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Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR

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Abstract

Denitrification, the reduction of nitrate to nitrous oxide or dinitrogen, is the major biological mechanism by which fixed nitrogen returns to the atmosphere from soil and water. Microorganisms capable of denitrification are widely distributed in the environment but little is known about their abundance since quantification is performed using fastidious and time-consuming MPN-based approaches. We used real-time PCR to quantify the denitrifying nitrite reductase gene (*nirK*), a key enzyme of the denitrifying pathway catalyzing the reduction of soluble nitrogen oxide to gaseous form. The real-time PCR assay was linear over 7 orders of magnitude and sensitive down to 10^2 copies by assay. Real-time PCR analysis of different soil samples showed *nirK* densities of 9.7×10^4 to 3.9×10^6 copies per gram of soil. Soil real-time PCR products were cloned and sequenced. Analysis of 56 clone sequences revealed that all cloned real-time PCR products exhibited high similarities to previously described *nirK*. However, phylogenetic analysis showed that most of environmental sequences are not related to *nirK* from cultivated denitrifiers.

Keywords: Nitrite reductase; *NirK*; Real-time PCR; Soil; Denitrification

1. Introduction

Denitrification is a respiratory process in which oxidized nitrogen compounds are used as alternative electron acceptors for energy production when oxygen is limited. It is the major mechanism by which fixed nitrogen returns to the atmosphere from soil and water, thus completing the N-cycle. This removal of soluble nitrogen oxide from the biosphere is of great importance in agriculture, where it can account for significant losses of nitrogen fertilizer from soil, and also in wastewater treatment.

Denitrification has also received considerable interest recently because it leads to N_2O emissions, it is an

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important greenhouse gas (Lashof and Ahuja, 1995) and a natural catalyst of stratospheric ozone degradation (Bange, 2000). Bacteria capable of denitrification are widely distributed in the environment and exhibit a high taxonomic diversity (Tiedje, 1988).

Denitrification consists of four reaction steps by which nitrate is reduced into dinitrogen gas by the metalloenzymes nitrate reductases, nitrite reductases, nitric oxide reductases and nitrous oxide reductase. The nitrite reductase is the key enzyme of this respiratory process since it catalyzes the reduction soluble nitrite into gas. Thus, previous studies have used probes or antibodies against this enzyme to identify denitrifying isolates (Coyne et al., 1989; Ward, 1995; Ward and Cockcroft, 1993). Two types of nitrite reductase that are different in terms of structure and prosthetic metal have been characterized: a copper nitrite reductase encoded by the *nirK* gene and a cytochrome cd_1-nitrite reductase encoded by the *nirS* gene (Zumft, 1997). Since 1998, several studies have reported the use of *nirK* or *nirS* as molecular markers of the denitrifying bacteria to study their diversity in various environments (Avrahami et al., 2003; Braker et al., 1998, 2000, 2001; Hallin and Lindgren, 1999; Liu et al., 2003; Prieme et al., 2002; Yoshie et al., 2004). However, abundance of denitrifiers in the environment is still determined by MPN and only the *nirS* gene has been used to quantify this functional community using competitive PCR and real-time PCR as cultivation-independent approaches (Michotey et al., 2000; Gruntzig et al., 2001). Since denitrification is not restricted to cytochrome cd_1-nitrite reductase denitrifiers, we developed a real-time PCR assay targeting the *nirK* gene in order to quantify in soils the denitrifiers having the copper nitrite reductase.

2. Materials and methods

2.1. Environmental samples and bacterial strains

Six different soils were selected for their contrasting physicochemical characteristics (Table 1). All samples were obtained from the first 20-cm top layer. La Bouzule soil was collected from a wheat planted plot in an experimental field of the ENSAIA domain of La Bouzule (Meurthe et Moselle, North East of France). This soil was amended either with C 150 μg C g soil^{-1} day^{-1} or water. Vannecourt soil was collected from a winter wheat agricultural field (Moselle, North East of France). Paris soil was obtained from garden soils near Paris. Soil from Kenya was collected in a glade of the Kakamega rainforest located in the highlands of western Kenya. Termite nests (*Cubitermes* sp) from Burkina Faso were collected in a savannah landscape located in Tiogo in the west part of Burkina Faso. Rennes soil was collected in a maize planted field amended with ammo-nitrate (110 kg N ha^{-1} year^{-1}). The strains used in this study are listed in Table 2.

Table 1
Properties of the soils analysed

Soils	% of:					pH
	Clay	Sand	Silt	N	C org	
Bouzule	33.3	15.4	51.3	N.D.	15.3	5.8
Kenya	35.6	33.8	32.3	0.27	3.77	N.D.
Paris	13	77	10	0.009	1.1	7.7
Rennes	14	19.3	66.6	1.04	9.41	5.89
Termite mound Burkina	25.9	22.5	51.2	0.29	3.46	N.D.
Vannecourt	22.5	33.2	44.3	0.2	1.6	6

2.2. DNA extraction

DNA was extracted from three 250-mg aliquots of soil samples (Martin-Laurent et al., 2001). Briefly, samples were homogenized in 1 ml of extraction buffer during 30 s at 1600 rpm in a minibead beater cell disrupter (Mikro-DismembratorS; B. Braun Biotech International). Soil and cells debris were eliminated by centrifugation (14,000×*g* for 5 min at 4 °C). Afterwards, 5 M sodium acetate was used to remove the proteins and nucleic acids were precipitated using cold ethanol. DNA was washed with 70% ethanol and purified through a sepharose 4 B spin column. At the end, DNA was quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany) and by comparison to DNA standard in 1% (wt/v) agarose gel electrophoresis.

2.3. nirK primers development and testing

To design the *nirK* primers, *nirK* sequences from cultivated strains, from complete and unfin-

Table 2
Bacterial strains used in this study and test of the *nirK* primer sets to amplify the copper nitrite reductase

Strains	Nir type	Result of PCR[a]
Alcaligenes faecalis ATCC8750	Cu (2,0)	+
Achromobacter cycloclastes ATCC21921	Cu (0,0)	+
Bradyrhizobium japonicum 526	Cu (0,2)	+
Escherichia coli JM109	None	−
Rhizobium meliloti	Cu (1,0)	+
Rhodobacter sphaeroides DSM158	Cu (2,0)	+
Pseudomonas fluorescens C7R12	cd_1	0

Numbers of mismatches of the *nirK* sequences from reference strains with forward and reverse primers are indicated in parenthesis.

[a] +, visible band of the expected size; −, no visible band; 0, non-specific bands.

ished bacterial genomes and from environmental *nirK* libraries, were aligned using the ClustalX software V.101 (Thompson et al., 1997) and compared to select conserved regions by eye. Two degenerated primers (5′–3′) nirK876 (ATYGGCGGVAYGGCGA) and nirK1040 (GCCTCGATCAGRTTRTGGTT) were designed to amplify a 165-bp fragment (*nirK* from *Sinorhizobium meliloti* 1021 was used as reference sequence for numbering). The nirK1040 primer is based on the sequence of the *nirK* primer designed by Braker et al. (1998) and Hallin and Lindgren (1999). Several copper nitrite reductase and cytochrome cd_1 denitrifiers and non-denitrifying strains were used as positive and negative control to test the specificity of the primer set (see Table 2).

2.4. Real-time PCR assay

Amplification of real-time PCR products was carried out with a Smart Cycler (Cypheid®, USA) using SYBR Green as detection system in a reaction mixture of 25 μl containing: 0.5 μM of each primer for *nirK*, 12.5 μl of SYBR Green PCR master mix, including HotStar Taq™ DNA polymerase, Quanti Tec SYBR Green PCR Buffer, dNTP mix with dUTP, SYBR Green I, ROX and 5 mM $MgCl_2$ (QuantiTect™ SYBR® Green PCR Kit, QIAGEN, France), 1.25 μl of DNA diluted template corresponding to 12.5 ng of total DNA, and Rnase-free water to complete the 25-μl volume.

The conditions for nirK real-time PCR were 120 s at 50 °C, 900 s at 95 °C for enzyme activation as recommended by the manufacturer (QuantiTect™ SYBR® Green PCR Kit, QIAGEN); afterwards six touchdown cycles were performed: 15 s at 95 °C for denaturation, 30 s at 63 °C for annealing, 30 s at 72 °C for extension and 15 s at 80 °C for a final data acquisition step. The annealing temperature was progressively decreased by 1 °C down to 58 °C. Finally, a last cycle with an annealing temperature of 58 °C was repeated 40 times. One last step from 60 to 95 °C with an increase of 0.2 deg/s was added to obtain a specific denaturation curve. Purity of amplified products was checked by the observation of a single melting peak and the presence of a unique band of the expected size in a 3% agarose gel stained with ethidium bromide.

2.5. Quantification of nirK from soil samples

Two independent real-time PCR assays were performed on each of the three replicate soil DNA extracts. The standard curve was created using 10-fold dilution series of three linearized plasmids containing the different *nirK* genes from environmental samples. The detection limit of our assay in soils was determined using 10-fold dilutions of soil DNA. Soil DNA was also tested for inhibitory effect of coextracted substance by determining the *nirK* copy number in 10-fold dilutions of soil DNA and by adding 10^6 copies of the target gene in the lowest dilution of soil DNA.

To check specificity, real-time PCR products from one replicate of each environment were cloned into the pGEM-T Easy Vector System (Promega, France) according to the instructions of the manufacturer. Approximately eight clones from each soil were then randomly chosen for sequencing using DTCS-1 kit (Beckman, Coulter) with universal primer T7 in a Ceq 2000 XL sequencer. The resulting sequences were deposited in GenBank under accession numbers AY675449-AY675504.

2.6. Calculation and statistical analysis

A one-way analysis of variance was performed to compare the *nirK* abundance between the different

soil samples. Means were compared using the least significant difference (LSD) test at $P<0.05$.

3. Results

3.1. nirK primers specificity

DNA from denitrifying strains containing either the copper or the cytochrome cd_1 nitrite reductase gene and from a non-denitrifying strain was used to test the specificity of the designed primers. No non-copper nitrite reductase strain gave a PCR amplification (Table 2). Application of the designed primers to real-time PCR assay using DNA extracted from various soil environments as template results in a single band of the expected size of approximately 160 bp except in the Kenya soil exhibiting two non-specific faint bands (Fig. 1). Analysis of data from RT-PCR showed that a single melting peak corresponding to the standard DNA was observed for all soil samples (data not shown).

3.2. Performance of standard curves and detection limit

Plasmids containing cloned *nirK* genes were used to draw a standard curve relating Ct to the added mass of linearized plasmid DNA and the number of gene copies. The same linear response was observed with the three tested plasmids for 7 orders of magnitude, ranging from 10^2 to 10^8 *nirK* gene copies (r^2=0.999) (Fig. 2).

The sensitivity of the assay was determined using a dilution series of extracted soil DNA. The overall sensitivity in soil samples was 10^2 targets per assay, corresponding to the same order of magnitude when compared to DNA standard curve. After addition of 10^6 copies of the standard DNA to soil samples diluted below the detection limit, 1.9×10^6 (Standard Error: 1.8×10^4) copies were obtained out of the 1.3×10^6 (Standard Error: 3.2×10^5) expected. The absence of inhibitory substance at the dilution used was also confirmed by the similar amplification efficiencies obtained with the 10-fold dilution of soil DNA extracts.

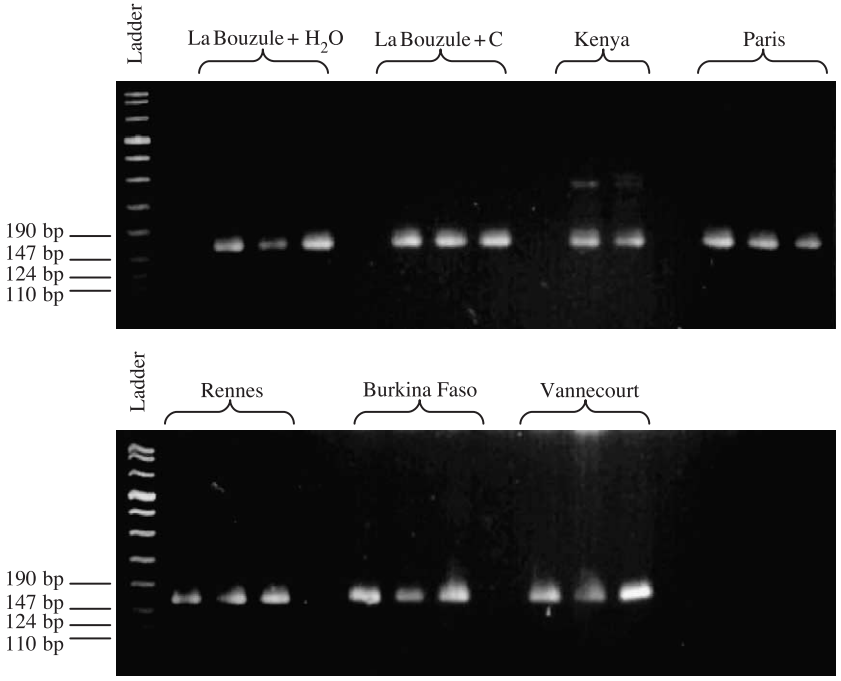

Fig. 1. Agarose gel (3%) electrophoresis of the real-time PCR products using DNA extracted from the different soil samples as template. Molecular size marker VIII from Boehringer Mannheim was used as ladder.

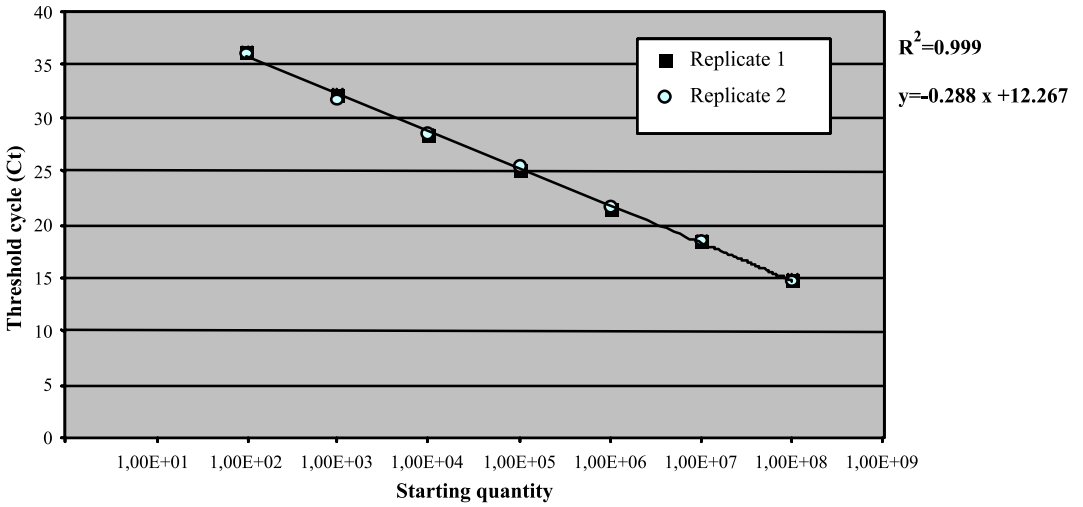

Fig. 2. Calibration curve plotting log starting *nirK* copy numbers versus threshold cycle. Data point represent duplicate measurement performed during two independent real-time PCR using dilutions of one of the plasmid containing *nirK* as template.

3.3. Quantification of nirK in soil samples

For evaluation of the method, five soils exhibiting contrasted physicochemical characteristics in terms of soil structure and organic content and a soil amended either with water or 150 μg C g^{-1} soil day^{-1} during 3 weeks were analysed. Two independent real-time PCR measurements were performed on triplicate DNA extraction for each soil. The number of *nirK* target molecules ranged between 9.7×10^4 and 3.9×10^6 copies per gram of soil (Table 3). A higher *nirK* abundance (approximately 3.9×10^6 copies per gram of soil) was observed in the agricultural soil from La Bouzule amended with C. Comparison of the soil amended with C or H_2O revealed a significant four fold increase of the number of *nirK* copies in the studied soil.

Table 3
nirK copy number in the different soil samples

Soils	*nirK* gene copy number per nanogram of DNA	*nirK* gene copy number per gram of soil
Bouzule amended with water	9.7×10^1 $(1.1\times10^1)^a$	8.9×10^5 $(1.0\times10^5)^a$
Bouzule amended with C	4.2×10^2 $(1.1\times10^2)^c$	3.9×10^6 $(9.8\times10^5)^c$
Kenya	8.9×10^1 $(2.2\times10^1)^a$	2.1×10^5 $(5.3\times10^4)^a$
Paris	1.9×10^2 $(4.4\times10^1)^b$	9.7×10^4 $(2.5\times10^4)^a$
Rennes	7.7×10^1 $(4.0\times10^1)^a$	4.2×10^5 $(5.3\times10^4)^a$
Termite mound Burkina Faso	5.1×10^1 $(2.5\times10^1)^a$	2.2×10^5 $(2.2\times10^5)^a$
Vannecourt	3.0×10^1 $(1.2\times10^1)^a$	1.4×10^6 $(5.1\times10^5)^b$

Values followed by the same letter within columns do not significantly differ according to LSD test ($P<0.05$).
Standard errors indicated in parenthesis.

3.4. Phylogenetic diversity of the nirK real-time PCR products

A total of 56 clones from five libraries obtained by cloning the real-time PCR products from the analysed soil samples were randomly chosen and sequenced. Comparison of the obtained sequences with the NCBI database by using a BLAST search revealed that all the sequences exhibited similarities ranging between 60% and 80% with the closest known *nirK* sequence. The copper nitrite reductase from *Neisseria meningitidis* was used as an outgroup for phylogenetic analysis. Neighbor-joining tree showed that the majority of the cloned real-time PCR products are distributed in a major cluster containing mainly *nirK* sequences from the α-Proteobacteria (Fig. 3). However, some sequences from the soil of Paris are related to *nirK* from the β-Proteobacteria *Nitrosomonas*. Most of the NirK sequences from cultivated denitrifying bacteria are present in a cluster, which did not contain environmental clones.

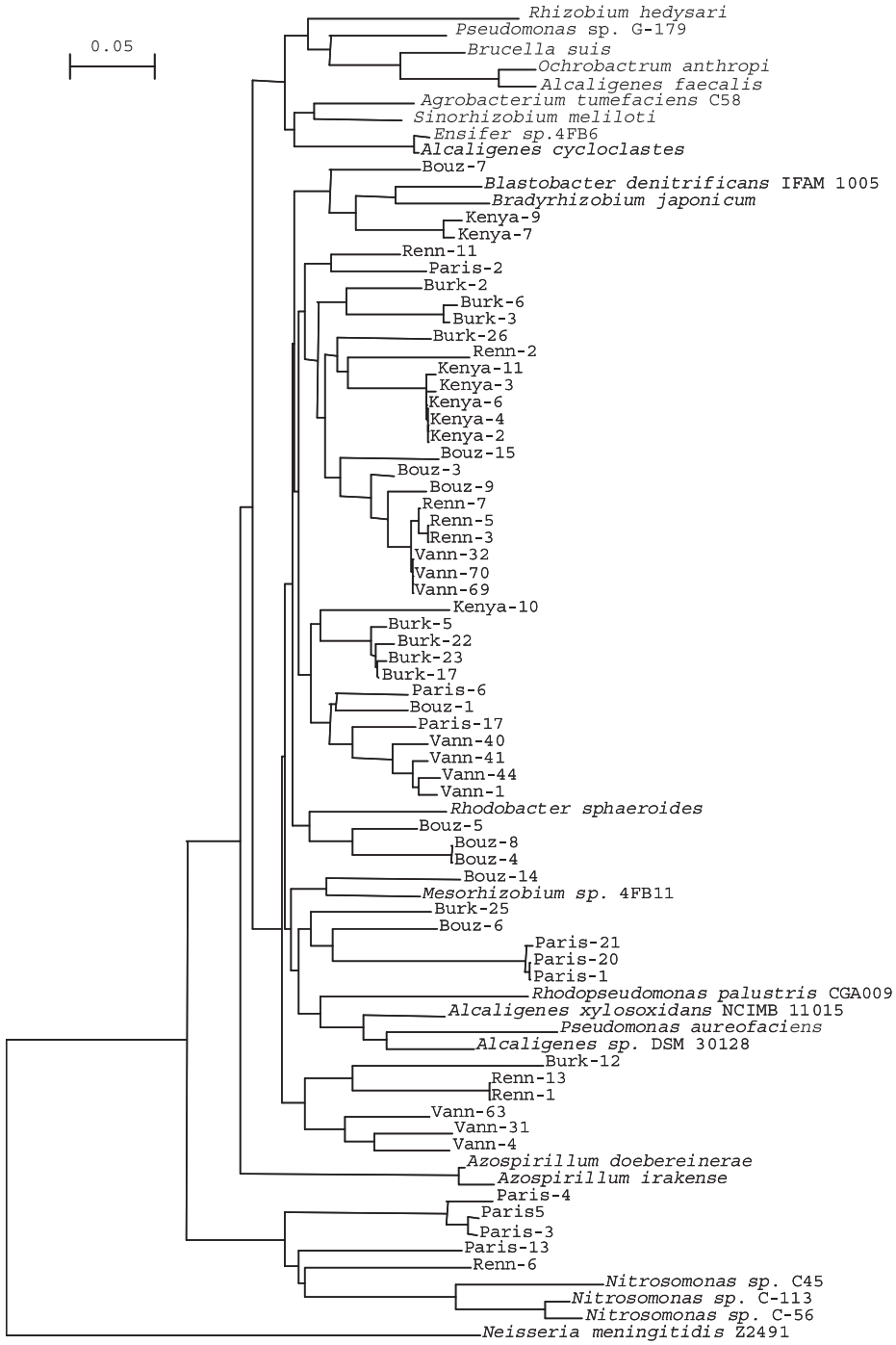

Fig. 3. Phylogenetic neighbor-joining (NJ) tree of *nirK* genes (partial, around 165 bp) from environmental clones obtained in this study and from known bacteria.

4. Discussion

Quantification of bacteria capable of denitrification is important for a better understanding of denitrifying activity and N_2O fluxes in the environment. Commonly used methods such as MPN are biased by unculturability of many microorganisms. Therefore, in this study, a real-time PCR assay was developed to quantify the denitrifying bacteria using the *nirK* gene encoding the copper nitrite reductase, a key enzyme of the denitrifying pathway.

In addition to strains from culture collections and genome sequences, we selected cloned *nirK* sequences from various environmental libraries to design *nirK* primers more accurate for application in the environment. In order to be able to amplify most of the Cu nitrite reductase denitrifiers, it was necessary to design degenerated primers, increasing the risk of non-specific amplification. However, a good specificity of our set of primers was observed with the cultured strains (Table 1).

Application of the *nirK* primers to environmental samples was performed using SyberGreen as detection system as discussed by Stubner (2002). In contrast to the TaqMan™ detection system, SyberGreen detection does not need the development of additional probes which is unrealistic for the *nirK* gene due to its high polymorphism between the different taxonomic group of denitrifiers (Philippot, 2002). Our real-time PCR assay was linear over 7 orders of magnitude and sensitive down to 10^2 copies by assay, similar to the results obtained in other studies (Bach et al., 2002; Gruntzig et al., 2001; Kolb et al., 2003; Lopez-Gutierrez et al., 2004; Stubner, 2002).

Environmental soil samples analysed by real-time PCR displayed a range of 2 orders of magnitude of *nirK* abundance between the different soil samples (Table 3). The higher density was observed in the agricultural soil from La Bouzule amended with C. Interestingly, the real-time PCR assay developed in this study is sensitive enough to detect a significant increase ($P<0.05$) in the density of the denitrifying community between a soil amended with a mix of different carbon substrates compared to a soil amended with water (Table 3).

Microorganisms capable of denitrification are widely distributed in the environment with densities estimated using MPN-method ranging between 10^4 and 10^6 bacteria g^{-1} soil (Cheneby et al., 2000; Gamble et al., 1977; Vinther et al., 1982; Weier and MacRae, 1992). In contrast to 16S rDNA, the *nirK* gene copy number can be directly correlated to cell numbers since only one copy of the *nirK* gene has been identified in denitrifying bacteria up to now (Philippot, 2002). Therefore, we can assumed that densities of copper nitrite denitrifiers reported in this study are in the range of 10^4–10^6 bacteria g^{-1} soil (Table. 3). Considering that only a part of the denitrifying community has been taken into account in this study—the copper nitrite reductase containing denitrifiers—while MPN count both types of denitrifiers, our results confirmed that MPN underestimated number of denitrifiers as previously observed (Michotey et al., 2000). Unfortunately, the proportion of copper nitrite reductase denitrifiers among the total denitrifying community in nature is still unknown. However, previous study based on the analysis of a collection of isolated denitrifiers reported that while cytochrome cd_1 nitrite reductase dominated (between 64% and 92%), regardless of soil type or geographic origin, the Cu type was found in more taxonomically unrelated strains (Coyne et al., 1989).

Besides verifying that application of our real-time PCR assay results in a single band, specificity of the assay was also evaluated by cloning and sequencing of the real-time PCR products obtained from the different soil samples. Phylogenetic analysis revealed that the clone sequences are distributed over the whole *nirK* tree confirming the validity of our primers (Fig. 3). While all the clone sequences exhibited similarities to *nirK*, most of them are not closely related to *nirK* from cultivated bacteria as previously observed (Prieme et al., 2002). No strong soil-specificity among the environmental clones was observed.

Previous studies have developed PCR-based assay to quantify denitrifying bacteria using the genes encoding the cytochrome cd_1 nitrite reductase as molecular marker. Thus, a real-time PCR study targeting the *nirS* gene has been published by Gruntzig et al. (2001). However, the designed primers were specific to *Pseudomonas stutzeri* and therefore cannot be used to quantify this taxonomically diverse functional community. More recently, Michotey et al. (2000) developed a competitive PCR assay to also quantify *nirS*. The designed primers were more universal but competitive PCR is fastidious and cannot be used for rapid analysis of multiple samples.

In summary, to our knowledge, this is first PCR-based approach enabling a rapid quantification of the copper nitrite reductase denitrifiers in the environment. In the future, combination of quantitative PCR-based approaches targeting the *nirK* and *nirS* genes would be useful to determine both the total number denitrifying bacteria using cultivation-independent method and the proportion of Cu and cytochrome cd_1 denitrifiers among the total denitrifying community. Thanks to the quantitative PCR approaches, the effect of agricultural practices or of other factors on the size of the denitrifying community can now be studied using a rapid cultivation-independent technique.

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