RESEARCH ARTICLE

First exploration of *Nitrobacter* diversity in soils by a PCR cloning-sequencing approach targeting functional gene *nxrA*

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Abstract

Nitrite oxidoreductase (NXR) is the key enzyme responsible for the oxidation of NO_2^- to NO_3^- in nitrite-oxidizing bacteria. For the first time a molecular approach for targeting the *nxrA* gene was developed, encoding the catalytic subunit of the NXR, to study diversity of *Nitrobacter*-like organisms based on the phylogeny of *nxrA* gene sequences in soils. *NxrA* sequences of the *Nitrobacter* strains analysed (*Nitrobacter hamburgensis*, *Nitrobacter vulgaris*, *Nitrobacter winogradskyi*, *Nitrobacter alkalicus*) by PCR, cloning and sequencing revealed the occurrence of multiple copies of *nxr*A genes in these strains. The copy number and similarity varied among strains. The diversity of *Nitrobacter*-like *nxr*A sequences was explored in three soils (a French permanent pasture soil, a French fallow soil, and an African savannah soil) using a cloning and sequencing approach. Most *nxr*A sequences found in these soils (84%) differed from *nxr*A sequences obtained from *Nitrobacter* strains. Moreover, the phylogenetic distribution and richness of *nxr*A-like sequences was extremely variable depending on soil type. This *nxr*A tool extends the panel of functional genes available for studying bacteria involved in the nitrogen cycle.

Introduction

Nitrification is of fundamental importance for the functioning of terrestrial and aquatic ecosystems. This is a key process of the nitrogen cycle that oxidises ammonium to nitrate. Nitrification thus strongly influences nitrogen plant nutrition, and water pollution (eutrophication). Nitrification is a two-step microbial process. The first step is the oxidation of ammonia to nitrite, and the second step is the oxidation of nitrite to nitrate. Each step is performed by distinct microorganisms: ammonia-oxidizing bacteria (AOB) (Teske *et al.*, 1994) or archaea (AOA) (Francis *et al.*, 2005; Treusch *et al.*, 2005) and nitrite-oxidizing bacteria (NOB) such as *Nitrobacter* (for a review see Prosser, 1989).

In addition to cultivation biases, the very slow growth rate of organisms belonging to these microbial groups does not allow their diversity to be studied using isolation techniques. The use of molecular tools is thus necessary to evaluate their distribution, richness and relative abundances in natural environments. Most of the previous studies on the diversity of nitrifiers have focused on the AOB group, by studying the polymorphism of the 16S rRNA gene (Voytek & Ward, 1995; Stephen *et al.*, 1998; Kowalchuk *et al.*, 2000; Purkhold *et al.*, 2000; Patra *et al.*, 2005). The known NOB (*Nitrobacter*, *Nitrococcus*, *Nitrospina* and *Nitrospira* genera) is distributed among four phylogenetic groups (*Alpha-*, *Gamma-*, *Deltaproteobacteria*, and *Nitrospira*, respectively) (Watson & Waterbury, 1971; Bock *et al.*, 1990). Thus, when developing PCR-based studies of NOB diversity targeting rRNA genes, the use of several primer sets is required (Daims *et al.*, 2001; Freitag *et al.*, 2005). Moreover, Orso *et al.* (1994) found that 16S rRNA gene sequences of *Nitrobacter* genus showed more than 99% sequence conservation. In this case, the discriminant properties of 16S rRNA gene are thus too weak for successful analysis of the diversity of complex communities (Regan *et al.*, 2003).

It has been shown that functional genes can be good molecular markers for exploring the diversity within functional groups (Scala & Kerkhof, 1999; Poly *et al.*, 2001; Nogales *et al.*, 2002; Calvo *et al.*, 2005). A panel of functional genes has been used to study the bacterial diversity involved in different steps of the nitrogen cycle: *nar*G (Nogales *et al.*,

2002; Philippot *et al.*, 2002) for nitrate reduction, *nir*K, nirS (Braker *et al.*, 2000) and *nos*Z (Scala & Kerkhof, 1999) for denitrification, and *nif*H for nitrogen fixation (Kirshtein *et al.*, 1991; Poly *et al.*, 2001). For nitrification, the *amo*A gene encoding ammonia monooxygenase has been used successfully to characterize the diversity and phylogenetic characteristics of soil and water AOB communities (Rotthauwe *et al.*, 1997; Kowalchuk *et al.*, 2000; Purkhold *et al.*, 2000; Calvo *et al.*, 2005; O'Mullan & Ward, 2005) and more recently AOA communities (Francis *et al.*, 2005; Treusch *et al.*, 2005). However, until recently, no equivalent tool targeting a functional gene was available for the NOB communities.

In *Nitrobacter*, the oxidation of nitrite to nitrate is performed by the nitrite oxidoreductase (NXR), encoded by the *nxr* operon (Starkenburg *et al.*, 2006) formerly named *nor* (Kirstein & Bock, 1993). Sequences of *nxrA* genes encoding the catalytic subunit of NXR in *Nitrobacter* species have been deposited in Genbank in 2001 by Degrange *et al.* (pers. commun.). More recently, Vanparys *et al.* (2007) expanded this database with partial *nxrA* sequences (308 bp) from five pure cultures and 34 environmentally derived sequences. In addition, the *nxrA* sequences are available from three complete genomes of *Nitrobacter* and from the genome of *Nitrococcus mobilis* currently sequenced (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Until now, however, no information on the diversity and the distribution of *nxr*A sequences in environmental samples were available.

This study presents a molecular approach for targeting the *nxr*A gene. A PCR and cloning/sequencing approach was developed to explore the variability of multiple *nxr*A gene copies in different species of *Nitrobacter* and the diversity of this gene in soils.

Materials and methods

Bacterial strains and DNA

All strains used in this study are listed in Table 1. *Nitrobacter* cultures were grown aerobically in 1-L stirred batch cultures at 28 °C. *Nitrobacter winogradskyi* and *Nitrobacter alkalicus* strains were cultivated in an autotrophic medium (Schmidt *et al.*, 1973). *Nitrobacter vulgaris* and *Nitrobacter hamburgensis* strains were cultivated in a mixotrophic medium (Bock *et al.*, 1983).

Table 1. Bacterial strains used in this study

Type	Genus	Species	Strain	References
Nitrifiers	*Nitrobacter*	*hamburgensis*	X_{14}	DSM 10229
	Nitrobacter	*winogradskyi*	AG	ATCC 14123
	Nitrobacter	*vulgaris*	Z	DSM 10236
	Nitrobacter	*alkalicus*	AN4	Sorokin *et al.* (1998)

Soil samples

Soil samples were collected from the upper layer (0–10 cm) of the studied soils. The TV soil is a permanent pasture grazed by cows from the central part of France (Theix, ORE PCBB, Puy de Dôme). The E soil, from southwest of France (Clavier, Lot-et-Garonne), had been intensively cultivated with maize for more than 10 years followed by 7 years of fallow. The B soil was sampled in Côte d'Ivoire bushed savannah (Lamto reserve) (Abbadie *et al.*, 2006). Physicochemical characteristics of each soil are presented in Table 2.

DNA extraction

Genomic DNA from pure cultures of *Nitrobacter* was extracted using a DNA tissue kit (Qiagen) performed to the manufacturer's protocol. Environmental DNA was extracted from soils using the Fast DNA spin kit for soil (Bio101, Qbiogen, France).

Primer design

Partial nucleotide *nxr*A sequences of *N. hamburgensis* X_{14} (accession number AF344872), *N. vulgaris* Z (AF344875), *N. winogradskyi* AG (AF344874) and *Nitrobacter alkalicus* AN_4 (AF344873) were aligned using CLUSTAL X software (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) (Jeanmougin *et al.*, 1998). *NarG* sequences showing a great similarity with these *nxrA* sequences using BLAST software were selected (*Mycobacterium tuberculosis* BX842575, AE000516, *Escherichia coli* X16181, *Pseudomonas aeruginosa* Y15252, *Thermus thermophilus* Y10124, *Geobacter metallireducens* CP000148, *Anaeromyxobacter dehalogenans* NC_007760, *Arthrobacter* sp. CP000454, *Streptomyces coelicolor* AL939122). Conserved and variable regions between *nxr*A sequences and *nar*G sequences were identified to design the following primers: the forward primer F1norA 5′- CAG ACC GAC GTG TGC GAA AG-3′ and the reverse primer R1norA 5′- TCY ACA AGG AAC GGA AGG TC-3′ (Y = C or T) amplifying a fragment of 322 bp. *In silico* analysis, using the Functional gene pipeline/Repository website (http://flyingcloud.cme.msu.edu/fungene/index.jsp), was performed to compare designed primers with available *narG* sequences (1853 sequences) and confirm their specificity.

PCR amplification of partial *nxrA* sequences

PCR was carried out in 50 μL reaction volume with a T-Gradient thermal cycler (Biometra, France). The reaction mixture contained 20 ng of template DNA, 0.5 μM of each primer, 1 × PCR buffer (Invitrogen, France), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.05% of detergent W-1 solution provided by the manufacturer, 2.5 U Taq DNA polymerase (Invitrogen, France) and 5 ng μL^{-1} of T4 bacteriophage gene 32 product (QBiogene, France). For DNA strains, the PCR

Table 2. Soil characteristics

	Water pH	Clays (< 2 μm) (g kg^{-1})	Fine silt (g kg^{-1})	Coarse silt (g kg^{-1})	Fine sand (g kg^{-1})	Coarse sand (g kg^{-1})	Organic matter (g kg^{-1})	N total (g kg^{-1})	C/N	CEC (cmol kg^{-1})
Soil E	6.3	53	42	53	174	678	6.3	0.4	9.17	2.9
Soil B	6.2	75	58	82	294	480	20	0.68	16.89	ND
Soil TV	6.0	242	181	78	80	419	79.8	4.59	10	19.3

thermocycling program was: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s, and a final elongation step at 72 °C for 5 min. Using these conditions, a good amplification was obtained for E and TV soils, but PCR yield for the African soil was too low (data not shown). Thirty cycles were thus added to obtain amplicons and the temperature of the additional PCR cycles was increased to avoid the formation of unspecific products. To compare diversity results between soils, the same protocol, i.e. including additional cycles, was used to amplify all soils. Thus, for all soil DNA amplifications, two different annealing temperatures were used: the first 25 cycles were performed at 55 °C annealing temperature as before and then 35 additional cycles were performed at 58 °C. Additional 2.5 U of Taq DNA polymerase were added to each tube after the first 25 cycles.

Cloning of *nxrA* PCR products

*Nxr*A PCR products from DNA of *Nitrobacter* reference strains and soils were excised and purified from 2% agarose gels using the Qiaquick gel extraction kit (Qiagen, France) before cloning. PCR products were ligated into the pGEM-T vector (Promega corp, France). Ligated vectors were then transformed into chemical competent *E. coli* DH5α (Life Biotechnologies, France) in accordance with the manufacturer's instructions. Cells were grown on solid Luria–Bertani medium with ampicilline (100 μg mL^{-1}), β-X-Gal (80 μg mL^{-1}) and isopropyl-β-D-thiogalactoside (0.5 mM) at 37 °C for 15 h. Ten transformants (white colonies) for the libraries of *Nitrobacter* strains and between 27 and 110 transformants for soil libraries were randomly picked. Transformants were then subjected to PCR using F1norA and R1norA primers (under the conditions mentioned above for strains) to check for the presence of *nxrA* inserts.

Screening of clone libraries by restriction fragment length polymorphism (RFLP)

For each clone 200 ng of PCR product were digested with Sau96I and RsaI restriction enzymes (Biolabs). The resulting fragments were separated by electrophoresis in 3% Metaphor agarose gel (FMC BioProducts). The sizes of restriction fragments were determined using GEL COMPARE 2 software (Applied BioMath, Belgium) and clones were grouped into restriction groups or operational taxonomic units (OTU) based on 100% identity threshold of the restriction patterns for the two enzymes used. Rarefaction curves were computed based on the analytical approximation algorithm by applying the freeware program ARAREFACTION (v1.3) (http://www.uga.edu/strata/software/Software.html).

Sequencing of *nxrA* and phylogenetic analysis

In clone libraries obtained for each soil sample, a mean of three (two to five) clones per OTU were randomly selected for sequencing (minor OTUs i.e. less than three clones were not sequenced). Inserts from clones amplified with vector-specific primers (M13 forward and M13 reverse) were purified with the Qiaquick PCR purification kit (Qiagen) and *nxr*A fragments were sequenced (Genomexpress, Grenoble, France; DTAMB, Lyon, France). Sequences were run through a Mega BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) using nr database and were deposited in Genbank with the accession numbers DQ421329–DQ421378.

NxrA partial sequences were aligned using CLUSTAL X (Jeanmougin *et al.*, 1998) and a phylogenetic analysis was performed using three treeing methods: (1) parsimony method with 100 resampling and (2) neighbour-joining method with the HKY model of substitution (using 500 bootstraps) were performed with PHYLOWIN software (http://pbil.univ-lyon1.fr/software/phylowin.html) (Galtier *et al.*, 1996), and (3) maximum likelihood tree was calculated with PHYML software using the HKY model of substitution and four categories of substitutions with a estimated γ distribution parameter (http://atgc.lirmm.fr/phyml/) (Guindon & Gascuel, 2003). Then a consensus tree from the three trees obtained was computed with Consense in the PHYLIP package 3.66 (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1993). The consensus tree was drawn with the NJ plot (http://pbil.univ-lyon1.fr/software/njplot.html) (Perrière & Gouy, 1996).

Three criteria were used to define clusters: (1) in one cluster all sequences harboured a similarity >90% [but two sequences with a similarity >90% can be dispersed in two distinct clusters because the similarity and the phylogenetic distances are two different things (Koski & Golding, 2001)];

and (2) clusters had to be obtained using the three treeing methods and (3) clusters had to be supported by bootstrap values >80%.

Results

Strain and soil DNA amplifications

Amplification from *Nitrobacter* DNA using nxrA primers produced a single 322-bp fragment for the four tested strains. *In silico* analysis with complete *Nitrobacter* genomes confirmed that the primer F1norA perfectly matches all copies within these strains and the primer R1norA harboured a unique mismatch at the 5′ end. *In silico* analysis, using the Functional gene pipeline/Repository website, showed that the designed primer set theoretically cannot amplify *narG* sequences. At least, for the few pure cultures of nitrate reducing bacteria (harbouring *narG* gene) tested here no amplification was obtained (data not shown).

For both French soils (the permanent pasture TV soil and the fallow E soil), PCR amplification from soil DNA produced a unique band of 322 bp as expected. DNA from the African soil produced a weak amount of amplicons using the same PCR conditions probably due to the low number of counted NOB cells (< 500 cells g^{-1} dry soil determined by most probable number technique, data not shown). An additional 35 cycles were used to improve amplification yield. However, this extended PCR protocol (25+35 cycles), applied for all soil samples, resulted in the appearance of unspecific bands only for the African soil (data not shown), but the unspecific amplicons were not within the size range expected for *narG* or *nxrA*, and could not be confused with *nxrA* amplicons.

Cloning and identification of OTUs by RFLP analysis

Based on the analysis of the *nxr*A sequences of the four *Nitrobacter* pure cultures (Degrange *et al.* pers. commun.), the authors *in silico* selected the two enzymes RsaI and Sau96I for RFLP analysis. RFLP analysis of the *nxr*A PCR products in these four species allowed distinguishing four profiles (Fig. 1), but in some cases, the sum of restriction fragments exceeded the expected size of 322 bp. After cloning *nxr*A amplicons of the four referenced *Nitrobacter* strains, two different RFLP patterns was identified from *N. winogradskyi* AG and *N. alkalicus* AN_4 and three different RFLP patterns from *N. hamburgensis* X_{14} and *N. vulgaris* Z.

For the soils, a total of 191 *nxr*A clones (110 from TV soil, 54 from B soil, and 27 from E soil) were screened by RFLP, and 45 different OTUs were identified. The total number of OTUs varied from one for the E soil to 37 for the TV soil. Among these OTUs, those represented by more than one clone are presented in Fig. 2. The three soils did not share

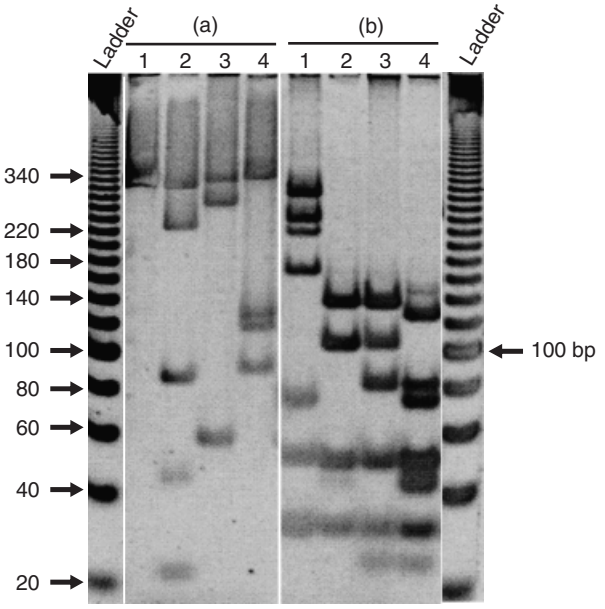

Fig. 1. RFLP profiles of *nxrA* gene amplicons of four *Nitrobacter* species digested with the restriction enzymes Sau96I (a) and RsaI (b): line 1, *Nitrobacter hamburgensis* X_{14}; line 2, *Nitrobacter alkalicus* AN_4; line 3, *Nitrobacter winogradskyi* AG; line 4, *Nitrobacter vulgaris* Z.

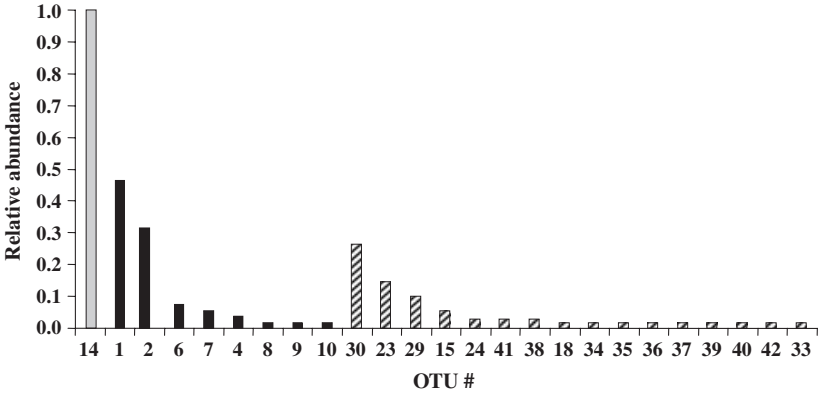

Fig. 2. Frequency distribution of OTUs from the *nxr*A gene libraries from TV, B and E soils. OTU definition based on RFLP patterns obtained by digestion with Sau96I and RsaI restriction endonucleases. OTUs obtained from E, B and TV soil samples were represented by , □, ■, ▨ and respectively. Note that for TV and B soils, the sum of relative abundance is <1 because OTUs represented by one clone only are not shown.

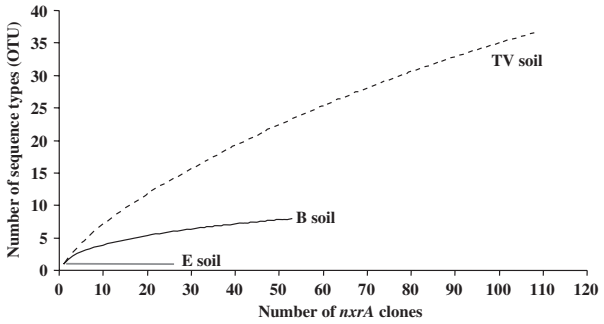

Fig. 3. Rarefaction curves obtained for each soil, i.e. number of OTUs represented by different RFLP patterns as a function of clone number in each *nxrA* gene library.

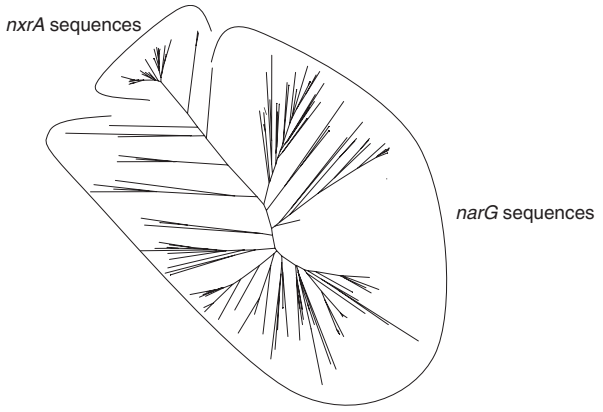

Fig. 4. Unrooted tree obtained from phylogenetic analysis of 72 partial *nxr*A and 419 *narG* nucleic acid sequences (322 bp) available in functional gene pipeline/Repository data-base (http://flyingcloud.cme.msu.edu/fungene/index.jsp). This tree was constructed by the neighbour-joining method of Saitou & Nei (1987) with the HKY distance.

common OTU, except for OTU #2 found in both TV and B soils but with different relative abundances (31.5% vs. 0.9%, respectively).

In the E soil, all 27 clones belonged to OTU #14. In the B soil, OTU #1 and #2 represented more than 77% of all the clones. In contrast, in the TV soil the two dominant OTUs (#30 and #23) represented $< 31\%$ of clones. Rarefaction analysis showed that the level of richness of *nxr*A sequences was higher in the TV soil than in the B and E soils (Fig. 3) and for the TV soil this curve indicated a far greater diversity than sampled.

Phylogenetic analysis

All analysed sequences retrieved from *Nitrobacter* pure cultures and soils were *nxr*A-like sequences. As expected from RFLP results, cloning and sequencing revealed that more than one gene copy was harboured by the *Nitrobacter* strains (two sequences for *N. alkalicus* AN_4, and three for *N. vulgaris* Z, *N. winogradskyi* AG, and *N. hamburgensis* X_{14}). For *N. winogradskyi* one copy among the three was not detected by RFLP. The similarity between *nxr*A copies within each *Nitrobacter* strain was $> 97.8\%$, 98.2%, 93.2% and 86.5% for the AG, AN_4, X_{14} and Z strains, respectively.

Between *Nitrobacter* species, the highest similarity of partial *nxr*A sequences was 93.2% between *N. alkalicus* and *N. winogradskyi* pure cultures and the lowest similarity was 83.3% between *N. hamburgensis* and *N. vulgaris*.

From soil DNA, 38 partial *nxr*A sequences were obtained. All sequences were confirmed as *Nitrobacter*-like *nxr*A sequences by comparison to the EMBL database using BLAST search and by their gathering in *nxrA* cluster distinct from *narG* sequences in the phylogenetic tree using *narG* sequences (419) (Fig. 4) that are known to be close neighbours. The *nxrA* phylogenetic analysis (Fig. 5) grouped the sequences belonging to the four *Nitrobacter* species into four different branches defining four clusters (named 1, 2a, 2b and 3) and the soil sequences into five distinct clusters (4, 5, 6, 7 and 8). *NxrA* sequences of *N. vulgaris* (Z1, Z2 and Z3) grouped in the same branch but could not form a cluster due to (1) a similarity $<90\%$ (Z2–Z3 vs. Z1) and (2) the branch including Z2 and Z3 was not consistent with the three treeing methods (not visible in the consensus tree). This lack of consistency was also observed for *nxrA* sequences of *Nitrobacter* sp. Nb-311 and *N. winogradskyi* Nb-255 whose locations in the tree were not stable. All *nxr*A sequences obtained from the B soil were closely related with *N. vulgaris*, whereas from TV soil two clones only (TV 72 and TV 147) showed high similarity with *N. alkalicus* sequences and other TV clones were not related to any known *Nitrobacter* species. No *nxr*A sequences retrieved from the studied soils were closely related to *nxr*A sequences belonging to *N. hamburgensis* and *N. winogradskyi* species.

The similarity between the partial *nxr*A sequences retrieved from soils and *nxr*A sequences of strains ranged from 82% to 99% and the similarity values for the cluster 7 and 8 with no similar sequence in the database to the closest pure culture sequence were $< 89\%$.

Discussion

For quite some time, oxidation of nitrite in soil has been considered as a very specific reaction catalysed by bacteria exhibiting low taxonomic diversity from which *Nitrobacter* was the most commonly isolated nitrite-oxidizing genus. Recently, molecular tools based on 16S rRNA gene (Freitag *et al.*, 2005) suggested a higher diversity of NOB in soils. However, due to the polyphyletic distribution of NOB in several classes of *Proteobacteria* and in *Nitrospira* phylum, and the high similarity of 16S rRNA gene sequences within *Nitrobacter* genus, investigating the diversity of NOB remained difficult using this gene as the phylogenetic marker.

Fig. 5. Phylogenetic analysis of partial *nxr*A nucleic acid sequences (322 bp) obtained from *Nitrobacter* pure cultures and soils. A consensus tree of the parsimony, neighbour-joining and maximum-likelihood methods is shown. The *nar*G sequences were used as outgroup for tree calculations. Clones obtained from E, B and TV soils were designated (in bold) E ▭, B ■ and TV ▨, followed by their number in the clone library and their accession number. Clones obtained from *Nitrobacter hamburgensis* X_{14}, *Nitrobacter vulgaris* Z, *Nitrobacter winogradskyi* AG and *Nitrobacter alkalicus* AN_4 were designated as X, Z, G and N, respectively, followed by their number in the clone library. Bootstrap values obtained from the likelihood method above 70% are indicated. Stars indicated *nxrA* sequences retrieved from complete genomes of *Nitrobacter* and *Nitrococcus*.

Here, the *Nitrobacter* diversity in soils was investigated by targeting the functional gene *nxrA* encoding an essential enzyme for nitrite oxidation, the NXR. It can be assumed that the PCR and cloning sequencing approach that was presented here can successfully be used to provide a more comprehensive view of diversity of *Nitrobacter*-like *nxr*A sequences in soils.

Kirstein & Bock (1993) reported an important similarity between the β subunit of the NXR (*nxr*B) of *N. hamburgensis* and the β subunit of the dissimilative nitrate reductase (*nar*H, *nar*Y) of *E. coli*. This similarity was also confirmed by BLAST between *nxr*A sequences and *nar*G sequences of nitrate reducers and by Starkenburg *et al.* (2006) showing similarity of NXR with respiratory nitrate reductases. For this reason, in this study, *nxr*A sequences were compared with *nar*G sequences to design specific primers and to construct phylogenic trees. The specificity of the designed primers was confirmed by *in silico* analysis and the fact that no sequences obtained from soils DNA were related to *narG* sequences, but grouped with sequences of *Nitrobacter* pure cultures (Fig. 4).

The RFLP patterns obtained for the *nxr*A amplicons from the *Nitrobacter* strains suggested the existence of multiple copies of the *nxr*A gene (with different sequences) in the genome of the four *Nitrobacter* species (*N. hamburgensis, N. vulgaris, N. winogradskyi*, and *N. alkalicus*). The presence of multiple copies of the *nxr*A gene in *Nitrobacter* genomes was confirmed by cloning and sequencing, and was consistent with information provided by the complete genome sequences of three *Nitrobacter* strains now available (http://www.ncbi.nlm.nih.gov/). Duplicated genes in bacterial genomes are not scarce. For instance, multiple copies of

functional genes were encountered in the genome of numerous bacteria such as for *rbc*LS genes (encoding ribulose 1, 5-bisphosphate carboxylase/oxygenase) in *Thiobacillus ferroxidans* (Kusano *et al.*, 1991), *pmo*CAB genes (encoding methane monooxygenase) in *Methylosinus trichosporium* and *Methylocystis* sp. (Gilbert *et al.*, 2000), *nif*H genes (encoding nitrogenase) in *Nostoc* sp. (Raymond *et al.*, 2004) or *nir*S genes (encoding nitrite reductase) in *Thauera* sp. (Etchebehere & Tiedje, 2005). Other nitrifiying bacteria, such as *Nitrosospira, Nitrosolobus, Nitrosomonas* or *Nitrosovibrio*, harbour multiple copies of functional genes involved in nitrogen cycle (*hao* gene encoding hydroxylamine oxidoreductase, or *amoCAB* genes encoding ammonia monooxygenase) (McTavish *et al.*, 1993; Bergmann *et al.*, 1994; Norton *et al.*, 1996; Klots *et al.*, 1997; Hommes *et al.*, 1998). The physiological significance of multiple copies of genes is still unclear but may be involved in functional traits of strains (e.g. a copy could be expressed differentially to another in response to environmental conditions), as suggested for *amo* genes of *Nitrosomonas europaea* (Hommes *et al.*, 2001) or for *nir* genes of Thauera sp. (Etchebehere & Tiedje, 2005). Similarities of partial *nxr*A sequences between tested *Nitrobacter* species varied from 83% to 93%, showing a higher variability than that observed for 16S rRNA gene sequences (above 98.8%) within *Nitrobacter* genus (Orso *et al.*, 1994; Sorokin *et al.*, 1998). For environmental retrieved sequences, the multiple gene copies have to be taken into account and complicate the taxonomic interpretability of the results. But this variability of these multiple gene copies could be highly interesting if the presence of one copy could be related to specific functional traits. In this case, the use of *nxr*A gene could be a marker for estimating the functional traits of *Nitrobacter* in the environment. The diversity of *Nitrobacter* and other NOB in soil has been explored using immuno-detection (Josserand & Cleyet-Marel, 1979; Stanley & Schmidt, 1981; Grundmann & Normand, 2000; Bartosch *et al.*, 2002) and rRNA molecular tools (Burrell *et al.*, 1998; Grundmann & Normand, 2000; Grundmann *et al.*, 2000; Freitag *et al.*, 2005). Immunodetection or molecular approaches based on 16S rRNA gene imply the use of several antibodies or primers targeting different serotypes (Navarro *et al.*, 1992) or genera (Freitag *et al.*, 2005). A high percent similarity exists between 16S rRNA gene sequences of *Nitrobacter* and species phylogenetically closely related (*Bradyrhizobium japonicum*, *Rhodopseudomonas palustris* or *Afipia* spp.) (Regan *et al.*, 2003). This impaired an adequate differentiation between *Nitrobacter* and non-NOB species using partial 16S sequences (Regan *et al.*, 2003; Freitag *et al.*, 2005) even if specific probes targeting *Nitrobacter* 16S could be designed (Wagner *et al.*, 1996). Screening of clone libraries is thus necessary to eliminate non *Nitrobacter*-like sequences that can represent up to 48% of the sequences retrieved by these molecular tools (Freitag *et al.*, 2005). By targeting 16S rRNA gene and 23S rRNA gene intergenic spacer, Grundmann *et al.* (2000) obtained a higher resolution for phylogenetic analysis of *Nitrobacter* species isolated from soil or obtained in enrichment medium, but not directly obtained from soils. These results show that the functional gene *nxr*A may be a universal and at the same time, more discriminating target to detect different strains of *Nitrobacter* and possibly other NOB in soils. Despite the small length of the amplified fragment (322 bp), the phylogenetic resolution of this region is sufficient to differentiate species of *Nitrobacter* and *nxrA* copies within the genome of a strain. Moreover this fragment of 322 bp presents the advantage to be a length compatible for the development of fingerprint approaches such as DGGE.

In this study, most of the *nxr*A sequences obtained from soils differed from those obtained for strains. This shows that this new tool allowed the assessment of a previously unknown diversity of *Nitrobacter*-like *nxr*A sequences in soils. The fallow soil (formerly intensively cultivated with maize) presented very low diversity of *nxr*A sequences with only one OTU detected. For the two other soils, the diversity of *nxr*A sequences was higher (Figs 2 and 3). Moreover, these results showed a contrasting phylogenetic distribution of the *Nitrobacter*-like *nxr*A sequences between fallow, permanent pasture and savannah soils. The *nxr*A sequences of the fallow soil grouped in a distinct cluster (cluster 4) (Fig. 5), whereas in the savannah soil all *nxrA* sequences retrieved were included in the branch clustering *N. vulgaris* sequences. In contrast, *nxrA* sequences from the TV soil were spread among five clusters, with four of these clusters including only sequences from the TV soil (Fig. 5). In this TV soil *nxrA* diversity observed was larger than expected and the rarefaction curve indicated that this diversity was far from the real diversity. The authors note that richness of *Nitrobacter*-like *nxrA* sequences in soils seemed to increase with the organic matter content and it may be assumed that soil management can influence *Nitrobacter* diversity and phylogenetic distribution in soil. Similar observations were carried out by Bruns *et al.* (1999) and Kowalchuk *et al.* (2000) concerning the diversity and distribution of AOB in soils. To confirm correlation between *Nitrobacter*-like *nxrA* sequences diversity and soil characteristics and management, more studies are needed on a larger suite of soils exhibiting a larger range of physico-chemical characteristics.

In conclusion, a molecular tool was developed based on functional gene *nxrA* to explore the diversity of *Nitrobacter* in soils. The application of this tool showed that the diversity and phylogenetic distribution of *Nitrobacter*-like *nxr*A sequences may differ according to soil type. This new tool is promising for detecting new *Nitrobacter* species and for studying the *Nitrobacter*-like *nxr*A sequences in soils.

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