NOTE

High-resolution phylogenetic analysis of NO₂⁻-oxidizing *Nitrobacter* species using the *rrs-rrl* IGS sequence and *rrl* genesGeneviève L. Grundmann,¹ Marc Neyra² and Philippe Normand¹Author for correspondence: Philippe Normand. Tel: +33 4 72 43 13 77. Fax: +33 4 72 43 12 23.
e-mail: normand@biomserv.univ-lyon1.fr¹ Ecologie Microbienne,
U.M.R. CNRS 5557, 43
Boulevard du 11 Novembre
1918, Université Claude
Bernard, Lyon I, 69622
Villeurbanne, France² Laboratoire de
Microbiologie, IRD, BP
1386 Dakar, Sénégal**A high-resolution phylogenetic analysis of *Nitrobacter* strains and their neighbours was made using the *rrs-rrl* intergenic spacer sequence and the hypervariable part of the *rrl* gene. The phylogenetic tree obtained was consistent with that which was obtained previously but was much more discriminating, permitting the design of genus-specific primers.****Keywords:** *Nitrobacter*, phylogeny, *rrs-rrl* IGS, *rrl* gene

Nitrobacter is a key microbial group in terms of nitrate production, which affects both soil fertility and water pollution. *Nitrobacter* is the only NO₂⁻-oxidizing genus isolated from soil and freshwater (Watson *et al.*, 1989). It belongs to the α -subclass of the *Proteobacteria*. Four species are mentioned in the literature: *Nitrobacter winogradskyi*, which has standing in nomenclature, '*Nitrobacter hamburgensis*' (Bock *et al.*, 1983) and '*Nitrobacter vulgaris*' (Bock *et al.*, 1990), which are commonly considered as species (Navarro *et al.*, 1992a), and the recently described '*Nitrobacter alkalicus*' (Sorokin *et al.*, 1998). The other NO₂⁻-oxidizing bacteria, members of the genera *Nitrospira*, *Nitrospina* and *Nitrococcus*, are commonly found in seawater and were shown to be phylogenetically very distant from members of the genus *Nitrobacter* since they belong to other subclasses of the *Proteobacteria* (Teske *et al.*, 1994). Because of these traits, it is of interest to study the phylogeny of this group in more detail. Moreover, the ecological importance of nitrate production by NO₂⁻-oxidizers calls for molecular tools such as PCR primers and probes that can be routinely used in ecological studies. It was decided to pursue both goals by using a relevant DNA sequence.

A phylogenetic analysis (Orso *et al.*, 1994) based on *rrs* sequences has shown that the genus *Nitrobacter* is particularly consistent, there being 99% similarity between the three sequences studied (which encom-

passed two of the three species described at the time). They are very closely related to *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris*, in the α -subclass of the *Proteobacteria*: *B. japonicum* has 21–26 differences in the *rrs* compared to *Nitrobacter* spp., corresponding to 98.1–98.5% similarity. *B. japonicum* and *R. palustris* are prevalent in certain soils and thus may constitute false positives. Such close relatedness is an important shortcoming for ecological studies in soil. In fact, it has become increasingly clear that complex environments comprise thousands of uncharacterized bacterial taxa and that amplification using universal primers yields new sequences (Stackebrandt *et al.*, 1993). Moreover, Normand & Chapelon (1997) showed that undescribed sequences were obtained when using a single genus-specific primer targeting the *rrs* gene, highlighting the need for two specific primers with several specific nucleotides at the 3' end for specific targeted PCRs in complex samples. Taken together, previous studies of the *rrs* region suggest that the discriminatory properties are too weak to be used successfully for the high-resolution phylogenetic analysis required for the genus *Nitrobacter*. There are, however, some alternatives in terms of phylogenetic analysis and probe and primer design. The *rrs-rrl* intergenic spacer sequence (IGS) is by far the most variable region of the ribosomal genes, especially for bacteria of the α -subclass of the *Proteobacteria*, in which its length varies from 800 to 1200 nt (Normand *et al.*, 1996). It is at least twice as variable as the *rrl* gene and four times as variable as the *rrs* gene. It has been used for identification purposes in a number of bacteria (East & Collins, 1993; Honeycutt *et al.*, 1995; Minnick *et al.*, 1994; Rouvier *et al.*, 1996; Riffard *et al.*, 1998). The *rrs-rrl* IGS region of members of the genus *Nitrobacter* has also been used profitably

Abbreviations: IGS, intergenic spacer sequence; s/s, substitutions per site. The GenBank/EMBL/DBJ accession numbers for the sequences described in this work are as follows: *Nitrobacter winogradskyi* ATCC 25391^T, AJ005010; '*Nitrobacter* sp.' DE30, AJ005008; *Nitrobacter winogradskyi* 'agilis', AJ005009; '*Nitrobacter* sp.' LL, AJ005014; '*Nitrobacter hamburgensis*' X14, AJ005011; '*Nitrobacter vulgaris*' Z, AJ005012; *Rhodopseudomonas palustris* DSM 123, AJ005013.

Table 1. List of bacterial strains used in the present study

Accession numbers are given for the strains used for phylogenetic analysis.

Taxon	Strain*	Source/reference*	EMBL no.
<i>Nitrobacter winogradskyi</i>	ATCC 25391 ^T		AJ005010
' <i>Nitrobacter</i> sp.'	Strain DE30	Navarro <i>et al.</i> (1992a)	AJ005008
<i>Nitrobacter winogradskyi</i>	Strain 'agilis'	Navarro <i>et al.</i> (1992a)	AJ005009
' <i>Nitrobacter</i> sp.'	Strain LL	Navarro <i>et al.</i> (1992a)	AJ005014
' <i>Nitrobacter hamburgensis</i> '	Strain X14	Bock <i>et al.</i> (1983)	AJ005011
' <i>Nitrobacter vulgaris</i> '	Strain Z	Bock <i>et al.</i> (1990)	AJ005012
<i>Rhodopseudomonas palustris</i>	DSM 123		AJ005013
<i>Azospirillum lipoferum</i>	Strain 4B	Bally <i>et al.</i> (1983)	
<i>Burkholderia cepacia</i>	ATCC 25416 ^T		—
<i>Azorhizobium caulinodans</i>	ORS 571	Dreyfus <i>et al.</i> (1988)	
<i>Sinorhizobium meliloti</i>	NZP 4027		—
<i>Arthrobacter globiformis</i>		Berger <i>et al.</i> (1996)	—
<i>Agrobacterium tumefaciens</i>	C58	CFBP	—
<i>Bradyrhizobium japonicum</i>	SMGS1	Pinochet <i>et al.</i> (1993)	
<i>Bradyrhizobium japonicum</i>	G49	Fernandez-Flouret & Cleyet-Marel (1987)	—
<i>Bradyrhizobium japonicum</i>	GMB1	Fernandez-Flouret & Cleyet-Marel (1987)	—
<i>Bradyrhizobium japonicum</i>	USDA110		Z35330

* Source/reference: ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NZP, Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; CFBP, Collection Française des Bactéries Phytopathogènes, INRA, Angers, France; INRA, Institut National de la Recherche Agronomique.

for the characterization of natural populations using PCR-RFLP analysis (Navarro *et al.*, 1992b) and has been found to be highly discriminating, even between strains with *rrs* sequences differing by less than 15 positions (99% similarity; Orso *et al.*, 1994).

The *rrl* (23S) gene also has characteristics that make it an interesting target for phylogeny analysis and probe design. Not only is it significantly longer than the *rrs* gene (approx. 3000 nt instead of 1500 nt), it is also highly variable in the first quarter of the gene (Selenska-Pobell & Evguenieva-Hackenberg, 1995; Roller *et al.*, 1992; Hönerlage *et al.*, 1994); the number of total sequences available for comparison has increased to more than 2500 (at February 2000).

The hypervariable regions of the *rrs-rrl* (16S–23S IGS) intergene and the 5' end of the *rrl* (23S rRNA gene) appeared appropriate (Selenska-Pobell & Evguenieva-Hackenberg, 1995) for improving the resolution of phylogenetic analysis of the genus *Nitrobacter* and for defining favourable regions for tool design. The potential discriminating properties of the region necessary to achieve both of these goals were evaluated; sequencing was carried out in the context of phylogenetic analysis and probe design. Potential routine applications for *Nitrobacter* population studies on environmental samples were tested by using PCR-RFLP analysis of these regions and comparing the results with phylogenetic analysis of the same regions (to validate the more simple PCR-RFLP approach).

Six *Nitrobacter* strains were studied (Table 1). Cells

Fig. 1. Primer positions in the *rrs*-IGS-*rrl* region. Only the last number of each primer name is indicated.

were grown at 28 °C in mineral medium with NO₂⁻ as the energy source (Schmidt & Belser, 1994), except for '*N. hamburgensis*' strain X14 and '*N. vulgaris*' strain Z, which were grown according to Bock *et al.* (1983) and Bock *et al.* (1990), respectively. *B. japonicum* cells were grown in YEM medium. The DNA of *R. palustris* was provided by A. Vermeglio (CEA, Cadarache, France).

Total DNA from pure cultures was extracted as described before (Navarro *et al.*, 1992a) for amplification and sequencing.

Double-stranded amplification was performed on the *rrs-rrl* IGS spacer and on the 5' part of the *rrl* gene by using a modification of the PCR procedure of Mullis & Faloona (1997). The set of primers (the positions of which are indicated in Fig. 1) used for amplification of the IGS spacer comprised FGPS1490-72 (5'-TGCGGCTGGATCCCC-TCCTT-3') and either FGPL50'-73 (5'-ATCGGCT-CGAGGTGCCAAGGGATCCA-3') or FGPL132'-38 (5'-CCGGGTTTCCCCATTTCGG-3'). The set of

primers used for amplification of the 5' part of the 23S gene comprised FGPL43-544 (5'-AGAGGCGAT-GAAGGACGT-3') and FGPL580'-545 (5'-ACCTT-TCCCTCACGGTCAT-3'). The PCR reactions were carried out in a final volume of 50 µl containing template DNA, reaction buffer [10 mM Tris/HCl (pH 8.3), 1.5 mM MgCl₂, 50 µM KCl and 10% (w/v) gelatin], 200 µM of each dNTP, 0.5 µM oligomers and 2 U *TaqI* DNA polymerase (Gibco-BRL). The amplification reactions were carried out for 35 cycles as follows: denaturation of DNA at 95 °C for 1 min, annealing for 1 min at 52, 55 or 58 °C and extension at 72 °C for 2 min. To analyse the amplification products, 5 µl reaction mix was separated by electrophoresis on a 2% (w/v) NuSieve (FMC) agarose gel. The *Nitrobacter*-specific PCR was performed with primers FGPI149-457 (5'-CGGCGCTTGTGCTCA-3') and FGPL420'-458 (5'-CCTGACAGGTTCTGG-3').

The *rrs-rrl* IGS and *rrl* amplicons (obtained with primers FGPS1490-72/FGPL50'-73 and FGPL143-544/FGPL580'-545, respectively) were sequenced according to Sanger *et al.* (1977) using the dideoxy chain-termination sequencing procedure and the amplification primers. The sequences were deposited in the EMBL sequence database (Table 1).

GenBank was scanned for related sequences by using the algorithm BLAST and the closely related *B. japonicum* (strain USDA 110) sequence (accession no. Z35330) found was included in the following analyses. Sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997) and indel-containing regions were excluded from the analyses. Matrix pairwise comparisons were corrected for multiple base substitutions by the two-parameter method of Kimura (1980). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and parsimony (Kluge & Farris, 1969) methods. A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance-tree topologies obtained (Felsenstein, 1985). Graphic representations of the resulting trees were produced using NJPLOT (Perrière & Gouy, 1996) and PHYLO_WIN (Galtier *et al.*, 1996) software.

The resulting amplicons of the region comprising part of the *rrs-rrl* IGS and part of *rrl* obtained with *Nitrobacter*-specific primers FGPI149-457 and FGPL420'-458 were restricted with restriction enzymes *Nla*III, *Mse*I, *Aci*II, *Cac*81, *Sau*3aI, *Hinf*I and *Msp*I according to the manufacturer's instructions (Boehringer). The digested fragments were electrophoresed in 2.5% (w/v) Metaphor agarose (FMC) with TBE buffer for 3 h at 30 V cm⁻¹. The photographs, taken at 300 nm, were scanned and the bands sizes determined and compared.

Intergenic spacer sequences

The six *Nitrobacter* strains and *R. palustris* (Table 1) yielded abundant 700 nt amplicons. In the case of *R. palustris*, the amplicon obtained with primers

FGPS1490-72 and FGPL50'-73 was only 300 nt long and its sequence indicated that it was not homologous to the other IGSs. Only when primer FGPL50'-73 was replaced by primer FGPL132'-38 was an amplicon of the correct length obtained (Fig. 1). The sequence of *R. palustris* was examined at the target site for primer FGP50'-73, and, as expected, one mismatch was present at the 3' part which was not the case in the *B. japonicum* and *N. winogradskyi* strain 'agilis' sequences. The putative isoleucine and alanine tRNAs at coordinates 278-355 and 389-464 were found to be conserved in all sequences examined.

The *rrl* gene sequences

The *rrl* alignment is convincing except in the hyper-variable part of the *rrl* gene at coordinates 992-1060 (the coordinates of *B. japonicum* strain USDA110; accession no. Z35330).

Distances

The distances calculated for the IGS and the *rrl* gene are given in Table 2. The mean genetic distance between IGS sequences is 0.133 substitutions per site (s/s), which is an underestimation given the fact that only positions without gaps are considered, and 0.047 s/s when only *Nitrobacter* sequences are considered. Those distances are much lower in the *rrl* gene (0.041 s/s; 0.022 s/s when only *Nitrobacter* sequences are considered).

Phylogenetic analysis

Phylogenetic trees derived from these sequences were in good agreement, irrespective of whether the *rrl* gene or the IGSs were used (not shown). Thus, both sets of data were pooled to derive a unique tree (Fig. 2). '*N. hamburgensis*' strain X14 was at an intermediate position between *B. japonicum* and the other *Nitrobacter* spp. *N. winogradskyi* was consistently closer to *N. winogradskyi* strain 'agilis' and the '*Nitrobacter* sp.' strain DE30 (Navarro *et al.*, 1992a) isolate, whereas '*Nitrobacter* sp.' strain LL, described as a putative genomic species by Navarro *et al.* (1992a), was closer to '*N. vulgaris*' strain Z. Bootstrap results and parsimony and maximum-likelihood analyses all concurred in grouping together all of the *Nitrobacter* sequences.

Furthermore, '*Nitrobacter* sp.' strain LL was suggested as a genomic species (Navarro *et al.*, 1992a), although this could not be determined by DNA-DNA hybridization against '*N. vulgaris*' strain Z. These strains were both very closely related at the level of rDNA sequences. The DNA-DNA homology (%) determined in a previous study on *N. winogradskyi*, '*Nitrobacter* sp.' strain LL, *N. winogradskyi* strain 'agilis', '*Nitrobacter* sp.' strain DE30 (Navarro *et al.*, 1992a) and '*N. hamburgensis*' strain X14 and the identity (%) in the hypervariable ribosomal regions obtained in the present work were highly correlated

Table 2. Distances (in substitutions/site, s/s) in the IGS (above the diagonal) and in the *rrl* gene (below the diagonal) calculated with the no-gap option and according to Kimura (1980)

Mean IGS distances: 0.133 s/s (not considering *Rhodopseudomonas palustris* 0.047 s/s). Mean partial 23S distances: 0.041 s/s (not considering *Rhodopseudomonas palustris* 0.022 s/s).

Taxon	Strain	Nagil	NDE30	Nwino	Nvulg	NspLL	Nhamb	Bjapo	Rhodo
<i>N. winogradskyi</i>	Strain 'agilis'	—	0.024	0.033	0.067	0.065	0.081	0.187	0.298
' <i>Nitrobacter</i> sp.'	Strain DE30	0.007	—	0.044	0.069	0.069	0.087	0.193	0.312
<i>N. winogradskyi</i>		0.012	0.005	—	0.057	0.056	0.088	0.185	0.291
' <i>N. vulgaris</i> '	Strain Z	0.012	0.009	0.014	—	0.026	0.070	0.174	0.277
' <i>Nitrobacter</i> sp.'	Strain LL	0.012	0.009	0.014	0.009	—	0.070	0.183	0.284
' <i>N. hamburgensis</i> '	Strain X14	0.039	0.037	0.037	0.031	0.036	—	0.179	0.283
<i>B. japonicum</i>	USDA 110	0.087	0.085	0.082	0.082	0.077	0.082	—	0.241
<i>R. palustris</i>	DSM 123	0.085	0.077	0.080	0.080	0.077	0.080	0.074	—

Fig. 2. Neighbour-joining phylogenetic tree based on the IGS+*rrl* sequences. Bootstrap results (%) are encircled. Those groupings also obtained by parsimony (P) and by maximum-likelihood (L) methods are indicated by the appropriate letter below the node.

($r^2 = 0.82$, $P < 0.01$). The three strains *N. winogradskyi*, *N. winogradskyi* strain 'agilis' and '*Nitrobacter* sp.' strain DE30 which belong to the same genomic species (Navarro *et al.*, 1992a) had identity levels of between 95.6 and 97.6%. The 97.4% identity that was obtained between '*Nitrobacter* sp.' strain LL and '*N. vulgaris*' strain Z showed that they presumably belong to the same species, confirming the existence of four species: *N. winogradskyi*, '*N. vulgaris*', '*N. hamburgensis*' and '*N. alkalicus*' (Bock *et al.*, 1990; Navarro *et al.*, 1992a; Sorokin *et al.*, 1998). In addition, '*N. hamburgensis*' strain X14 and '*N. vulgaris*' strain Z, both of which share the ability to grow heterotrophically (Bock *et al.*, 1983, 1990), were less related to one another than '*N. vulgaris*' strain Z and '*Nitrobacter* sp.' strain LL (Fig. 2), which is mostly lithotrophic (Gay *et al.*, 1983). Thus, bacteria that are mostly lithotrophic with little heterotrophic capability and bacteria with a strong heterotrophic capability can be found in

the same species. This observation also indicates that heterotrophy has not been a meaningful evolutionary step. Furthermore, the present study confirms the results of our previous study, based on the 16S gene, which showed that '*N. hamburgensis*' strain X14 diverged early from the *Bradyrhizobium*–*Rhodopseudomonas*–*Nitrobacter* ancestor.

The evolution of the *rrs*–*rrl* IGS and the *rrl* sequence appears to have been rapid and composed of both substitutions and insertion/deletion events, which may obscure distant relationships but nevertheless permit discrimination and phylogenetic studies of closely related strains, a task impossible otherwise in the dense *Bradyrhizobium*–*Rhodopseudomonas*–*Nitrobacter* line of descent. All these factors point to that region as the most appropriate for the design of specific primers, especially in the study of bacteria of the α -subclass of the *Proteobacteria*.

Fig. 3. Relationship between total DNA-DNA homology (%) among *Nitrobacter* species (Navarro *et al.*, 1992a) and identity (%) derived from PCR-RFLP analysis and sequence analysis of amplicons obtained with *Nitrobacter*-specific primers. □, DNA-DNA versus RFLP ($r^2 = 0.78$); ◆, DNA-DNA versus sequences ($r^2 = 0.89$).

Specific primers

Predictions of primer specificity are hard to make: even though more than 19800 ribosomal sequences have already been deposited in DNA sequence databases, several others remain unknown. Most of the studies that target the soil or other such complex environments with universal *rrs* primers have yielded new sequences (Borneman *et al.*, 1996; Jurgens *et al.*, 1997; Stackebrandt *et al.*, 1993; Ward *et al.*, 1990; Wise *et al.*, 1997). Specific primers can only be designed relative to the sequences known at the time and should be checked regularly against new sequences. Thus, primer specificity can only be thought of as a dialectic dynamic process with successive rounds of improvement.

The alignment of the *rrs-rrl* IGS and *rrl* region was used to design genus-specific primers. The most discriminating sequences had three nucleotides that were conserved for all *Nitrobacter* spp. but different from *B. japonicum* and *R. palustris* sequences; one was found in the IGS and one was in the *rrl* gene. The resulting primers with the three nucleotides at the 3' part of the target, designated as FGPI149-457 and FGPL420'-458, were then tested on DNA from *Nitrobacter* pure cultures at the temperature calculated for annealing (52 °C) and at 56, 58 and 60 °C, to improve stringency. An amplicon of the expected length (approximately 1050 nt) was obtained from all of the *Nitrobacter* spp. tested at these temperatures, the optimum temperature being 58 °C (data not shown). No amplicon was obtained at 58 °C with the bacterial isolates not belonging to the genus *Nitrobacter* (*R. palustris* DSM123, *Azospirillum lipoferum* strain 4B, *Burkholderia cepacia* ATCC 25416^T, *Azorhizobium caulinodans* ORS 571, *Sinorhizobium meliloti* NZP 4027, *Arthrobacter globiformis* SI55, *Agrobacterium tumefaciens* C58, *B. japonicum* SMGS1, *B. japonicum* G49 and *B.*

japonicum GMB1) (references are given in Table 1). A weak amplicon was obtained from *B. japonicum* G49 at 52 °C only. Furthermore, these primers were compared with GenBank by using BLAST software and no similar sequences could be found. Thus the primers FGPI149-457 and FGPL420'-458 can be defined as *Nitrobacter*-specific.

PCR-RFLP analysis

RFLP analysis was performed on amplicons obtained with primers FGPI149-457 and FGPL420'-458 on *Nitrobacter* pure cultures, yielding patterns made up of from three to six bands larger than 90 nt (the resolution threshold of Metaphor gels). The set of seven enzymes used allowed the construction of a tree (not shown) similar to that which was obtained by sequence analysis. The comparison of DNA-DNA homology (Navarro *et al.*, 1992a) and similarity levels obtained with RFLP analysis yielded a high correlation coefficient value ($r^2 = 0.78$, $P < 0.01$) (Fig. 3), as did the correlation between RFLP-analysis-derived similarity and sequence similarity levels ($r^2 = 0.81$). Thus, PCR-RFLP analysis of this region seems to be a valid, discriminating tool suitable for routine use with *Nitrobacter* isolates and complex samples.

The *rrs-rrl* IGS and the contiguous *rrl* area of the ubiquitous ribosomal region were found to be highly discriminating even between strains with *rrs* sequences differing by less than 15 positions (> 99% similarity) (Orso *et al.*, 1994), permitting high-resolution phylogenetic analysis and specific tool design for monitoring the genus *Nitrobacter* and for diversity analysis. One of the aims of microbial ecology is to clarify how bacterial populations fluctuate in response to environmental factors; this requires a fast, cheap, discriminating and reliable method for strain identification. PCR-RFLP analysis of this region, using the two specific primers that have been defined and tested, meets these criteria and has potential in the design of specific probes.

References

- Bally, R., Thomas-Bauzon, D., Heulin, T., Balandreau, J., Richard, C. & De Ley, J. (1983). Determination of the most frequent N_2 -fixing bacteria in a rice rhizosphere, *Can J Microbiol* **29**, 881–887.
- Berger, F., Morellet, N., Menu, F. & Potier, P. (1996). Cold shock and cold acclimation proteins in the psychrophilic bacterium *Arthrobacter globiformis* SI55, *J Bacteriol* **178**, 2999–3007.
- Bock, E., Koops, H. P., Moller, U. C. & Rudert, M. (1990). A new facultatively nitrite-oxidizing bacterium, *Nitrobacter vulgaris* sp. nov., *Arch Microbiol* **153**, 105–110.
- Bock, E., Sundermeyer-Klinger, H. & Stackebrandt, E. (1983). New facultative lithotrophic nitrite-oxidizing bacteria, *Arch Microbiol* **136**, 281–284.
- Borneman, J., Skroch, P. W., O'Sullivan, K. M., Palus, J. A., Rumjanek, N. G., Jansen, J. L., Nienhuis, J. & Triplett, E. W. (1996). Molecular microbial diversity of an agricultural soil in Wisconsin, *Appl Environ Microbiol* **62**, 1935–1943.

- Dreyfus, B., Garcia, J. L. & Gillis, M. (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*, *Int J Syst Bacteriol* **38**, 89–98.
- East, A. K. & Collins, M. D. (1993). Molecular characterization of DNA encoding 23S rRNA and 16S–23S rRNA intergenic spacer regions of *Aeromonas hydrophila*, *FEMS Microbiol Lett* **106**, 129–133.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach, *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap, *Evolution* **39**, 783–791.
- Fernandez-Flouret, D. & Cleyet-Marel, J. C. (1987). Identification par test ELISA de souches de *Bradyrhizobium japonicum* en culture et dans les nodosités de soja (*Glycine max* (L.) Merr.), *C R Acad Agric Fr* **73**, 163–171.
- Galtier, N., Gouy, M. & Gautier, C. (1996). SEAVIEW and PHYLO-WIN, two graphic tools for sequence alignment and molecular phylogeny, *CABIOS* **12**, 543–548.
- Gay, G., Josserand, A. & Bardin, R. (1983). Growth of the two strains of *Nitrobacter* in mixotrophic and chemoorganotrophic conditions, *Can J Microbiol* **29**, 394–397.
- Hönerlage, W., Hahn, D., Zepp, K., Zeyer, J. & Normand, P. (1994). A hypervariable 23S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*, *Syst Appl Microbiol* **17**, 433–443.
- Honeycutt, R. J., Sobral, B. W. & McClelland, M. (1995). tRNA intergenic spacers reveal polymorphisms diagnostic for *Xanthomonas albilineans*, *Microbiology* **141**, 3229–3239.
- Jurgens, G., Lindstrom, K. & Saano, A. (1997). Novel groups within the kingdom Crenarchaeota from boreal forest soil, *Appl Environ Microbiol* **63**, 803–805.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, *J Mol Evol* **16**, 111–120.
- Kluge, A. G. & Farris, J. S. (1969). Quantitative phyletics and the evolution of anurans, *Syst Zool* **18**, 1–32.
- Minnick, M. F., Strange, J. C. & Williams, K. F. (1994). Characterization of the 16S–23S rRNA intergenic spacer of *Bartonella bacilliformis*, *Gene* **143**, 149–150.
- Mullis, K. B. & Faloona, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction, *Methods Enzymol* **155**, 335–350.
- Navarro, E., Fernandez, M. P., Grimont, F., Clays-Josserand, A. & Bardin, R. (1992a). Genomic heterogeneity of the genus *Nitrobacter*, *Int J Syst Bacteriol* **42**, 554–560.
- Navarro, E., Simonet, P., Normand, P. & Bardin, R. (1992b). Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer, *Arch Microbiol* **157**, 107–115.
- Normand, P. & Chapelon, C. (1997). Direct characterization of *Frankia* and of close phyletic neighbors from an *Alnus viridis* rhizosphere, *Physiol Plant* **99**, 722–731.
- Normand, P., Ponsonnet, C., Nesme, X., Neyra, M. & Simonet, P. (1996). ITS analysis of procaryotes. In *Molecular Microbial Ecology Manual* section 3.4.5, pp. 1–12. Edited by A. D. L. Akkermans, J. D. van Elsas & F. J. De Bruijn. Dordrecht: Kluwer.
- Orso, S., Gouy, M., Navarro, E. & Normand, P. (1994). Molecular phylogenetic analysis of *Nitrobacter* spp, *Int J Syst Bacteriol* **44**, 83–86.
- Perrière, G. & Gouy, M. (1996). WWW-Query: an on-line retrieval system for biological sequence banks, *Biochimie* **78**, 364–369.
- Pinochet, X., Arnaud, F. & Cleyet-Marel, J. C. (1993). Competition for nodule occupancy of introduced *Bradyrhizobium japonicum* strain SMG1S in French soils already containing *Bradyrhizobium japonicum* strain G49, *Can J Microbiol* **39**, 1022–1028.
- Riffard, S., Lo Presti, F., Normand, P., Forey, F., Reyrolle, M., Vandenesch, F. & Etienne, J. (1998). Species identification in *Legionella* via intergenic 16–23S ribosomal spacer PCR analysis, *Int J Syst Bacteriol* **48**, 723–730.
- Roller, C., Ludwig, W. & Schleifer, K. H. (1992). Gram-positive bacteria with a high DNA G + C content are characterized by a common insertion within their 23S rRNA genes, *J Gen Microbiol* **138**, 1167–1175.
- Rouvier, C., Prin, Y., Reddell, P., Normand, P. & Simonet, P. (1996). Genetic diversity among *Frankia* strains nodulating members of the family *Casuarinaceae* in Australia revealed by PCR and restriction fragment length polymorphism analysis with cruhed root nodule, *Appl Environ Microbiol* **62**, 979–985.
- Saitou, R. & Nei, M. (1987). A neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol Biol Evol* **44**, 406–425.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors, *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schmidt, E. L. & Belser, L. W. (1994). Autotrophic nitrifying bacteria. In *Methods of Soil Analysis. Part 2, Microbiological and Biochemical Properties* (SSSA Book Series no. 5), pp. 159–177. Edited by R. W. Weaver, J. S. Angle & P. S. Bottomley. Madison, WI: Soil Science Society of America.
- Selenska-Pobell, S. & Evguenieva-Hackenberg, E. (1995). Fragmentation of the large-subunit rRNA in the family *Rhizobiaceae*, *J Bacteriol* **177**, 6993–6998.
- Sorokin, Y. S., Muyzer, G., Brinkhoff, T., Kuenen, J. G. & Jetten, M. S. M. (1998). Isolation and characterization of a novel facultatively alkaliphilic *Nitrobacter* species, *N. alkalicus* sp. nov., *Arch Microbiol* **170**, 345–352.
- Stackebrandt, E., Liesack, W. & Goebel, B. M. (1993). Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis, *FASEB J* **7**, 232–236.
- Teske, A., Alm, E., Regan, J. M., Toze, S., Rittmann, B. E. & Stahl, D. A. (1994). Evolutionary relationships among ammonia- and nitrite-oxidizing bacteria, *J Bacteriol* **176**, 6623–6630.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acids Res* **25**, 4876–4882.
- Ward, D. M., Weller, R. & Bateson, M. M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community, *Nature* **344**, 63–65.
- Watson, S. W., Bock, E., Harms, H., Koops, H. P. & Hooper, A. B. (1989). Nitrifying bacteria. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, pp. 1808–1834. Edited by J. C. Holt, J. T. Staley, M. P. Bryant & N. Pfennig. Baltimore: Williams & Wilkins.
- Wise, M. G., McArthur, J. V. & Shimkets, L. J. (1997). Bacterial diversity of a Carolina bay as determined by 16S rRNA gene analysis: confirmation of novel taxa, *Appl Environ Microbiol* **63**, 1505–1514.