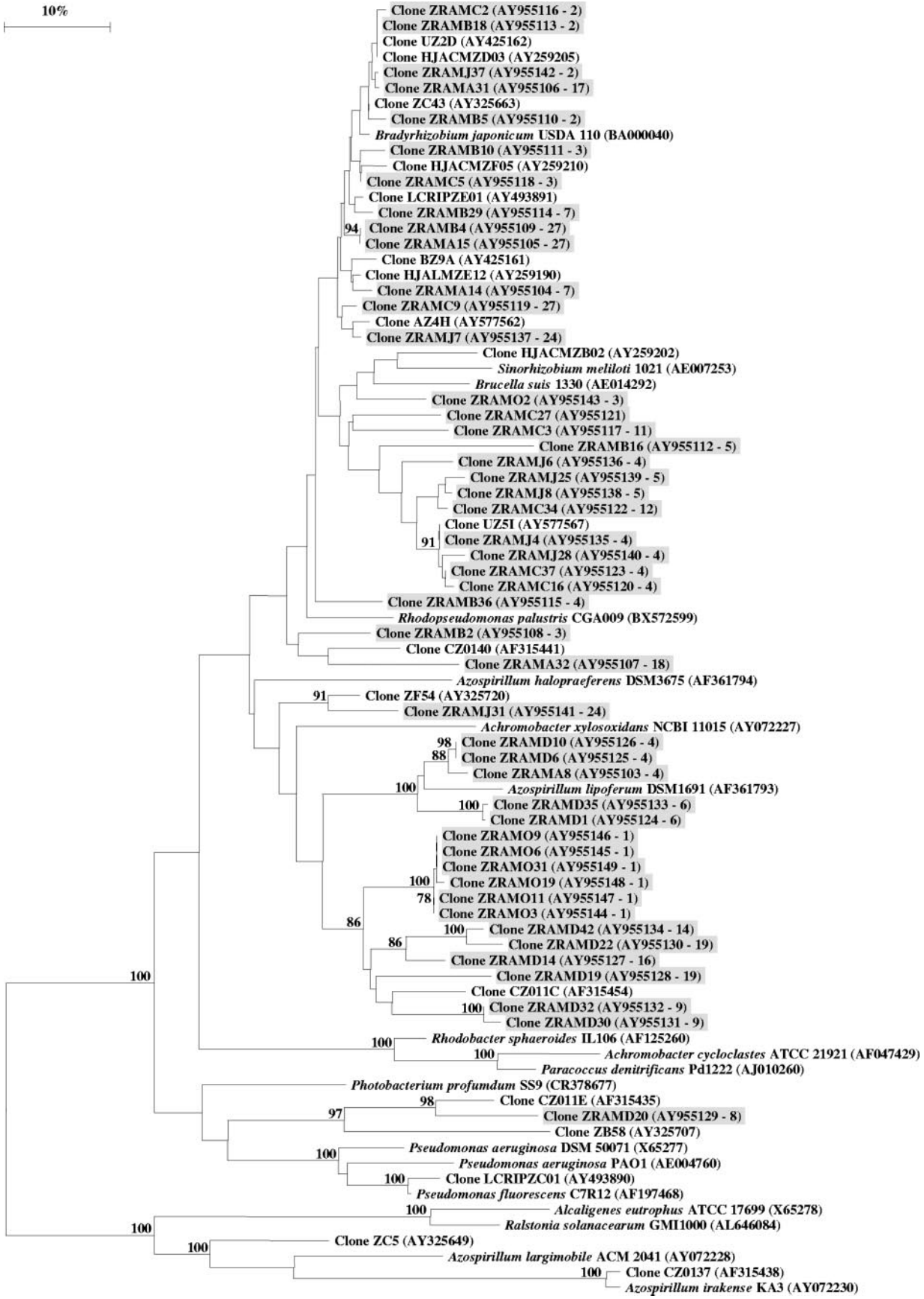


FIG. 4. Neighbor-joining phylograms of partial *nosZ* genes (400 bp) translated into amino acid sequences. Percent bootstrap values supporting more than 750 (of 1,000) iterations are shown at the nodes. Clones from this study are shaded in gray. Accession numbers for the *nosZ* genes are shown in parentheses together with the numbers of the ZRAM RFLP pattern types.

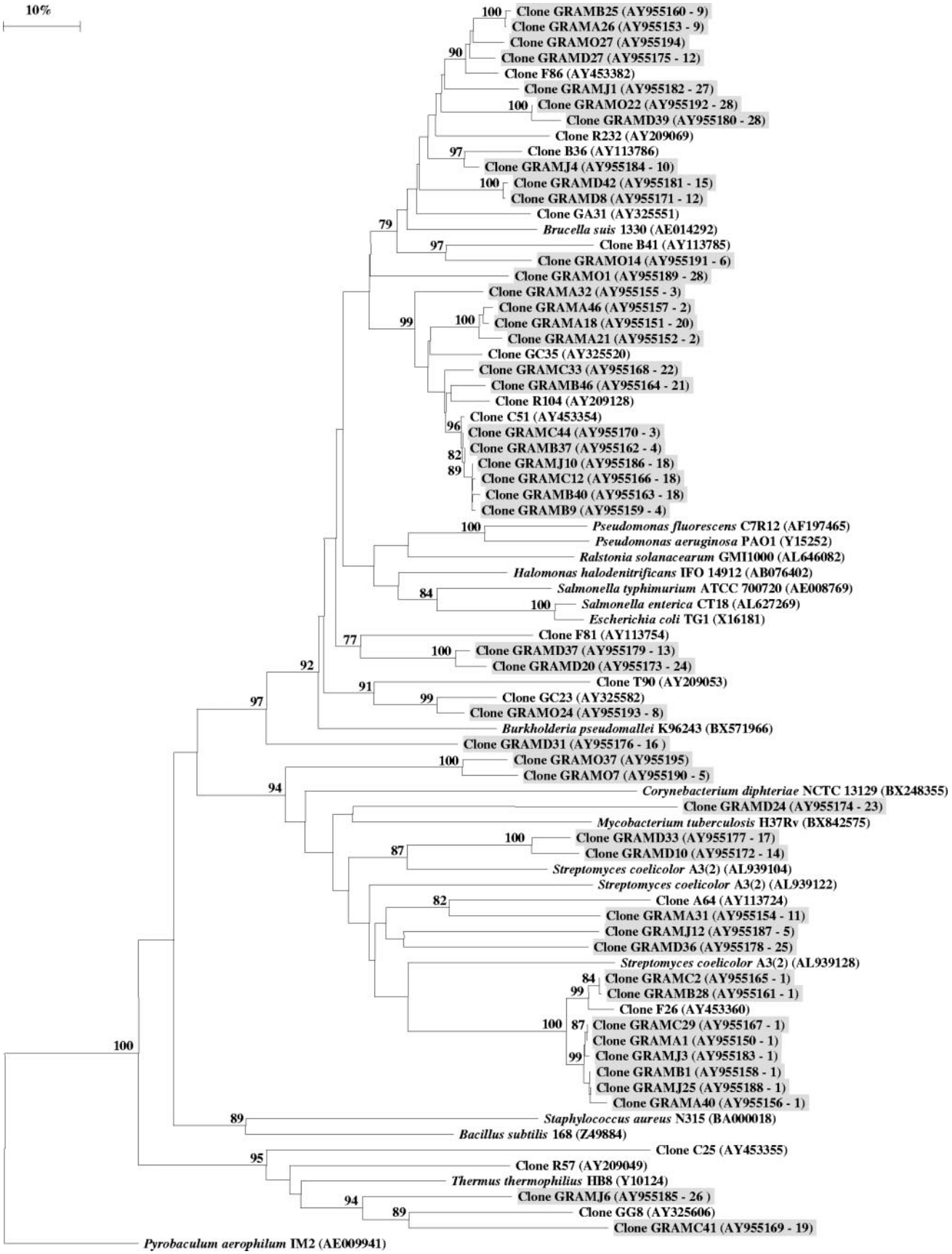


FIG. 5. Neighbor-joining phylograms of partial *narG* genes (650 bp) translated into amino acid sequences. Percent bootstrap values supporting more than 750 (of 1,000) iterations are shown at the nodes. Clones from this study are shaded in gray. Accession numbers for the *narG* genes are shown in parentheses together with the numbers of the GRAM RFLP pattern types.

after 20 years of fertilization with acid generating ammonium salts also suggest this. Recently, it has been shown that pH can affect the composition of the denitrifying communities in soil (9). However, no strong relationship between soil pH and the measurements of microbial activity was observed in our study. The basal respiration measurements did not reflect any pH effect at all, but the potential denitrification activity was lower in the ammonium-sulfate treatment (D; pH 3.97) than the plots fertilized with calcium nitrate (C; pH 6.26). This could, however, be explained by the higher carbon content in the latter. The effect of pH on potential denitrification in soil is not clear, and contradictory results have been reported (32).

Potential denitrification and soil respiration rates were significantly higher in the field plots amended with organic fertilizers (J and O), and these higher rates were correlated with the total organic carbon concentration. Similarly, Rochette et al. (29) observed a stimulation of the denitrifying enzyme activity in soil after a long-term application of organic fertilizers, and denitrification is, in general, correlated to the soil organic carbon content (5). Analysis of the composition of the bacterial communities showed that between the two organic fertilization treatments (J and O), only the sewage sludge treatment (O) differed from mineral fertilization treatments. In contrast, the denitrifying community composition differed between fields amended with mineral or cattle manure fertilizers in a Danish study (40). However, no field replicates were included and the differences reported could have been related to site-specific properties. The differences in composition of the bacterial communities between the sewage sludge (O) treatment and the other treatments could have been caused by factors other than pH and carbon, such as the heavy metal content, which may have had a long-term impact on the denitrifying and total bacterial communities. The compositions of the communities analyzed did not show a significant difference between the plots with (B) and without (A) crops, while some differences in soil respiration and denitrifying activity were observed. Nevertheless, rhizosphere effects on both the total bacterial and denitrifying community compositions have been shown by others (25, 33). The lack of an effect from the rhizosphere in our study might be explained by the sampling in between rows.

Manure (J) and sewage sludge (O) treatments had the highest potential denitrification and basal respiration rates compared to the other four treatments (Fig. 1 and 2), while the compositions of the denitrifying and the bacterial communities differed the most in the ammonium sulfate (D) and sewage sludge (O) treatments (Fig. 3a to c). These results suggest that potential activity was uncoupled to community composition, at least concerning the denitrifiers. For the bacterial community, the lack of a link could be due to the fact that both bacterial and fungal respiration were taken into account for respiration rate measurements, while the fingerprint showed only the bacterial community. In agreement with this study, Rich and Myrold (28) reported that the composition of the denitrifying communities was not related to potential rates in two different soils and one sediment. In contrast, they appeared to be linked in another study of two highly different soil ecosystems (27). The lack of agreement between the composition and potential activity of the denitrifying community is not surprising, since other factors, such as the density of denitrifiers, are likely to be of importance for potential activity (23). Even though composition of the denitrifying community may not be a major factor driving potential denitrification activity, it could be of importance for the in situ denitrification activity. Different denitrifying populations have contrasting physiological characteristics, such as growth kinetics or sensitivity of enzymes to oxygen, which are not taken into account when potential denitrifying activity measurements are being used (6, 12). In conclusion, a long-term fertilization regimen can differentially affect the activity and composition of the denitrifying community. The simultaneous assessment of denitrifying community composition and ecological functioning, including the identification of specific populations, is still in its infancy. Understanding of forces shaping denitrifying communities is critical for linking these communities to ecosystem-scale processes and sustainable ecosystem management.

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