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Original article

Effect of common bean (*Phaseolus vulgaris* L.) on the community composition of ammonia-oxidizing bacteria in soil previously cultivated with *Medicago sativa*

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ABSTRACT

The community composition of ammonia-oxidizing bacteria (AOB) was studied during four stages of plant development in soil cultivated with *Phaseolus vulgaris* in comparison to unplanted soil, using an alfisol previously harboring the legume *Medicago sativa*. Denaturing gradient gel electrophoresis (DGGE) patterns of 16S rRNA gene and clone libraries of the same gene suggested that bacteria related to *Nitrosospira* cluster 3 were dominant in both planted and unplanted soil. Bacteria related to *Nitrosomonas* cluster 8 (*Nitrosomonas communis* cluster) were found at all times in planted soil, but appeared only randomly in unplanted soil. Analysis of PCR products of the gene encoding the alpha-subunit of ammonia monooxygenase (*amoA*) by DGGE and clone libraries only detected *Nitrosospira* cluster 3-like organisms, but failed to detect sequences related to *Nitrosomonas*. The results suggest that *P. vulgaris* does not affect the dominant members of AOB communities (*Nitrosospira* cluster 3), but could have an effect on the prevalence of *Nitrosomonas* cluster 8 in this type of legume-planted alfisol.

1. Introduction

Ammonia-oxidizing bacteria (AOB) are chemolitoautotrophic bacteria responsible for the oxidation of ammonia to nitrite, which is the rate-limiting step in autotrophic nitrification and a central component of the biogeochemical nitrogen cycle [34]. Several factors such as temperature [4], pH [12], management practices [23,45] or fertilization with NH_4^+ studied in microcosms [31], experimental plots [2] or experimental fields [3,9,31], have shown to affect the structure of AOB communities in soil. Recently, it has been observed that changes involving alterations in the level of N, CO_2, temperature and precipitation at a global scale can also affect the structure of nitrifying bacterial communities [14].

In the soil, plants and AOB communities can interact in several ways. In systems where oxygen is a limiting factor (e.g. flooded rice rhizosphere) elevated abundances of AOB [7,29] and specific association of certain *Nitrosomonas* spp. [6] have been linked to oxygen leakage from the roots [6]. In other cases in which the role of oxygen is expected to be less relevant, AOB communities, heterotrophic bacteria and plants compete for ammonium that becomes available from mineralization-immobilization of nitrogen in soil or from fertilization [40]. This second type of interaction is less understood. In most of the cases studied until now, the plant and the AOB were expected to compete for NH_4^+ [6], leading to the selection of a particular community composition. Leguminous plants are a model that has never been considered before to investigate the relationship between plants and the composition of AOB communities. Legumes are a group of plants that establish a symbiosis with rhizobial bacteria to obtain mineral N via N_2 fixation in specialized structures called root nodules [32]. Since legumes can obtain their mineral N via this symbiosis, they are expected not to compete for NH_4^+ with AOB in soil, and processes such as leaching of NH_4^+ or rhizodeposition [25], could influence the composition and activity of nitrifying bacterial communities.

The aim of this study was to establish whether the composition of ammonia-oxidizing communities in soil is affected by *Phaseolus vulgaris* (common bean). A soil of the alfisol order, which was used previously for the culture of the legume *Medicago sativa*, was selected for the experiments. This soil has shown to bear indigenous rhizobial populations able to induce the formation of nodules in *Phaseolus vulgaris* that were favored by the presence of their symbiotic host [16]. In comparison with other soil orders also found in the central area of Chile (e.g. Mollisols and Inceptisols), the

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alfisols selected in the present study are relatively poor in organic matter and the presence of plants might be an important element to modify their native microbial communities. To study the effect of common bean the community composition of AOB in legume-planted was compared to unplanted soil at different times during plant development. PCR amplification, DGGE and clone libraries of the 16S rRNA genes and the gene encoding the alpha-subunit of the ammonia monooxygenase (*amoA*) were used to characterize the community composition of AOB in soil.

2. Materials and methods

2.1. Experimental design and sampling

The soil used for culturing *Phaseolus vulgaris* was collected from the organic layer (0–20 cm) of a field that was previously used to cultivate the legume *Medicago sativa*. The soil belonged to the Alfisol order, which is found covering some areas of the periphery of the central region of Chile near Santiago [10]. Soil was sieved through a 10 mm mesh prior to use. Twenty individual pots were prepared containing 5 kg of sieved soil. The 20 pots were divided into two sets of 10 pots. In the first set, designated P for planted soil, seedlings of *P. vulgaris* were planted (one plant per pot). The second set, designated S, was kept unplanted. The pots were incubated in the laboratory at 28 °C in summer day-length light regime (14 h light). Inoculation with rhizobial bacteria was not conducted in order to maintain the indigenous soil bacterial community composition. Considering the previous use of the soil to grow legume plants, it was expected to bear natural symbiotic populations of rhizobia capable to establish symbiosis with common bean. Every second day each pot was watered with 150 mL of water, maintaining an average soil humidity of 10%.

It is widely accepted that the addition of combined N such as NH_4^+, NO_3^-, and urea reduces the potential of the legumes to fix atmospheric N [33]. Thus different nitrogen treatments, consisting in a nutrient solution containing or not nitrate, were also tested, in order to evaluate whether a reduction in N_2 fixation potential in the legume plant has an effect on the AOB communities in soil. For this, once a week one subset of four pots from the sets P and S was watered with 10 ml of Hoagland nutrient solution containing nitrate in the form of 0.4 mM KNO_3, as well as 0.4 mM $MgSO_4$, 0.4 mM KH_2PO_4, 0.4 mM $Ca(NO_3)_2 4H_2O$ (treatment N). Another subset of four pots from the sets P and S was watered with 10 ml of the same solution but supplying 0.4 mM $CaCl_2$ and 0.4 mM KCl instead of nitrate (treatment H). Two pots of each set were maintained without addition of nutrient solution, adding water instead (treatment W).

About 2 cm^3 of soil were collected at 3 (T0), 20 (T1), 40 (T2) and 60 (T3) days after seeding. The soil was obtained from the area immediately surrounding the main root without removing the plant to maintain soil structure and was kept at −20 °C until DNA extraction.

2.2. Soil analysis

Physical and chemical characterization of the soil was carried out with the following methods of soil analysis at the Instituto Nacional de Investigaciones Agropecuarias (INIA, Chile): available nitrogen after KCl and Devarda extraction and distillation; available phosphorus by colorimetric assay after treatment with 0.5 M $NaHCO_3$; available potassium by atomic spectroscopy after treatment with 1.0 M NH_4AcO; organic matter by wet combustion and colorimetric assay; pH by potentiometric assay; the conductivity was measured at 25 °C after extraction in saturation with distilled water. The statistical analysis of the results was carried out using the Multivariate Statistical Software Package v3.1, which also calculates variance analysis (ANOVA).

2.3. DNA extraction and PCR

Total DNA from soil was extracted using the UltraClean Soil DNA kit (MoBio), following the manufacturer's guidelines. Concentration and quality of the DNA was checked by electrophoresis in 0.8% agarose gels stained with ethidium bromide. At the different time points, DNA from each replicate of the treatments was extracted separately. To reduce experimental bias due to sampling and DNA extraction, the DNA extracts from the replicates at each time point were pooled prior to PCR amplification. The purified DNA extracts were diluted with HPLC water to 1–2.5 ng mL^{-1} for PCR. For amplifying the 16S rRNA gene from AOB, a nested PCR approach was selected [43]. Initially, 16S rRNA gene was amplified using the bacterial primers Eub9_27 and Eub1542 [8]. PCR was set up in a total volume of 50 mL containing 1–2.5 ng DNA as template, 5 pmol of each primer, 1X PCR buffer (10 mM Tris–HCl; 15 mM $MgCl_2$; 50 mM KCl, pH 8.3), 10 nmol of each dNTP and 1 U of *Taq* polymerase (Roche). The amplification consisted of: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 45 s and extension at 72 °C for 90 s. These PCR products were diluted 100 times and used as templates for specific amplification of 16S rRNA gene from beta-AOB in a nested PCR with the primers NitA/NitB [41]. PCR was carried out with the same temperature program as mentioned above for Eub primers, but with an annealing temperature of 57 °C. For DGGE, these amplicons were diluted 100 times and used in a second nested PCR with the primers P3 (GC-clamped) and P2, using a touchdown temperature program [28]. All PCR amplifications were conducted in triplicates to reduce experimental biases.

The *amoA* gene was amplified with primers amoA34f [27] and amoA2r [37]. Magnesium concentration in the PCR master mix (see above) was increased to 3.8 mM $MgCl_2$. The same temperature program as for amplification of 16S rRNA gene was used with the annealing temperature set to 57 °C and a final extension at 72 °C for 7 min. To prepare the products for DGGE, these amplicons were used in a second nested PCR with the forward primer amoA121fgc and amoA360rC [17]. These primers were designed in our laboratory from an alignment of all available *amoA* sequences to match all AOB from the betaproteobacteria. For DGGE, a 41 bp GC-clamp [28] was added to the forward primer. A touchdown temperature program was run as described above, but with the annealing temperature decreasing from 55 to 45 °C.

2.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed according to the manufacturer's guidelines with the DCode System (BioRad) in 7.5% polyacrylamide gels with a gradient of 30–60% of denaturants (100% denaturants contained 420 g L^{-1} urea and 400 mL L^{-1} deionized formamide in 0.5X TAE) during 6 h at 200 V and 60 °C. Gels were silver stained [38] and scanned using a HP scanjet 5470c. The gels were run in duplicates to ensure reproducibility of the patterns obtained.

2.5. Cloning and sequencing

16S rRNA gene PCR products amplified with the primers NitA/NitB from the samples PWT3 (planted, sampled after 60 days) and SNT3 (unplanted, sampled after 60 days), and *amoA* products amplified with the primers amoA1F/amoA2R [37] from the sample PWT1 (planted, sampled after 20 days), were selected for cloning and sequencing. These samples were selected because in the DGGE analysis they displayed a band pattern that included most of the bands observed in the different samples. PCR was carried out in a total volume of 50 mL with the proof-reading *Pfu* DNA polymerase (Promega) according to the manufacturer's guidelines. PCR was carried out using an annealing temperature of 60 °C. For cloning the Zero Blunt PCR cloning kit (Invitrogen) was used according to the manufacturer's guidelines. From each sample 48 clones were selected and checked for inserts of the expected size by PCR with plasmid-specific primers M13f/M13r and agarose electrophoresis. For screening of these clones, one-shot sequencing using M13f primer was performed using the BigDye terminator v3.1 cycle sequencing kit, and analyzed with an ABI 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's recommendations. For complete sequencing of the unique clones (see next section), both strands were sequenced with overlap using M13f and M13r and additional internal primers [24].

2.6. *DNA-sequence analyses*

The nucleotide sequences were compared with those in the GenBank using BLASTN [1] on the NCBI's homepage (http://www.ncbi.nlm.nih.gov/blast/Blast). According to the similarities in the BLAST hits and alignments from all the sequences obtained, the sequences were grouped into 17 groups (11 groups of sequences from clones of 16S rRNA gene and five groups of *amoA*). A single clone from these 17 groups was selected for sequencing both strands and the sequences were deposited in GenBank under the accession numbers AY944201–AY944217. Phylogenetic analyses were carried out with the ARB program (http://www.arb-home.de) as described elsewhere [36]. Briefly, 16S rRNA gene and *amoA* sequences were integrated and aligned into the respective database available for ARB, correcting the alignments by visual inspection. For *amoA*, the nucleotide sequences were translated into protein and re-aligned with ARB to correct the nucleotide alignment. The phylogenetic tree was constructed using the PHYLIP subroutine in ARB by the neighbor-joining algorithm using a distance matrix calculated with Kimura (16S rRNA gene) or the Jones–Taylor–Thornton (JTT) (*amoA*) as substitution model. 1000 bootstraps were calculated to test the robustness of the clades obtained. Equivalent topologies were found using also the treeing methods neighbor-joining, and maximum-likelihood from the respective tools in the ARB package (data not shown).

3. Results

3.1. *Effect of the plants on soil N content and nitrogen fixation activity*

The addition of nitrate did not have any effect on the soil parameters measured nor on the N_2 fixation potential in the plants, therefore the analysis was focused on the comparison between planted and unplanted soil. Concentrations of available phosphorous and potassium, as well as pH and conductivity, remained about the same in planted and unplanted soil during the experiment (Table 1). Percentage of organic matter increased in both planted and unplanted soil, whereas available N (NO_3^- plus NH_4^+) in planted soil was significantly lower compared to unplanted soil at the end of the experiment. Decreased available N in planted soil was not due to absence of nitrogen fixation in the plants, since all the plants presented actively fixing root nodules (acetylene reduction rates in nodules of 1570 ± 50 nmol C_2H_4 g^{-1} nodule dry weight day^{-1}).

3.2. *Composition of AOB communities analyzed by DGGE patterns and DNA-sequence analysis of 16S rRNA genes*

The 16S rRNA gene DGGE patterns obtained from PCR products amplified with primers targeting AOB within the betaproteobacteria are shown in Fig. 1. Pure cultures of AOB used as standards formed two migration groups in the DGGE: *Nitrosomonas europaea*, *Nitrosomonas eutropha* and *Nitrosomonas marina* with bands between 35 and 40% of denaturants, and *Nitrosospira briensis* with a band between 50 and 53%. Soil samples showed bands at positions similar to these groups, as well as additional bands not related to any of the standards (Fig. 1).

Table 1
Soil properties at the beginning (T0) and at the end of the experiment (T3 = 60 days).

Soil treatment	Time	pH	Organic matter (%)	Conductivity (dS/m)	Available nitrogen (mg/kg)	Available phosphorous (mg/kg)	Available potassium (mg/kg)
Initial	T0	8.0	3.9	1.3	10.0	37.0	199.0
Planted	T3	8.0	4.5	2.5	7.0	47.2	168.2
Unplanted	T3	7.9	4.7	2.4	26.5	52.5	182.8

All values at T0 are the average of three independent determinations. Values at T3 are the average for the individual replicates of the combined watering regimes of planted and unplanted soil.

Some bands in the DGGE migrated to the same position as those from sequenced clones. These bands, which appeared reproducibly in replicated gels, were assigned to seven migration classes (indicated by letters A–G in Fig. 1). The position in the gradient of migration classes corresponding to those clones matching sequences of recognized AOB (Table 2), like *Nitrosomonas communis* (clones SNT3-50-16S and SNT3-55-16S), *Nitrosospira multiformis* (SNT3-95-16S), and *Nitrosospira* NpAV (SNT3-61-16S), were coincident with the position of DGGE bands from reference strains of these genera.

Some of these migration groups differed among the soil samples. The migration group A (related to *Nitrosomonas communis*) was observed in all the samples from planted soil, but it was not detected in some samples from unplanted soil (SH-T0, SH-T2, SN-T0, SN-T1, and SW-T2). In contrast, the migration groups E and F (related to *Nitrosospira* spp.) appeared in all the samples, independent from the plant. The other migration groups, corresponding to non-nitrifying bacteria, were not obviously correlated with the plant.

In the phylogenetic analysis (Fig. 2), 12 of the clones matching sequences from nitrifying bacteria by BLAST clustered together with recognized AOB isolated from soil. The clone SNT3-95-16S (and another identical clone) clustered into the 16S rRNA gene cluster 3 of the *Nitrosospira* lineage [35,36], whereas the clone SNT3-55-16S (and nine identical clones), clustered with the 16S rRNA gene cluster 8 of the *Nitrosomonas* lineage, which corresponds to *Nitrosomonas communis*/*Nitrosomonas nitrosa* [35,36]. Although the clones SNT3-50-16S, SNT3-57-16S, SNT3-74-16S and SNT3-94-16S (which represented another 21 clones) matched sequences from nitrifying bacteria in BLAST, in the phylogenetic analysis, they were related to uncultured beta proteobacteria in an additional cluster more distantly related to AOB (Fig. 2).

The remaining clones were grouped with sequences from different betaproteobacterial genera such *Leptothrix* spp., *Comamonas* spp. and *Aquabacterium* spp. that are not autotrophic nitrifiers but are phylogenetically related to AOB. For example, the closest relative by BLAST of the dominant clone PWT3-1-16S (and 26 identical clones) was related to the MTBE-degrading bacterium PM1 isolated from a compost biofilter.

3.3. *Composition of AOB communities analyzed by the functional gene amoA*

PCR products of *amoA* were obtained only from some of the soil samples. DGGE patterns varied very little between the samples (Fig. 3). All the 29 sequences of *amoA* from the clone library were related to different uncultured AOB retrieved from soil samples and clustered with the *Nitrosospira* lineage (Fig. 4). Within this lineage,

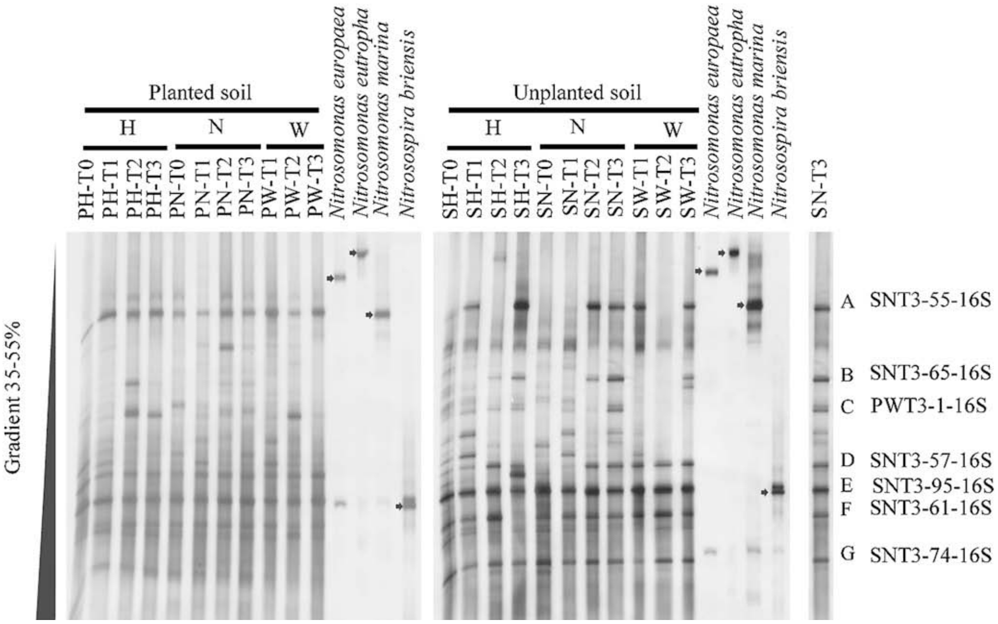

Fig. 1. Composition of AOB communities in the rhizosphere of *Phaseolus vulgaris* analyzed by DGGE of 16S rRNA gene PCR products amplified with the NitA/NitB primer set. P = soil planted with *Phaseolus vulgaris*; S = unplanted soil. Three experiments from each treatment are indicated by the letters H, N and W. Samples were taken at 3 (T0), 16 (T1), 40 (T2), and 60 (T3) days after seeding. DGGE groups established in the clone libraries are indicated with letters on the right using the sample SN-T3 as reference. Arrows show the DGGE bands from the reference strains *Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosomonas marina*, and *Nitrosospira briensis*.

the *amoA* clones were divided into two subclusters. Clones PWT1-70-amoA and PWT1-82-amoA were grouped with several uncultured AOB clones from the previously described AmoA cluster 3a [2]. The other clones were grouped into an additional cluster together with several environmental clones and *Nitrosospira* sp. Nsp17, which was designated as the new *amoA* subcluster 3c. This new subcluster was supported by a high bootstrap value.

4. Discussion

Chemical characterization of the soil after harvesting the plants (60 days) revealed a decrease in available nitrogen (NH_4^+ plus NO_3^-) in the soil planted with *P. vulgaris*. This decrease occurred not only in comparison with unplanted soil but also in comparison with the initial conditions (Time T0, Table 1). Since all plants studied here possessed several active nodules and their growth rates did not indicate nitrogen limitation (data not shown), it is presumable that the decrease in nitrogen content was not due to absence of nitrogen fixation in nodules. Although a negative balance of nitrogen in soil seems surprising, it has been observed that the net nitrogen balance in soil planted with legumes is often low (and in some instances negative), once the quantities of nitrogen involved in plant growth, nitrogen fixation, and seed formation are taken into account [33]. The soil analyzed in the present study had been used for the culture of another legume, *Medicago sativa*. For this species a high potential for net mineralization of nitrogen in soil has been observed [11], probably explaining the increase in total nitrogen observed in unplanted soil. By comparison, the results suggest immobilization of nitrogen in soil in the presence *P. vulgaris*.

The analysis of the microbial communities involved in nitrogen fixation (including the rhizobial bacteria symbionts of *P. vulgaris*) in this same alfisol [16], indicates that *P. vulgaris* stimulates specifically its native symbiotic partner, compared to unplanted soil. The present study provides evidence that *P. vulgaris* also affects the prevalence of some members of the AOB communities in soil. The analysis of DGGE band patterns of 16S rRNA gene revealed that AOB related to *Nitrosomonas* cluster 8, also called *Nitrosomonas communis* cluster 8, were present in planted soil during the whole experiment but more randomly in unplanted soil. To this date, *Nitrosomonas* cluster 8 includes species isolated from soil inhabiting environments with relatively high concentration of NH_4^+ [2], and therefore the prevalence of this cluster in soil samples planted with *P. vulgaris* seems surprising considering the decrease in total available nitrogen. However, sequences related to *Nitrosomonas* cluster 8 have rarely been detected in environmental clone libraries [18,20] and consequently, extrapolations of habitat preference from the cultured strains to environmental members of this cluster should be considered with caution. The physiology of these environmental members of *Nitrosomonas* cluster 8 is a topic that should be studied in more detail in the future.

Table 2
First match in BLAST corresponding to closest relative for the 16S rRNA gene clones.

DGGE group	Clone	No of clones	Nearest described relative	Accession no.	Identity (%)
A	SNT3-50-16S	9	*Nitrosomonas communis*	AJ298732	92
A	SNT3-55-16S	10	*Nitrosomonas communis*	AJ298732	98
B	SNT3-65-16S	1	*Burkholderia cepacia*	AF244133	94
C	PWT3-1-16S	27	*Caenibacterium thermophilum*	AJ512945	95
E	SNT3-95-16S	2	*Nitrosospira multiformis*	AB070984	98
D	SNT3-57-16S	3	*Leptothrix* sp. MBIC3364	AB015048	94
F	SNT3-61-16S	1	*Nitrosospira* sp. NpAV	Y10127	99
G	SNT3-74-16S	10	*Bulkholderia multivorans*	AY48372	91
G	SNT3-94-16S	3	*Leptothrix* sp. MBIC3364	AB015048	93
G	PWT3-12-16S	3	*Burkholderia cepacia*	AF244133	95

Position of the respective clones in DGGE profiles from which they were isolated is indicated in Fig. 1.

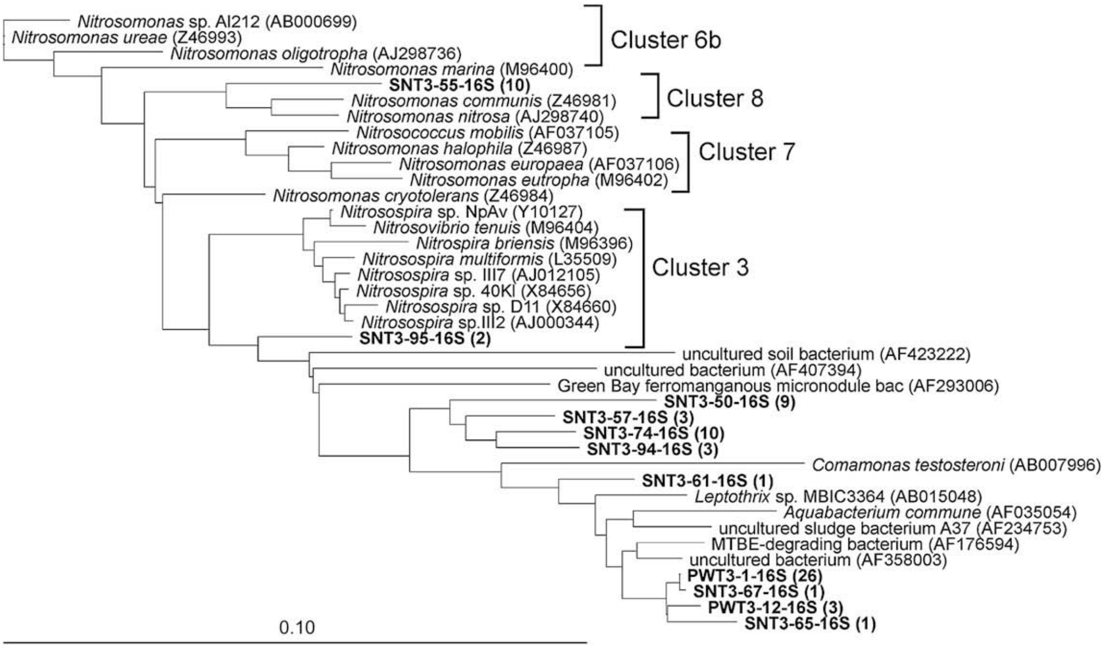

Fig. 2. Phylogenetic tree showing the relationships of 16S rRNA gene retrieved as cloned PCR products prepared with primers NitA and NitB from soil. Tree constructed using the PHYLIP subroutine in ARB by the neighbor-joining algorithm using a distance matrix calculated with Kimura index. Scale bar indicates 0.1 substitutions/site. The number of identical clones is indicated in brackets.

The low representation of sequences related to *Nitrosomonas* cluster 8 is surprising considering that they match with high similarity the sequence of most primers commonly used to amplify the 16S rRNA gene from AOB in environmental samples [18]. An explanation for this is a low abundance of this type of AOB in the environment. Nested PCR, especially in the case of the 16S rRNA gene, has been suggested in the past as an alternative to overcome the challenge of detecting poorly-represented AOB [13,18,19,30,42,43]. In the present study, the detection of *Nitrosomonas* cluster 8-like AOB using a pre-amplification step with bacterial primers prior to PCR with the specific 16S rRNA gene AOB primers NitA/NitB is probably a good example. However, the successive PCR amplifications render the quantitative evaluation of the results unreliable, limiting the analysis to a qualitative observation.

Based on comparison of sequences in public databases, it has been suggested that PCR primers recommended for specific amplification of 16S rRNA gene of AOB could also amplify non-AOB [35,39]. A slight lack of specificity may shift the spectrum of sequences amplified by PCR towards phylogenetically related but physiologically and ecologically different organisms [5,18]. In contrast, molecular approaches targeting *amoA* analyze more specifically the composition of AOB because this gene has been observed only in microorganisms oxidizing ammonia [26,37]. The results from 16S rRNA and *amoA* coincided by indicating the presence and possible dominance of *Nitrosospira* cluster 3 in the soil studied here. This cluster contains highly versatile and adaptable species [4,44] and it has been detected as the dominant ammonia-oxidizer group in a number of neutral-pH and arable soils [9,14,21,22].

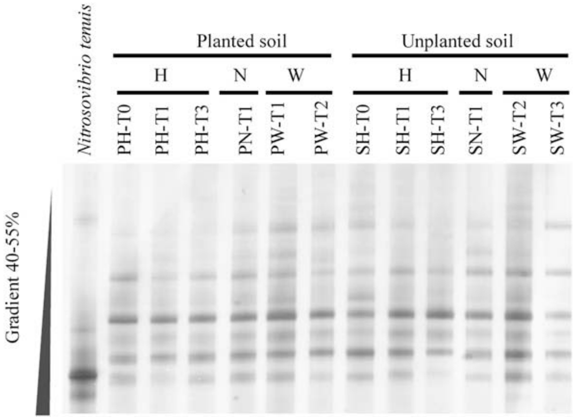

Fig. 3. Composition of AOB communities in soil analyzed by DGGE of the *amoA* PCR products amplified with the primers amoA121fgc and amoA306rC. Coding of the samples as in Fig. 1.

The results with the 16S rRNA and *amoA* genes did not show changes in the presence of *Nitrosospira* cluster 3-like species over time and between the different samples. Similarly, short-term experiments with agricultural soil incubated under different conditions of temperature and fertilization did not show considerable changes in DGGE band patterns for *amoA* [4]. As in our study, Avrahami et al. [4] detected *Nitrosospira*-like, but failed to detected *Nitrosomonas*-like *amoA* sequences.

A study of rice rhizosphere [6] suggests that the underestimation of *Nitrosomonas*-like *amoA* can be attributed to lower efficiency of DNA extraction from *Nitrosomonas* spp, and/or possible biases during PCR amplification. In our study, amplification due to biases in the primers is a less likely explanation since a comparison with sequences in GenBank revealed that the primers used match the *amoA* gene of *Nitrosomonas* better than that of *Nitrosospira*-like [17]. Therefore, differences in the sensitivity of the *amoA* amplification versus the nested PCR16S rRNA gene approach appear to be a more plausible explanation.

Using *amoA* as a marker allowed a better resolution within the *Nitrosospira* cluster than it was possible with the 16S rRNA gene. While all the 16S rRNA gene sequences were grouped into a single cluster, *amoA* sequences allowed to differentiate three subclusters: the *amoA Nitrosospira* clusters 3a and 3b [4] and the new *Nitrosospira* cluster 3c, which contains the majority of our clonal *amoA* sequences (Fig. 4). Most of the *amoA* sequences in this cluster

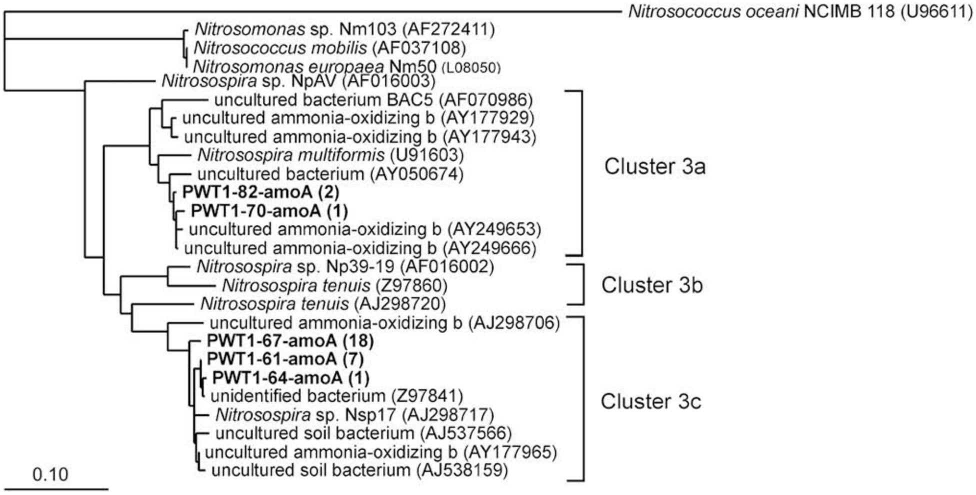

Fig. 4. Phylogenetic tree showing the relationships of clonal nucleotide sequences of *amoA* from soil, AOB cultures and the most similar BLAST hits in GenBank. Tree constructed using the PHYLIP subroutine in ARB by the neighbour-joining algorithm using a distance matrix calculated with the Jones–Taylor–Thornton (JTT) as substitution model. Scale bar indicates 0.1 substitutions/site. The number of identical clones is indicated in brackets.

correspond to uncultured soil bacteria and the cultured strain *Nitrosospira* sp. Nsp17 [36]. This strain was isolated from soil in Iceland and it was recently observed associated with nodal roots in rice [15], suggesting a possible preference for rhizospheric habitats.

In summary, this study shows that the presence of the legume *P. vulgaris* affects the prevalence not only of its rhizobial symbiotic partner [16], but also of some members of other microbial groups involved in the nitrogen cycle, particularly the AOB communities in soil. Since the results from this study were obtained from one particular soil (Alfisol), it would be important to carry out similar studies in other soils and with other legume species in order to better understand how legumes in general affect the different microbial groups involved in nitrogen cycling in soil.

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References

[1] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miler, D.J. Lipman, Gapped Blast and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[2] S. Avrahami, R. Conrad, Patterns of community change among ammonia oxidizers in meadow soils upon long-term incubation at different temperatures, Appl. Environ. Microbiol. 69 (2003) 6152–6164.

[3] S. Avrahami, R. Conrad, G. Braker, Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers, Appl. Environ. Microbiol. 68 (2002) 5685–5692.

[4] S. Avrahami, W. Liesack, R. Conrad, Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers, Environ. Microbiol. 5 (2003) 691–705.

[5] H. Bothe, G. Jost, M. Schloter, B.B. Ward, K.-P. Witzel, Molecular analysis of ammonia oxidation and denitrification in natural environments, FEMS Microbiol. Rev. 24 (2000) 673–690.

[6] A.M. Briones, S. Okabe, Y. Umemiya, N.B. Ramsing, W. Reichardt, H. Okuyama, Ammonia-oxidizing bacteria on root biofilms and their possible contribution to N use efficiency of different rice cultivars, Plant Soil 250 (2003) 335–348.

[7] A.M. Briones, S. Okabe, Y. Umemiya, N.B. Ramsing, W. Reichardt, H. Okuyama, Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice, Appl. Environ. Microbiol. 68 (2002) 3067–3075.

[8] J. Brosius, M.L. Palmer, P.J. Kennedy, H.F. Noller, Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli, Proc. Natl. Acad. Sci. U.S.A. 75 (1978) 4801–4805.

[9] M.A. Bruns, J.R. Stephen, G.A. Kowalchuk, J.I. Prosser, E.A. Paul, Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils, Appl. Environ. Microbiol. 65 (1999) 2994–3000.

[10] M. Casanova, W. Vera, W. Luzio, O. Salazar, Manual de edafologia, 74 pp. Universidad de Chile, Facultad de Ciencias Agronomicas, Departamento de Ingenieria de Suelos, Santiago de Chile, 2004.

[11] R.H. Fox, R.J.K. Myers, I. Vallis, The nitrogen mineralization rate of legume residues in soil as influenced by their polyphenol, lignin, and nitrogen contents, Plant Soil 129 (1990) 251–259.

[12] R.C. Hastings, C. Butler, I. Singleton, J.R. Saunders, A.J. McCarthy, Analysis of ammonia-oxidizing bacteria populations in acid forest soil during conditions of moisture limitation, Lett. Appl. Microbiol. 30 (2000) 14–18.

[13] R.C. Hastings, J.R. Saunders, G.H. Hall, R.W. Pickup, A.J. McCarthy, Application of molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake, Appl. Environ. Microbiol. 64 (1998) 3674–3682.

[14] H.P. Horz, A. Barbrook, C.B. Field, B.J. Bohannan, Ammonia-oxidizing bacteria respond to multifactorial global change, PNAS 101 (2004) 15136–15141.

[15] M. Ikenaga, S. Asakawa, Y. Muraoka, M. Kimura, Bacterial communities associated with nodal roots of rice plants along with the growth stages: estimation by PCR-DGGE and sequence analyses, Soil Sci. Plant Nutr. 49 (2003) 591–602.

[16] P. Junier, T. Junier, K-P. Witzel, M. Caru, Composition of diazotrophic bacterial assemblages in bean-planted soil compared to unplanted soil. J. Soil Biol, in press [Available online 28 November 2008].

[17] P. Junier, O.-S. Kim, V. Molina, P. Limburg, T. Junier, J.F. Imhoff, K.-P. Witzel, Comparative in silico analysis of PCR primers suited for diagnostics and cloning of ammonia monooxygenase genes from ammonia-oxidizing bacteria, FEMS Microbiol. Ecol. 64 (2008) 141–152.

[18] P. Junier, O.S. Kim, O. Hadas, J.F. Imhoff, K.P. Witzel, Evaluation of PCR primer selectivity and phylogenetic specificity by using amplification of 16S rRNA genes from betaproteobacterial ammonia-oxidizing bacteria in environmental samples, Appl. Environ. Microbiol. 74 (2008) 5231–5236.

[19] O.-S. Kim, P. Junier, J.F. Imhoff, K.-P. Witzel, Comparative analysis of ammonia-oxidizing bacterial communities in two lakes in North Germany and the Baltic Sea, Arch. Hydrobiol. 167 (2006) 335–350.

[20] H.-P. Koops, A. Pommerening-Roeser, Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species, FEMS Microbiol. Ecol. 37 (2001) 1–9.

[21] G.A. Kowalchuk, J.R. Stephen, Ammonia-oxidizing bacteria: a model for molecular microbial ecology, Annu. Rev. Microbiol. 55 (2001) 485–529.

[22] G.A. Kowalchuk, J.R. Stephen, W. De Boer, J.I. Prosser, T.M. Embley, J.W. Woldendorp, Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments, Appl. Environ. Microbiol. 63 (1997) 1489–1497.

[23] G.A. Kowalchuk, A.W. Stienstra, G.H. Heilig, J.R. Stephen, J.W. Woldendorp, Molecular analysis of ammonia-oxidising bacteria in soil of successional

grasslands of the Drentsche A (The Netherlands), FEMS Microbiol. Ecol. 31 (2000) 207–215.

[24] D.J. Lane, 16S/23S rRNA sequencing, in: E. Stackebrandt, M. Goodfellow (Eds.), Nucleic Acids Techniques in Bacteria Systematics, J. Wiley, Chichester, 1991 Vol.

[25] J. Mayer, F. Buegger, E.S. Jensen, M. Schloter, J. Hess, Estimating N rhizodeposition of grain legumes using a 15N in situ stem labelling method, Soil Biol. Biochem. 35 (2003) 21–28.

[26] T.A. Mendum, R.E. Sockett, P.R. Hirsch, Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the beta subdivision of the class proteobacteria in arable soils to nitrogen fertilizer, Appl. Environ. Microbiol. 65 (1999) 4155–4162.

[27] V. Molina, O. Ulloa, L. Farías, H. Urrutia, S. Ramírez, P. Junier, K.-P. Witzel, Ammonia-oxidizing β-proteobacteria from the oxygen minimum zone off northern Chile, Appl. Environ. Microbiol. 73 (2007) 3547–3555.

[28] G. Muyzer, E.C. de Waal, A.G. Uitterlinden, Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microbiol. 59 (1993) 695–700.

[29] M.H. Nicolaisen, N. Risgaard-Petersen, N.P. Revsbech, W. Reichardt, N.B. Ramsing, Nitrification-denitrification dynamics and community structure of ammonia oxidizing bacteria in a high yield irrigated Philippine rice field, FEMS Microbiol. Ecol. 49 (2004) 359–369.

[30] G.D. O'Mullan, B.B. Ward, Relationship of temporal and spatial variabilities of ammonia-oxidizing bacteria to nitrification rates in Monterey Bay, California, Appl. Environ. Microbiol. 71 (2005) 697–705.

[31] Y. Okano, K.R. Hristova, C.M. Leutenegger, L.E. Jackson, R.F. Denison, B. Gebreyesus, D. Lebauer, K.M. Scow, Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil, Appl. Environ. Microbiol. 70 (2004) 1008–1016.

[32] E.J. Patriarca, R. Tate, M. Iaccarino, Key role of bacterial NH_4^+ metabolism in *Rhizobium*-plant symbiosis, Microbiol. Mol. Biol. Rev. 66 (2002) 203–222.

[33] M.B. Peoples, D.F. Herridge, Nitrogen fixation by legumes in tropical and subtropical agriculture, Adv. Agron. 44 (1990) 155–223.

[34] J.I. Prosser, Autotrophic nitrification in bacteria, Adv. Microb. Physiol. 30 (1989) 125–181.

[35] U. Purkhold, A. Pommerening-Roser, S. Juretschko, M.C. Schmid, H.P. Koops, M. Wagner, Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity surveys, Appl. Environ. Microbiol. 66 (2000) 5368–5382.

[36] U. Purkhold, M. Wagner, G. Timmermann, A. Pommerening-Roser, H.P. Koops, 16S rRNA and *amoA*-based phylogeny of 12 novel betaproteobacterial ammonia-oxidizing isolates: extension of the dataset and proposal of a new lineage within the nitrosomonads, Int. J. Syst. Evol. Microbiol. 53 (2003) 1485–1494.

[37] J.H. Rotthauwe, K.-P. Witzel, W. Liesack, The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing population, Appl. Environ. Microbiol. 63 (1997) 4704–4712.

[38] C.J. Sanguinetti, E.D. Neto, A.J.G. Simpson, Rapid silver staining and recovery of PCR products separated by polyacrylamide gels, Biotechniques 17 (1994) 915–918.

[39] J.B. Utaker, I.F. Nes, A qualitative evaluation of the published oligonucleotides specific for the 16S rRNA gene sequences of the ammonia-oxidizing bacteria, Syst. Appl. Microbiol. 21 (1998) 72–88.

[40] F.J.M. Verhagen, P.E.J. Hageman, J.W. Woldendorp, H.J. Laanbroek, Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization, Soil Biol. Biochem. 26 (1994) 89–96.

[41] M.A. Voytek, B.B. Ward, Detection of ammonium-oxidizing bacteria of the beta-subclass of the class Proteobacteria in aquatic samples with the PCR, Appl. Environ. Microbiol. 61 (1995) 1444–1450.

[42] B.B. Ward, D.P. Martino, M.C. Diaz, S.B. Joye, Analysis of ammonia-oxidizing bacteria from hypersaline Mono Lake, California, on the basis of 16S rRNA sequences, Appl. Environ. Microbiol. 66 (2000) 2873–2881.

[43] B.B. Ward, M.A. Voytek, K.-P. Witzel, Phylogenetic diversity of natural populations of ammonia oxidizers investigated by specific PCR amplification, Microb. Ecol. 33 (1997) 87–96.

[44] G. Webster, T.M. Embley, T.E. Freitag, Z. Smith, J.I. Prosser, Links between ammonia oxidizer species composition, functional diversity and nitrification kinetics in grassland soils, Environ. Microbiol. 7 (2005) 676–684.

[45] G. Webster, T.M. Embley, J.I. Prosser, Grassland management regimens reduce small-scale heterogeneity and species diversity of beta-proteobacterial ammonia oxidizer populations, Appl. Environ. Microbiol. 68 (2002) 20–30.