APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1995, p. 2093–2098 Vol. 61, No. 6
0099-2240/95/$04.00+0

Detection and Counting of *Nitrobacter* Populations in Soil by PCR

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Received 14 September 1994/Accepted 23 March 1995

Although the biological conversion of nitrite to nitrate is a well-known process, studies of *Nitrobacter* populations are hindered by their physiological characteristics. This report describes a new method for detecting and counting *Nitrobacter* populations in situ with the PCR. Two primers from the 16S rRNA gene were used to generate a 397-bp fragment by amplification of *Nitrobacter* species DNA. No signal was detected from their phylogenetic neighbors or the common soil bacteria tested. Extraction and purification steps were optimized for minimal loss and maximal purity of soil DNA. The detection threshold and accuracy of the molecular method were determined from soil inoculated with 10, 10^2, or 10^3 *Nitrobacter hamburgensis* cells per g of soil. Counts were also done by the most-probable-number (MPN)-Griess and fluorescent antibody methods. PCR had a lower detection threshold (10^2 *Nitrobacter* cells per g of soil) than did the MPN-Griess or fluorescent antibody method. When PCR amplification was coupled with the MPN method, the counting rate reached 65 to 72% of inoculated *Nitrobacter* cells. Tested on nonsterile soil, this rapid procedure was proved efficient.

A better understanding of the nitrification process could help to reduce excess nitrate production and ensuing losses due to denitrification or leaching of water-soluble nitrate anions (2, 21). Improved control of nitrification should lead to better plant productivity with fewer risks of eutrophication and domestic water pollution. The microorganisms involved in this process have been classically grouped into the *Nitrobacteriaceae* family (44). One group of bacteria oxidizes ammonia to nitrite, and another oxidizes nitrite to nitrate (36, 44). An important member of the latter group, the genus *Nitrobacter*, is present in freshwater, seawater, and soil, where it is the only known nitrite oxidizer (36, 44). Species of this genus grow chemolithotrophically as well as chemo-organotrophically (44).

There have been several studies on the metabolism, survival, and growth of nitrite oxidizers in pure cultures and on their nitrifying activity in various environments (13, 20), but fewer studies have dealt with natural *Nitrobacter* populations. Although the biological conversion of nitrite to nitrate is a well-known process, studies of *Nitrobacter* populations are currently hampered by inadequate methods of detection and counting.

This failure is due in part to the unfavorable physiological characteristics of these bacteria, namely, slow growth, small biomass, and susceptibility of cultures to contamination (2, 36). The only way to count *Nitrobacter* populations in soil, the most-probable-number (MPN)-Griess procedure, is time consuming and selective (4, 16), and there is no antibody against the whole *Nitrobacter* genus (18, 25, 27).

The PCR (24) is a technique which amplifies a few target DNA sequences to make them detectable and quantifiable. This method has been used to detect pathogenic microorganisms in food (14), in clinical samples (19), and, more recently, in soil and sediment (9, 33, 42). It has been used in conjunction with the MPN method to count *Agrobacterium* populations in soil (33). Molecular biology techniques have also been used to provide genomic, taxonomic, and phylogenetic data on the genus *Nitrobacter* (26, 28, 31). This approach is a potential alternative to existing methods for the study of these microorganisms in situ (25).

This work was designed to develop a procedure for counting *Nitrobacter* populations in the soil, based on genomic tools, by PCR coupled with the MPN method (PCR-MPN). We adapted a method of extracting and purifying *Nitrobacter* DNA from soil and identified the optimal conditions of amplification to ensure optimal counting precision.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. *Nitrobacter* strains were grown at 28°C in a mineral medium with 2 g of $NaNO_2$ $liter^{-1}$ (38); strain X14 was grown under mixotrophic conditions (3).

Soils. Two soils were used in this work. One was a sandy calcareous soil from the alluvial plain of the Ain and Rhône rivers (Loyettes, Rhône), likely to accumulate nitrites because of *Nitrobacter* activity inhibition (12). The other was an agricultural sandy loam soil (La Côte Saint André, Isère) (Table 2).

Oligonucleotide primers. The oligonucleotides used for PCR priming were selected by comparing the total 16S rRNA sequences of *Nitrobacter* species (*Nitrobacter winogradskyi*, *Nitrobacter hamburgensis*, and *Nitrobacter* genomic species strain 2) with the phylogenetic neighbors of this genus (*Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Photorhizobium thompsonianum*, and *Agrobacterium tumefaciens*) (31) (Table 1). The two primers selected were a *Nitrobacter*-specific primer (5′ TTTTTTGAGATTTGCTAG 3′ [FGPS1269′]) and a nonspecific primer (5′ CTAAAACTCAAAGGAATTGA 3′ [FGPS872] [31]). (FGPS refers to the small subunit 16S rRNA gene, and the number is the 5′ coordinate of the oligonucleotide corresponding to the sequences deposited in GenBank under the accession numbers LL11661, LL11662, and LL11663 [31]. A prime after the number indicates that the primer is complementary to the rRNA.) The synthetic oligonucleotides were obtained from the Centre de Génétique Moléculaire et Cellulaire, Université Lyon I, Lyon, France.

DNA extraction from pure cultures. DNA was extracted and purified as described by Brenner et al. (7), except that achromopeptidase (Wako Pure Chemical, Dallas, Tex.) was added to the lysis mixture (39).

PCR amplification of pure DNA. Pure DNA from *Nitrobacter* species (*N. winogradskyi*, *Nitrobacter agilis*, *N. hamburgensis*, and *Nitrobacter* genomic species strain 2), their phylogenetic neighbors (*Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Photorhizobium thompsonianum*, and *Agrobacterium tumefaciens*), and common soil bacteria (*Pseudomonas fluorescens*, *Burkholderia cepacia*, *Azospirillum lipoferum*, *Azospirillum brasilense*, *Flavobacterium* sp., *Xanthomonas albilineans*, *Rhizobium meliloti*, *Bacillus azotoformans*, and *Streptomyces lividans*) was amplified by PCR in a total volume of 50 μl under a layer of paraffin oil. One microliter of template DNA was added in a mixture of 1× PCR amplification buffer (10× buffer contains 100 mM Tris-HCl [pH 8.3], 500 mM

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TABLE 1. Bacterial strains used

Strain	Source or reference[a]
Nitrobacter winogradskyi W^T (=ATCC 25391^T)	ATCC
Nitrobacter agilis (=ATCC 14123)	ATCC
Nitrobacter hamburgensis	3
Nitrobacter genomic species strain 2 LL	18
Rhodopseudomonas palustris^T (=DSM123^T)	DSM
Bradyrhizobium japonicum USDA 110	USDA
Photorhizobium thompsonianum BTAi1	11
Agrobacterium tumefaciens C58	CFBP
Pseudomonas fluorescens AK15	45
Burkholderia cepacia (=ATCC 25416)	ATCC
Azospirillum lipoferum 4B	1
Azospirillum brasilense (=ATCC 29145)	ATCC
Flavobacterium sp. (=ATCC 33514)	ATCC
Xanthomonas albilineans (=LMG 494)	LMG
Rhizobium meliloti 2011	8
Bacillus azotoformans (=ATCC 29788)	ATCC
Streptomyces lividans TK24	18

[a] ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; USDA, U.S. Department of Agriculture, Beltsville, Md.; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; CFBP, Collection Française de Bactéries Phytopathogènes, Angers, France.

KCl, 15 mM $MgCl_2$, and 0.1% [wt/vol] gelatin), 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer, and 2.5 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md.). Amplifications were done with a Biometra Trio Thermoblock TB-1 (Gottingen, Germany) with the following program: initial denaturation for 3 min at 95°C and then 35 cycles of DNA denaturation for 1 min at 95°C, primer annealing for 1 min at 50°C, DNA extension for 1 min at 72°C, and a final extension for 3 min at 72°C.

Soil inoculations. The sandy loam soil was packaged into microcosms holding 5 g of dried soil and sterilized by two 25-kGy doses of gamma radiation from a ^{60}Co source (Ionosos, Dagneux, France). Checking of soil sterility was done by plate techniques on nutrient agar (bioMerieux) 12 days after the gamma radiation treatment. The gamma radiation-sterilized soil was stored for 8 weeks before use to stabilize it and to avoid remaining dead microbial bodies and residual enzyme activities (34, 22). The soil was moistened 5 days before use, and the humidity was adjusted to the field capacity (36% of dry weight) at the time of inoculation. *N. hamburgensis* X14 pure culture in the exponential phase was centrifuged at 22,000 × *g* for 30 min, and the cell pellet was washed several times with sterile phosphate buffer (2 mM, pH 7.3). Inoculum densities were adjusted optically at 580 nm. Soil samples were inoculated, in a volume of 800 μl, with 0 (control), 10, 10^2, or 10^3 *N. hamburgensis* X14 cells per g of soil. Microcosms were incubated for one night at 28°C in a water-saturated atmosphere. Each microcosm was then treated as follows: 1 g was reserved for PCR-MPN, and 4 g was used for the MPN-Griess method and immunofluorescence.

MPN-Griess counting. The protocol used for MPN-Griess counting was based on that of Both et al. (5). Four grams of soil microcosm was suspended in phosphate buffer (diluted 1:10), and bacteria were released from the soil with a Waring blender. The dispersed soil solution was subsampled before sedimentation for a series of dilutions (1:10) and inoculations of MPN plates with a 2× *Nitrobacter* autotroph medium with 5 mM $NaNO_2$. Samples were incubated for 104 days at 28°C in the dark, the Griess reagent was added, and the number of *Nitrobacter* cells per g of soil was determined with Cochran's tables (10).

Immunofluorescence. A modified procedure developed by Schmidt (37) was used to quantify fluorescent antibodies of microorganisms growing in soil (17). The bacteria were released from the soil with a Waring blender, soil colloids were sedimented out of suspension, and bacteria remaining in a known volume of suspension were concentrated on a membrane filter. The filter was stained with gelatin-rhodamine to reduce interfering fluorescence resulting from nonspecific adsorption of the antibody to the filter surface and soil particles. The primary antibacterial-specific antibody prepared in rabbits was followed by a second fluorescent anti-rabbit antibody. Reactive cells were examined and counted with a Zeiss Universal microscope equipped for epifluorescence.

TABLE 2. Characteristics of the soils used

Soil	Soil content (%)					
	Clay	Loam	Sand	Organic C	Water capacity (%)	pH
La Côte Saint André	17	39.2	40	2.6	40	6.4
Loyettes	7	28	65	4.44	31.2	8.1

Soil DNA extraction and purification. Protocols for soil DNA extraction were tested on nonsterile soils of La Côte Saint André and Loyettes (Picard et al. [33] and Nesme [29]). A direct method of extraction, including only physical treatments, was adopted.

The largest amounts of soil DNA were obtained from 600 mg of dried soil, extracted as three batches of 200 mg which were then pooled during purification. The dried soil was ground with a freezer-mill (SPEX, Metuchen, N.J.) and sonicated with a Cup Horn in extraction buffer (50 mM Tris, 20 mM disodium EDTA [pH 8.0], 100 mM NaCl, 1% [wt/vol] polyvinylpolypyrrolidone [Sigma Chemical Co., St. Louis, Mo.]) at 2/10 maximum power (600 W; Bioblock, Illkirch, France) for 4 min at 50% of active cycles. The cell debris was removed by centrifugation (3,835 × *g* for 5 min), and the supernatant containing DNA was saved. The pellet was resuspended in extraction buffer and subjected to three successive thermal shocks (liquid nitrogen and boiling water). The DNA was removed from the lysed pellet by three washes with extraction buffer. Finally, DNA was precipitated with ethanol and resuspended in 100 μl of TE buffer (pH 8; 10 mM Tris-HCl, 1 mM EDTA).

The crude DNA was purified through two types of columns, namely, Sephadex G-200 (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) (29, 43) and Elutip-d (Schleicher & Schuell, Dassel, Germany) (33). The Sephadex G-200 column was swollen in TE buffer (pH 8) and packed into sterile syringes with glass wool. Excess TE buffer was removed by centrifugation for 10 min at 1,100 × *g*. The 100 μl of DNA extract in TE buffer was placed on the top of the column, and the eluent was collected after two centrifugations for 10 min each at 1,100 × *g*. The eluates from three 200-mg soil samples were pooled and passed through an Elutip-d column as specified by the manufacturer. The purified DNA was precipitated with ethanol and resuspended in 30 μl of pure water.

PCR amplification of soil DNA. A sensitive PCR protocol was used to amplify very-diluted DNA target (33). Ten PCR cycles were first run with a primer concentration of 0.5×10^{-3} μM with the following program: initial denaturation for 3 min at 95°C and then 10 cycles of DNA denaturation for 1 min at 95°C, primer annealing for 1 min at 50°C, DNA extension for 1 min at 72°C, and a final extension for 3 min at 72°C. The primer concentration was then adjusted to 0.5 μM, and 60 PCR cycles were run with the following program: DNA denaturation for 1 min at 90°C, primer annealing for 1 min at 50°C, DNA extension for 1 min at 72°C, and a final extension for 3 min at 72°C.

Electrophoresis. Samples of DNA extracted from pure cultures or soil and PCR-amplified DNAs were checked by horizontal gel electrophoresis in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA [pH 8.0]) with 0.8 and 2% agarose (wt/vol), respectively. The gels were stained with ethidium bromide (0.4 mg/liter) and photographed under a 312-nm UV light source with Ilford FP4 film.

PCR-MPN counting. Serial dilutions of soil DNA were amplified to visualize extinction of signal amplification. The dilution rates were first 1:10 and then 1:3 with three repeats at each dilution. The number of *Nitrobacter* cells per g of soil was determined with Cochran's table (10).

RESULTS

Primer specificity. Pure DNA from strains belonging to *Nitrobacter* genomic species (*N. hamburgensis*, *N. winogradskyi* W^T, *N. agilis*, and *Nitrobacter* genomic species strain 2) and their phylogenetic neighbors (*Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Photorhizobium thompsonianum*, and *Agrobacterium tumefaciens*) were first amplified to determine the best conditions of amplification. The annealing temperature of 50°C and a primer concentration of 0.5 μM were optimal for amplification of *Nitrobacter* species DNA. The signal on the electrophoresis gel was a 397-bp band from all *Nitrobacter* species tested; neighboring genera yielded no band (Fig. 1). The specificity of the amplification for the genus *Nitrobacter* was checked by testing the two selected primers on common soil bacterial DNA samples. No band was obtained for any of the other bacteria tested (Fig. 1).

Detection and counting of *Nitrobacter* cells inoculated into sterile soil. (i) Extraction and purification of soil DNA. Extraction and purification procedures were tested on nonsterile noninoculated sandy loam soil and sandy calcareous soil. The total DNA extracted was estimated by gel electrophoresis. Its purity was checked by the dilution giving an amplification signal with the specific primers. The purified DNA solution, having lost its brownish color, provided the expected signal without preliminary dilution. The definite extraction and puri-

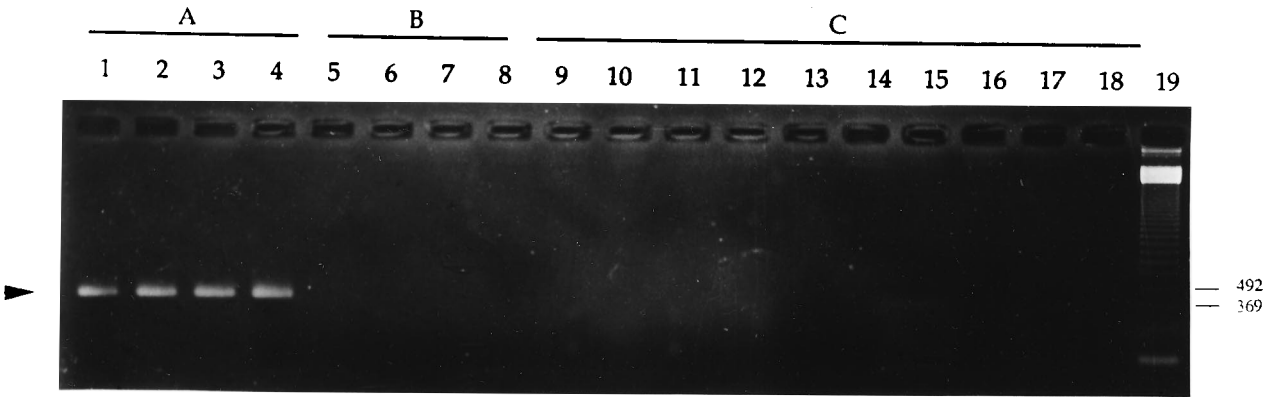

FIG. 1. Primer specificity. Amplification products of DNA from *Nitrobacter* species (A), phylogenetic neighbors (B), and usual soil bacteria (C). Lanes: 1 to 4, *N. winogradskyi* W^T, *N. agilis*, *N. hamburgensis*, and *Nitrobacter* genomic species strain 2 LL, respectively; 5 to 8, *Rhodopseudomonas palustris*^{T}, *Bradyrhizobium japonicum* USDA 110, *Photorhizobium thompsonianum* BTAi1, and *Agrobacterium tumefaciens* C58, respectively; 9 to 17, *Pseudomonas fluorescens* AK15, *Burkholderia cepacia*, *Azospirillum lipoferum* 4B, *Azospirillum brasilense*, *Flavobacterium* sp., *X. albilineans*, *Rhizobium meliloti*, *Bacillus azotoformans*, and *S. lividans* TK24, respectively; 18, negative control without DNA; 19, 123-bp ladder marker. The position of the amplification signal is consistent with the expected size of 397 bp.

fication procedure provided fragmented DNA, but the major part was longer than 600 bp (Fig. 2). DNAs extracted and purified from nonsterile sandy loam soil and sandy calcareous soil were always detected by gel electrophoresis. Although DNA extracted from sterile soil seeded with 10, 10^2, or 10^3 *N. hamburgensis* cells per g of soil was either invisible or detected very faintly on the gel, amplification gave the expected signal without any dilution.

(ii) Recovery of *Nitrobacter* cells inoculated in sterile soil by PCR-MPN. Undiluted extracted soil DNA was amplified by the sensitive PCR. Repeatedly diluted (1:3) soil DNA was amplified to determine a characteristic number. For instance, the PCR products shown in Fig. 3 were obtained from DNA extracted from soil inoculated with 10^2 *N. hamburgensis* cells per g of soil. The characteristic number was 321, and so 1 μl of sample diluted 1:9 contained 1.249 target sequences according to Cochran's tables. The volume used for this amplification corresponds to 30 μl of DNA solution extracted from 600 mg of dry soil. *Nitrobacter* cells have only one ribosomal operon (25) and therefore only one copy of this sequence per cell. Counting this sample by PCR-MPN gave $1.249 \times 9 \times 30 \times (1/0.6)$ or 5.62×10^2 *Nitrobacter* cells per g of dry soil (Table 3). Positive signals were always obtained by amplifying extracted DNA from a control sample, gamma radiation-sterilized sandy loam soil. The numbers of recovered *N. hamburgensis* cells in inoculated samples were computed after subtraction from the values obtained by PCR-MPN with inoculated samples of the noninoculated sample (Table 3).

The amplification signals for DNA extracted from sterile soil inoculated with 10 *N. hamburgensis* cells per g of soil and for DNA from control samples were essentially the same. But 65% of the bacteria were recovered from soil inoculated with 10^2 *N. hamburgensis* cells per g of soil and 72% were recovered from soil inoculated with 10^3 *N. hamburgensis* cells per g of soil.

(iii) Counting by MPN-Griess. No counts could be performed with 5 mM $NaNO_2$ in the medium used for MPN counting. All plates of MPN medium for suspensions of sterile soil inoculated with 10, 10^2, and 10^3 *Nitrobacter* cells per g of soil never completely lost the pink medium color, whatever the suspension dilution, after 104 days at 28°C.

(iv) Counting by immunofluorescence. No *N. hamburgensis* was detected in the control sample, gamma radiation-sterilized noninoculated soil. *N. hamburgensis* cells were detected on samples inoculated with 10, 10^2, and 10^3 bacteria per g of soil, but their numbers were not sufficient to be counted.

Detection and counting of *Nitrobacter* cells in noninoculated nonsterile soil. PCR-MPN gave 5×10^5 *Nitrobacter* cells per g in nonsterile dry sandy loam soil and 3.3×10^4 *Nitrobacter* cells per g in nonsterile dry sandy calcareous soil. With an estimated recovery of 65 to 72%, the final PCR-MPN count was 7×10^5 to 7.7×10^5 *Nitrobacter* cells per g in sandy loam soil and 4.6×10^4 to 5×10^4 *Nitrobacter* cells per g in sandy calcareous soil (Table 4).

DISCUSSION

The MPN viable counting technique (MPN-Griess) has, until now, been the only way to count the total population of *Nitrobacter* cells in situ. However, this procedure is known to underestimate the number of nitrifying bacteria (35). Moreover, representative counting would require the use of several different media and at least two nitrite concentrations (4) and would necessitate long incubation times (16). The immunofluorescence technique allows detection of *Nitrobacter* strains, but no antibody against the whole *Nitrobacter* genus exists and no fewer than 10 serotypes have been identified within this genus

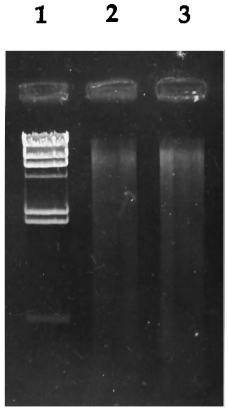

FIG. 2. DNA extracted and purified from soils of La Côte Saint André (lane 2) and Loyettes (lane 3). Lane 1 contains lambda *Hin*dIII-*Eco*RI size markers.

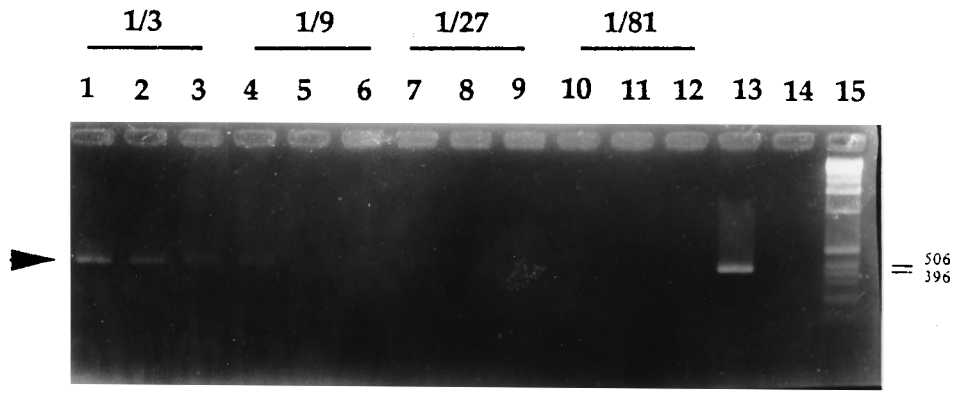

FIG. 3. Amplification products of DNA extracted from La Côte Saint André soil inoculated with 10^2 *N. hamburgensis* cells per g of wet soil. Lanes: 1 to 12, DNA serially diluted threefold and three repeats for each dilution; 13, positive control with pure *N. hamburgensis* DNA; 14, negative control without DNA; 15, 1-kb ladder marker.

(25). Hence, counting in nonsterile soil depends on obtaining fluorescent antibodies against all of the strains present.

For counting the total *Nitrobacter* population a conserved nucleic acid sequence could be a suitable tool. The sequence must exist in all species of the genus *Nitrobacter* but not in the genomes of other soil microorganisms if it is to be a specific primer. rRNAs have proved to be good targets for selective amplification. Although they are highly conserved, they have considerable sequence differences. Their high copy number per cell also makes direct tests more sensitive (40). We therefore examined sequences of 16S rRNA genes of *Nitrobacter* species and their phylogenetic neighbors. Orso et al. (31) showed that the sequences of the *Nitrobacter* species, *R. palustris*, and especially *B. japonicum* were very similar. Selecting a specific primer of the genus *Nitrobacter* was thus difficult. The PCR amplicon used for detection and especially counting of soil-extracted DNA also had to be not too long to allow counting of a partially fragmented extracted soil DNA. Several primers were tested, and two of them, a *Nitrobacter*-specific primer and a nonspecific primer, were selected. Amplifications of pure DNA from *Nitrobacter* species and a variety of common soil bacteria showed the specificity of the primer for the genus *Nitrobacter*. We used phylogenetic neighbors and other genera usually encountered in soils. The amplification conditions were set to obtain the best *Nitrobacter* signal intensity, without any signal from DNA of other genera.

The amplification of *Nitrobacter* DNA from soil proved to be more difficult. Extraction had to yield the largest amount of DNA with the least contamination risk, and purification should allow amplification of the less-diluted soil DNA. Several purification procedures have been efficiently developed, including Sephadex G-200 columns (43) or successive Elutip-d columns (33). Purification on Elutip columns was efficient and rapid, but the use of two Elutip columns resulted in a significant loss of DNA (33). We therefore used a Sephadex G-200 column and a single Elutip column to remove humic substances. Amplification was the best way to assess DNA purity. Previous purifications have often been partial, and positive amplifications could be obtained only for diluted DNA solutions (9, 33). The combination of Sephadex G-200 and Elutip-d columns gave highly purified DNA, since positive signal was always obtained without dilution of the DNA solution. Detection and counting by PCR-MPN could probably be improved by extracting DNA from a larger sample. The method adopted is therefore a balance between the sensitivity required and efficient, practical extraction and purification procedures. Some of the steps in soil DNA extraction and purification may have to be adjusted for different types of soils. One of the most important factors to be tested, as in the case of humic soils, is the pH, which at high values allows more-rapid DNA solubilization but also, more importantly, recovery of humic substances. In addition, the procedure developed here is a very drastic one. In the case of sandy soils, where DNA adsorption and quantity of humic substances are low, the necessity of some extraction steps, such as successive washes in extraction buffer, or purification steps, such as that with an Elutip-d column, may have to be tested.

The optimized soil DNA extraction, purification, and amplification steps were then used to determine the minimal number of *Nitrobacter* cells that could be detected by PCR and the efficiency of *Nitrobacter* counting by PCR-MPN. Gamma radiation-sterilized soil was inoculated with a small number of *N. hamburgensis* cells. Positive amplifications from controls were observed. The soil of La Côte Saint André used in our experiments contains many *Nitrobacter* cells, about 10^6 per g, and the

TABLE 3. PCR-MPN counts of *N. hamburgensis* cells inoculated into sterile soil

No. of *N. hamburgensis* cells/g of dry soil			*N. hamburgensis* cells recovered (%)
Inoculated	Counted by PCR-MPN	With control value[a] deducted	
0	4.97×10^2	0	
10	4.97×10^2	0	0
10^2	5.62×10^2	0.651×10^2	65
10^3	1.22×10^3	0.721×10^3	72

[a] The control value (4.97×10^2) is the value obtained by PCR-MPN for the noninoculated sample.

TABLE 4. *Nitrobacter* counts in noninoculated nonsterile soils, adjusted to 100% recovery

Nonsterile soil type	Range of *Nitrobacter* count by:	
	MPN-Griess	PCR-MPN
Sandy loam soil (La Côte Saint André)	8×10^5, 2.3×10^6 (12)[a]	7×10^5, 7.7×10^5
Sandy calcareous soil (Loyettes)	1.5×10^2, 9.5×10^2 (15)	4.6×10^4, 5×10^4

[a] Previous count.

amplified sequence was a short fragment. Gamma radiation produces double-strand breaks in the DNA at a rate sufficiently low to allow the preservation of fragments several kilobase pairs long (6). Therefore, after radiation, long fragments released in soil when cell lysis occurs could be protected against degradations by clay and sand (23, 32). It is thus not unreasonable that these DNA target sequences, persisting in soil samples treated with gamma rays, were extracted and detected by PCR. We therefore subtracted control values and then estimated the detection threshold of *Nitrobacter* cells in soil by PCR and the percentage of *Nitrobacter* cells inoculated counted by PCR-MPN. The PCR signals for the control and the soil inoculated with 10 *Nitrobacter* cells per g were the same. The PCR detected 10^2 *Nitrobacter* cells per g of soil in the DNA extracted from 0.6 g of dry soil. This is a satisfactory result when compared with the numbers of other microorganisms detected. Tsai and Olson (42) found a sensitivity of 5×10^2 cells per g of soil. Picard et al. (33) found positive amplification for 100 mg of soil inoculated with 10^3 bacteria. The detection threshold reached by Tebbe and Vahjen (41) was 10 cells per g, corresponding to 80 sequence copies after extraction of 5 g of soil, selection of the most-humic-acid-resistant *Taq* polymerase, and the addition of T4 gene 32 protein. But, *Nitrobacter* cells contain only a single copy of the target, and so detection was likely to be less favored. The percentage of *Nitrobacter* cells recovered by PCR-MPN was 65% for soil inoculated with 10^2 *Nitrobacter* cells per g and 72% for soil inoculated with 10^3 cells per g. These results are better than those of Picard et al. (33), who found a recovery ranging from 20 to 60%.

PCR-MPN was used to count naturally occurring *Nitrobacter* cells in sandy loam soil and sandy calcareous soil. For the sandy loam soil, cell numbers obtained by specific amplification agreed with MPN-Griess values (12). However, PCR-MPN gave an unexpectedly high value for the sandy calcareous soil (15). As stated above, the MPN-Griess method is likely to underestimate the total number of *Nitrobacter* cells in soil. The nitrifying activity in the sandy calcareous soil is also very low (12). Thus, PCR-MPN seems to allow the genomic exploration of *Nitrobacter* populations in some soils, such as the sandy calcareous soil, which are not accessible to standard counting methods.

We have, therefore, developed a rapid, efficient, and direct procedure for counting, at a low threshold, all bacteria of the genus *Nitrobacter* in soil. This constitutes a significant improvement relative to fluorescent antibody and MPN-Griess methods. This genomic method provides for a more exhaustive count of members of the genus *Nitrobacter* in situ.

ACKNOWLEDGMENTS

We are grateful to Philippe Normand for helpful discussions.

This work was supported by a grant from the Ministère de la Recherche et de l'Education.

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