# Detection and Enumeration of Bacteria in Soil by Direct DNA Extraction and Polymerase Chain Reaction

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In order to develop a rapid and specific detection test for bacteria in soil, we improved a method based on the polymerase chain reaction (PCR). Each step of the protocol, including direct lysis of cells, DNA purification, and PCR amplification, was optimized. To increase the efficiency of lysis, a step particularly critical for some microorganisms which resist classical techniques, we used small soil samples (100 mg) and various lytic treatments, including sonication, microwave heating, and thermal shocks. Purification of nucleic acids was achieved by passage through up to three Elutip d columns. Finally, PCR amplifications were optimized via biphasic protocols using booster conditions, lower denaturation temperatures, and addition of formamide. Two microorganisms were used as models: *Agrobacterium tumefaciens*, which is naturally absent from the soil used and was inoculated to calibrate the validity of the protocol, and *Frankia* spp., an actinomycete indigenous to the soil used. Specific primers were characterized either in the plasmid-borne *vir* genes for *A. tumefaciens* or in the variable regions of the 16S ribosomal gene for *Frankia* spp. Specific detection of the inoculated *A. tumefaciens* strain was routinely obtained when inocula ranged from $10^7$ to $10^3$ cells. Moreover, the strong correlation we observed between the size of the inocula and the results of the PCR reactions permitted assessment of the validity of the protocol in enumerating the number of microbial cells present in a soil sample. This allowed us to estimate the indigenous population of *Frankia* spp. at $0.2 \times 10^5$ genomes (i.e., amplifiable target sequences) per g of soil.

The polymerase chain reaction (PCR), which has the capacity to amplify specific sequences of DNA (5, 11, 25), has been successfully developed as a specific and sensitive diagnostic method for the direct detection of microorganisms in aquatic environments (2), food or dairy products (5), and clinical samples (6, 14).

Concerning environments such as soils or sediments, the utility of such a molecular approach is also very high. It can be used to detect fastidious microorganisms that are difficult or dangerous to culture in vitro (8, 9) and to determine the fates of selected or genetically engineered microorganisms (27, 33) and of particular genes disseminated by transfer to indigenous microbes. This technique can also be used to study the natural bacterial diversity in these complex environments, from which only a small percentage of the indigenous microorganisms can be isolated in vitro (30, 34).

However, purification of nucleic acids from soil microorganisms appears not to be as easy as it might be with organisms from other environments (13, 28). The humic acids and phenolic compounds present in soil are difficult to remove, making DNA purification a critical step in direct soil DNA extraction. These phenolic or humic compounds are known to reduce the efficiency of restriction or modification enzymes and even the specificity of hybridization (27). We have to keep in mind that specificity in a PCR process is based on the occurrence of hybridization between two oligonucleotides and template DNA. In positive hybridization, the *Taq* polymerase enzyme synthesizes the complementary strand, providing template DNAs for the following cycles and constituting a final stock of molecules which can

be visualized on an agarose gel (11, 19). In summary, if not removed, soil contaminants can affect hybridization specificity as well as *Taq* polymerase activity. The objective of this work was to develop a rapid and easy-to-use PCR protocol for directly detecting and enumerating microorganisms in soil. We wanted to take advantage of the properties of the PCR, including high specificity and sensitivity, as well as the fact that long DNA was not required to initiate the PCR process. The strategy we developed was to optimize and control each step of the protocol, including lysis of bacteria, DNA purification, and PCR amplification.

## MATERIALS AND METHODS

**Bacterial strains.** *Agrobacterium tumefaciens* C58, supplied by the Collection Française de Bactéries Phytopathogènes, Institut National de la Recherche Agronomique, Angers, France, was used as seed organism. Lytic treatments were also developed for spores produced by *Streptomyces lividans* TK64 (7).

**Soil inoculation.** Soil samples (100 mg) were inoculated with $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, or $10^1$ *A. tumefaciens* cells. Inoculum densities were optically adjusted at 600 nm and verified by standard plate counts. The cells were rinsed with sterile distilled water and suspended in less than 100 μl of water to avoid dilution of the soil sample.

**Bacterial enumeration by MPN-PCR.** Pure DNA or DNA extracted from soil solutions was serially diluted by a factor of 3 and amplified in triplicate according to the improved PCR protocol. The number of amplifiable target DNA sequences corresponding to the number of bacterial cells was determined according to the most-probable-number (MPN) technique.

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**Soil characterization.** Surface samples from a silt loam soil were collected and sieved (2-mm-mesh sieve), and 100-mg samples were placed in Eppendorf tubes prior to storage at 4°C. This soil was collected from an alder stand situated in La Toussuire, Savoie, France. It had a pH ($H_2O$) of 7.8, electrical conductivity of 0.2456 mS/cm, 59 g of organic matter · kg$^{-1}$, and C/N ratio of 8.9.

**Oligonucleotide primers.** Two *A. tumefaciens*-specific primers (44-*vir* primer 5'-TGCCGCATGGCGCGTTGTAG-3' and 14-*vir* primer 5'-GAACGTGTTTCAACGGTTCA-3') were characterized in the *vir* region located on the Ti plasmid and could amplify a 246-bp DNA fragment (12). Amplification occurred for nopaline-type pTi plasmids but not for pTi-free agrobacteria (12). *Frankia* DNA was specifically amplified by using the primers FGPS59 (5'-AAGTCGAGCGGGGA GCTT-3') and FGPS305' (5'-CCAGTGTGGCCGGTCGCCC TCTCAG-3') characterized according to Simonet et al. (23a), generating a 247-bp-long DNA fragment.

**PCR amplification.** PCR amplification was performed in a total volume of 50 μl in 0.5-ml Eppendorf tubes under a layer of paraffin oil by using a DNA thermal cycler (Dri-BlockR PHC-3', Techne Inc., Princeton, N.J.). All of the PCR components were UV irradiated for 5 min under a VL.80 G lamp (Vilbert-Lourmat, Paris, France) prior to the addition of the *Taq* polymerase enzyme and template DNA. The classical PCR experiment was done in 1× PCR amplification buffer (10× buffer contains 100 mM Tris HCl [pH 8.3], 500 mM KCl, 30 mM MgCl$_2$, and 0.1% [wt/vol] gelatin), 200 μM each deoxynucleoside triphosphates, 0.1 μM each primer, template DNA (1 μl), and 2.5 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md.). The PCR was run for 35 cycles, during which DNAs were denatured at 95°C for 1 min, primers were annealed at 55°C for 1 min, and DNAs were extended at 72°C for 1 min. An improved PCR protocol was also developed to repeatedly amplify very diluted target DNA solutions in which the concentration of primers was varied over the range 1 nM for the 10 first cycles to 1 μM for the following cycles in the presence of 1% formamide, the other components being maintained at the standard concentrations. These 10 PCR cycles were run according to the following protocol: initial denaturation, 95°C for 3 min; cyclic denaturation, 95°C for 1 min; annealing, 55°C for 1 min; extension, 72°C for 1 min. At the end of this first series of cycles, the primer concentration was adjusted to 0.1 μM and 60 new PCR cycles were run according to the following protocol: DNA denaturation, 90°C for 1 min; annealing, 55°C for 1 min; extension, 72°C for 1 min. PCR-amplified DNAs were detected by using 2% (wt/vol) horizontal agarose gel electrophoresis run in TBE buffer (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA [pH 8.0]) at 10 V · cm$^{-1}$ for 30 min. The gels were stained in an aqueous solution of 0.4 mg of ethidium bromide · liter$^{-1}$, destained in distilled water, and photographed with Ilford FP4 film and a 312-nm UV source.

**Inhibition of PCR amplifications by foreign DNA.** In order to assess the influence of total soil DNA on the efficiency of PCR in amplifying target DNA, amplifications of *A. tumefaciens* DNA were conducted in the presence of increased concentrations of sonicated calf thymus DNA, which was used as a source of foreign DNA.

**Total soil DNA extraction.** A 500-μl volume of TENP (50 mM Tris, 20 mM EDTA disodium salt [pH 8.0], 100 mM NaCl, 1% [wt/vol] polyvinylpolypyrrolidone [Sigma Chemical Co. St. Louis, Mo.]) buffer was added to each 100-mg soil sample, which was sonicated with a titanium microtip operated at a power setting of about 15 W (4/10 maximum power of a 600-W ultrasonicator; Bioblock, Illkirsh, France) for 5

min at 50% of active cycles. Cell debris was then sedimented by centrifugation (1 min, 12,000 × *g*). The supernatant was removed, and the microtube containing the pellet was then placed in a microwave oven, heated five times for 1 min each time (at 900 W), and then suspended in 100 μl of TENP buffer. Finally, three successive thermal shocks (liquid nitrogen, −196°C; boiling water, +100°C, 10 min) were applied to the soil suspension to achieve lysis. In order to remove all of the free DNA contained in the soil sample after lytic treatment, four successive washes including centrifugation and resuspension were conducted, with 100 μl of TENP buffer used for each wash.

**Visualization of DNA in solutions and in the soil pellet.** The soil pellet remaining after the lytic treatments and the four washes was resuspended in 100 μl of TENP buffer. Resuspended soil and DNA solutions were deposited on a 2% agarose gel containing 1.5 μg of ethidium bromide · ml$^{-1}$, electrophoresed at 10 V · cm$^{-1}$ for 30 min, and photographed. Treatments of various assay mixtures with DNase I were used as evidence that smears observed on photographs really indicated the presence of DNA.

**Purification of the DNA solutions.** The pooled supernatants were purified through a series of three successive Elutip d columns (Schleicher & Schuell, Dassel, Germany) as specified by the manufacturer. The DNA was precipitated with ethyl alcohol, and the pellet was resuspended in 10 μl of pure water.

## RESULTS AND DISCUSSION

**Direct lysis of soil bacteria.** A direct molecular analysis of the soil bacterial community via direct sequencing of 16S rRNA has shown that many eubacteria have never been cultured from this habitat (34). This confirms that different types of soils constitute the greatest reservoir of microorganism diversity. However, current methods for extracting DNA from bacteria had mainly been developed for proteobacteria and were not very efficient when applied to gram-positive bacteria (3, 24). According to recent reports, the most efficient techniques for gram-positive bacteria include thermal shocks or heating by microwave (3). Such treatments increase the yield of extraction over that of classical methods, resulting in DNA long and pure enough to be restricted, ligated, or hybridized. However, long DNA is not required to initiate the PCR process, so we decided to take advantage of sonication, a more drastic treatment previously developed for protein extraction which results in the complete lysis of the microbial cells (16, 22). Moreover, ultrasonication was described as the most efficient means of releasing the natural bacterial population attached to soil aggregates (17). This indicates that sonication could be effective not only in extracting DNA but also in releasing protected bacteria, making them available for subsequent lytic treatments. In order to optimize the lysis of microbial cells, we developed a lytic protocol including sonication, microwave heating, and thermal shocks successively. Furthermore, this protocol avoided the use of treatments with enzymes such as lysozyme or achromopeptidase, which may contain contaminant DNAs, and with particular detergents such as lauryl sulfate, described as a powerful inhibitor of *Taq* polymerase activity (36). The TENP buffer we developed for the lysis protocol contained Tris and EDTA to protect the DNA from nuclease activity produced by soil microorganisms, sodium chloride to provide a dispersing effect to the solution, and insoluble polyvinylpolypyrrolidone to remove humic acids and other phenolic impurities by adsorption.

**Efficiency in extracting soil microorganism DNA.** We have estimated the efficiency of each lytic treatment applied
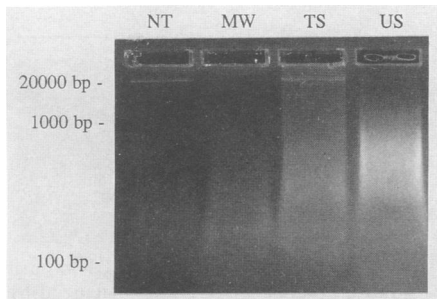
FIG. 1. Comparison of different lysis methods to extract soil DNA. DNA was extracted from 100 mg of soil in TENP buffer by no treatment (NT), microwave (MW; five times, 1 min each time), thermal shocks (TS; three at −196°C and +100°C), or ultrasonication (US; 5 min).
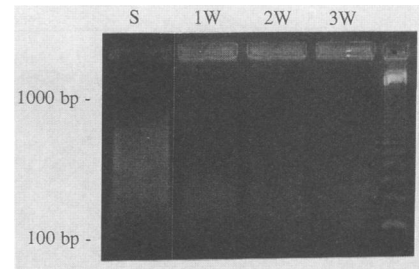


FIG. 2. Recovery of DNA extracted from soil. Lanes: S, supernatant after all lytic treatment (ultrasonication, microwave, and thermal shocks); 1W, 2W, and 3W, supernatant after first, second, and third wash, respectively, of the soil pellet with 100 μl of TENP buffer.

separately to different soil samples by visualizing the quantity of extracted DNA. An aliquot of the DNA solution cleared by centrifugation at 2,000 × g was deposited and electrophoresed on an agarose gel (Fig. 1). The soil sample which was not subjected to lytic treatment but only rinsed with TENP buffer exhibited a band on the agarose gel, indicating the presence of free DNA in the soil. This DNA was not degraded, being at least 20 kb long, which indicated that there was little contaminating nuclease activity in this soil. This DNA can originate from bacteria which naturally release their nucleic acids at death. The DNA can then be protected from nuclease activity on soil particles such as clay or sand, as described by Aardema et al. (1). It can also originate from bacteria lysed during incubation with TENP buffer. The three lysis methods increased the yield of DNA extracted compared with the control. This was particularly clear for the sonication treatment, which provided short DNA, but also for the thermal shock treatment, which liberated both long and short DNA. Power and duration of the sonication treatment (15 W, 50% active cycles, 5 min) were determined experimentally for a final use of the protocol providing the best compromise between the amount and size of the DNA extracted. Finally, the microwave heat treatment was found to be less efficient than the two other methods but was maintained in the final protocol because of the ease of this step and eventually to achieve the lysis of cells refractory to the other treatments. Indeed, the succession of these three treatments (sonication, microwave heating, and thermal shocks) was found to be necessary to achieve complete lysis of the *Streptomyces* spores used as controls for lysis efficiency (data not shown).

**Recovery of the DNA extracted from soil.** Following the three different lytic treatments, the soil DNA solution contained a mixture of soil particles and cell debris. A first purification step consisted in sedimenting the lysate by centrifugation to eliminate the biggest particles; the DNA remained in solution in the supernatant. However, in order to recover the DNA molecules which remained trapped in the debris, we washed the pellet three times more with 100 μl of TENP buffer. Our results indicate that this step has to be maintained, since small amounts of DNA were still recovered after the second and even the third wash (Fig. 2). The amount of DNA extracted from 100 mg of soil and the yield of recovery were estimated by comparing the values obtained for the supernatants resulting from the three washes with the amount of DNA extracted by electrophoresing the whole soil sample treated for lysis. Our results (Fig. 3) indicate that at least 90% of the soil DNA was extracted and recovered. The

efficiency of DNA recovery might, however, be specific to the tested soil in relation to its texture and the specific amount of microorganisms which may differ between soils. DNA in this soil was estimated to amount to about 50 μg/g of soil (data not shown), in complete agreement with results obtained by Selenska and Klingmüller (23) and Torsvick et al. (30), and five times greater than the amount recovered by Steffan and Atlas (27) or Sayler et al. (21).

**Sensitivity of PCR amplifications.** When pure chromosomal DNA corresponding to ≥$10^3$ cells (5 pg) of *A. tumefaciens* C58 was used as template DNA in standard PCR protocols, positive amplifications were routinely obtained, resulting in a band of the expected size on an agarose gel. However, such a band could not be routinely obtained for lower template DNA concentrations. In order to avoid such false-negative results, we developed a new PCR protocol to improve the sensitivity of the reaction. This protocol was based on previously published reports on the use of biphasic strategies when very dilute DNA samples have to be amplified (18). For instance, to overcome the problem of synthesis of primer dimers or other spurious products which consume the stock of primers and monopolize the enzyme, we applied the booster PCR, a biphasic method in which primers are strongly diluted in a first stage of 10 cycles before being brought up to 0.1 μM in a second stage for the remaining cycles (18). Moreover, according to a recent report, lowering the denaturation temperature after these initial 10 cycles also gives a much improved yield of products by preserving the enzyme *Taq* polymerase and enabling amplification to be performed for up to 70 cycles (37). Finally, we found that PCR amplifications conducted in the presence of 1% formamide resulted in marked improvement in yield. Formamide, previously described as improving the specificity of PCR (20), also acts in lowering the $T_m$, helping DNA be denatured
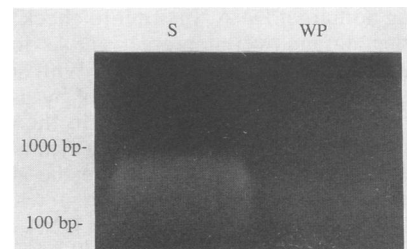


FIG. 3. Visualization of soil DNA contained in the supernatant (S) and the washed pellet (WP) after all lytic treatments and four washes.
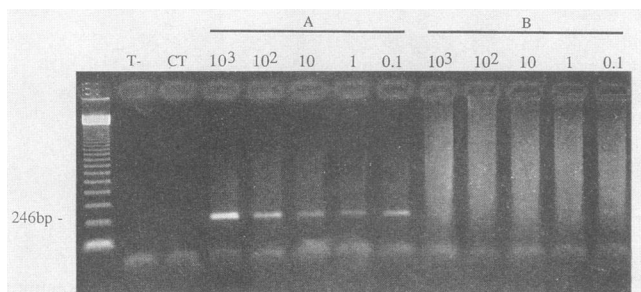
FIG. 4. Inhibition of PCR amplifications by nontarget DNA. PCR *vir* amplification of template DNA corresponding to $10^3$, $10^2$, 10, 1, or 0.1 cell of *A. tumefaciens* C58 in the presence of 0.5 μg (A) or 5 μg (B) of calf thymus DNA. T−, negative control without DNA; CT, negative control only with calf thymus DNA.
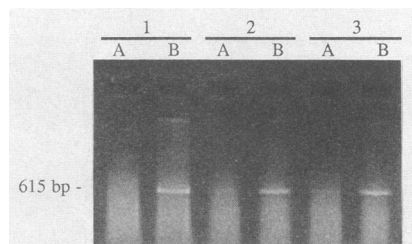


FIG. 5. Efficiency of recovery with soil DNA purified by three successive Elutip d columns. DNA was visualized by seeded soil samples with amplicons (DNA resulting from a PCR reaction) before (A) or after (B) sonication treatment and purified by one (lanes 1), two (lanes 2), or three (lanes 3) successive Elutip d columns.

when lower denaturation temperatures were applied in the second stage of PCR.

By using this new protocol, we improved the level of sensitivity by a factor of $10^3$, enabling us to amplify and routinely detect on an agarose gel plasmid DNA corresponding to approximately one bacterial cell (one amplifiable target *vir* sequence per 7 fg of genomic C58 DNA as determined by MPN-PCR, while the genome of one *Agrobacterium* cell is about 6 fg). However, such a protocol succeeded in amplifying relatively short fragments (246 bp for *A. tumefaciens*, 247 bp for *Frankia* DNA) but failed to amplify longer fragments (around 350 bp) (results not shown). In fact, these longer sequences require higher denaturation temperatures, preventing the use of such fragments for detection purposes when an improved PCR protocol was applied. This means that an optimized PCR protocol requires not only characterization of primers delimiting short segments but also empirical determination of optimum temperatures depending on the primers and the thermal cycler used.

The lysis procedure we developed to extract DNA from soil samples was based on disruption of the cell wall by sonication. However, this method resulted in breaking the DNA, which ranged in size from 100 to 500 bp (Fig. 2). In fact, sonication did not strongly affect the yield of the PCR, since the last positive amplifications were obtained with 50 fg of sonicated DNA compared with 7 fg when long DNA was used. Results may have been less consistent if longer segments had been targeted, since they would favor PCR-based detection protocols developed only with primers delimiting short fragments (i.e., <250 bp).

The microorganism population of soil is generally estimated to be around $10^{10}$ bacteria per g of soil (30), corresponding to about 85 μg of DNA (29). This indicates that DNA corresponding to specific bacteria (target DNA) must be highly diluted among nontarget DNA. In order to check whether the PCR can select target over nontarget DNAs in conditions similar to those which could be observed with soil samples, we developed the optimized PCR protocol by using as template sonicated C58 DNA (target DNA) in the presence of increased concentrations of sonicated calf thymus DNA (nontarget DNA). Our results (Fig. 4) indicate the ability of the PCR to select particular sequences over a wide range of foreign DNA, since amplification of target DNA corresponding to one *A. tumefaciens* cell was still positive in the presence of 0.5 μg of nontarget DNA and was inhibited only in the presence of 5 μg of nontarget DNA, which corresponds to the total DNA extractable from 100 mg of soil.

In conclusion, the PCR protocol we developed allowed us to routinely amplify template DNA corresponding to one bacterium. Moreover, this was achieved under conditions similar to those expected when DNA is extracted from soil, including use of sonicated DNA as template (because of the lytic treatment) and the presence of large amounts of nontarget DNA.

**Purification of DNA extracted from soil.** Contamination of PCR solutions by DNA is a serious problem for PCR amplification, especially when a small amount of DNA is being amplified. The success of detection by PCR amplification is also largely dependent on the degree of purity of the DNA solutions. So, the new method was devised to maximize the yields of DNA recovery to maintain the needed sensitivity while allowing the processing of many samples in a short time and minimizing the contamination risks. Thus, extraction and purification steps were reduced to a minimum. Such constraints eliminated the use of equilibrium density centrifugation on CsCl-ethidium bromide gradients, DNA-selective electrophoreses on agarose gels, and even phenol or chloroform extractions. On the other hand, purification and concentration of DNA can be generally achieved via selective adsorption on Elutip d columns. Such a purification step can be conducted safely in sterile conditions with DNA-free solutions, and a high recovery efficiency (>95%) would be expected when short DNA fragments were purified. Preliminary results obtained in our laboratory indicate that the yield of recovery strongly decreases when uncut DNA is eluted. That could explain the high loss (40%) found by Tsai and Olson (31) when an Elutip d column was used to purify long DNA extracted from soils and sediments. We have demonstrated that our lysis method sheared the DNA in fragments ranging in size from 100 to 500 bp, providing, in fact, the best conditions for a high recovery efficiency. The soil we used was rich in humic acids, since the use of one Elutip d column resulted in a still-brownish solution. However, the use of a second Elutip d column applied to purify this brownish solution usually cleared the DNA solution, though the use of a third column was sometimes necessary before a clearer solution was obtained. In order to estimate the efficiency of recovery of soil DNA purified in this way, we seeded the soil samples with amplicons (DNA resulting from a PCR reaction) before or after the sonication treatment. The results (Fig. 5), as visualized by electrophoresis on agarose gels of similar aliquots collected after each step of purification, indicated the highly efficient recovery of amplicons or indigenous soil DNA whatever the number of columns used. In order to confirm these preliminary results on the efficiency of Elutip d columns in purifying and recovering DNA, we developed a PCR test that used the optimized

TABLE 1. Threshold of inhibition detected by negative-positive
switch amplification[a]

| Type of DNA | Detection at dilution of: | | | | |
|---|---|---|---|---|---|
| | $10^0$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| Soil + sonicated C58 | − | − | − | − | + |
| Soil + sonicated C58 + Elutip purified | − | − | + | + | + |
| Sonicated C58 + Elutip purified | + | + | + | + | + |

[a] PCR amplification of 1 μl of the 10-μl DNA solution extracted from 100 mg of soil diluted as indicated. Quantity of C58 DNA at a dilution of $10^0$ is 0.5 μg. Symbols: +, amplification detected on agarose gel; −, amplification not detected on agarose gel.

TABLE 2. Enumeration of inoculated A. tumefaciens C58
bacteria by MPN-PCR

| | No. of cells/g of soil | |
|---|---|---|
| Inoculated | | As estimated by MPN-PCR in soil DNA extract |
| $10^4$ | | $0.35 \times 10^4$ |
| $10^5$ | | $0.6 \times 10^5$ |
| $10^6$ | | $0.2 \times 10^6$ |
| $10^7$ | | $0.5 \times 10^7$ |
| $10^8$ | | $1.2 \times 10^8$ |

protocol previously described. Template DNAs were derived from successive dilutions of a sonicated DNA solution of C58. Some of these templates were mixed with the soil lysate and then purified or not with three Elutip d columns (Table 1). Whether the amplification could be achieved with concentrated or highly diluted DNA solutions provided information on the efficiency of, respectively, the purification step (sufficient elimination of humic acids to remove inhibition of the *Taq* polymerase activity) and the recovery of DNA (presence or absence of at least one amplifiable target as estimated by a positive or negative amplification).

When concentrated DNA solutions were considered, the results of amplification were found to depend on the treatments. For instance, when the pure bacterial-DNA solutions were added to the soil lysate, the values of the first dilutions permitting amplification dropped from $10^{-2}$ for the Elutip-purified sample to $10^{-4}$ for the unpurified sample. For pure bacterial DNA, positive amplification was obtained even when the nondiluted sample was used as template (Table 1). This confirms the inhibitory effect of humic acids and the need to use Elutip d columns to achieve at least partial purification of the soil DNA solutions. However, our results indicate that even after passage through three Elutip d columns, positive amplifications could be obtained only for 100-fold-diluted DNA solutions. Purification of soil DNA solutions seems to remain a major problem (33) whatever the technique used and would prevent the DNA from being successfully amplified without dilution. Depending on this dilution factor, the actual threshold of a PCR detection test cannot decrease to less than $10^3$ target molecules per 100-mg soil sample (or $10^4$/g). A detection sensitivity of $10^4$/g is quite low for soil bacterial-population detection. The possibility of detection of a soil microorganism based on a specific DNA sequence is, however, real progress, for instance, for the detection of pathogenic agrobacteria that otherwise need, first, bacterial isolations on specific medium and, second, pathogenicity tests on several host plants (4). Detection sensitivity is partly due to specific soil properties, since a $10^3$/g threshold has been obtained with other soils (not shown). Nevertheless, we showed that 50 μg of nontarget DNA, which is equivalent to the DNA extracted from 100 mg of the soil we used, inhibits amplification. This indicates that even if all impurities could be properly removed, the threshold of detection would still be dependent on the amount of DNA and thus on the density of total microflora.

For the highest dilutions, the positive-negative amplification switch was obtained for values which were not found to be significantly different among the treatments (not shown), indicating that the final quantity of target DNA was not affected by the Elutip purification procedure. This confirms

the high efficiency obtained by Elutip d columns in recovering DNA.

**Efficiency of the method in enumerating inoculated bacteria.** In this experiment, 100-mg soil samples were inoculated with $10^7$, $10^6$, $10^5$, $10^4$, $10^3$, or $10^1$ A. *tumefaciens* cells. The lytic treatment was performed 1 h after inoculation. As previously described, positive PCR amplifications were obtained for 100-mg soil samples inoculated with $10^7$ to $10^3$ bacteria when DNA solutions were diluted at least 100 times to allow the enzyme to work. The number of A. *tumefaciens* cells (i.e., amplifiable target sequences) in the soil DNA extract was estimated by the MPN-PCR technique (Table 2). A good correlation was found to occur between MPN-PCR estimation and initial inoculum quantity. Moreover, soil bacteria such as *Agrobacterium* or *Rhizobium* spp. are known to be naturally present in soil at densities around $10^4$ to $10^7$/g of soil (4, 35). This indicates that the MPN-PCR technique, which has a range of sensitivity of $10^4$ to $10^8$ cells per g, may allow not only the detection but also the enumeration of naturally occurring microorganisms present in soil. However, enumeration by MPN-PCR was slightly underevaluated (Table 2), probably as a consequence of sonication treatments, as previously demonstrated with pure DNA. So, a correction factor should be introduced to improve the accuracy of the enumeration.

**Detection and enumeration of naturally occurring bacterial populations.** Microorganisms are known to colonize some soil components specifically by being adsorbed, for instance, on clays (15). This constitutes an ecological niche, protecting them from predation but making them more difficult to release. By specific binding with nucleic acids, clays and sand can also act in protecting extracted DNA against nuclease activity and release (10). In fact, the soil we used probably has a low capacity for nucleic acid binding and also enough clay and sand to protect the DNA. This could explain the high yields obtained in releasing bacteria and DNA. Inoculation experiments were conducted with A. *tumefaciens*, a proteobacterium for which complete lysis can be easily achieved. However, the lysis protocol used was found to be sufficient to achieve the lysis of more-refractory microorganisms such as spores of *S. lividans*. A similar lysis of *Frankia* hyphae and spores can be expected for cells present in soil samples treated by the lysis protocol. The soil we used for our study was collected in a stand that has been colonized by actinorhizal plants for more than 1 century. The level of the *Frankia* population in such a soil was expected to be high because of the numerous infective particles liberated at the death of the nodules and also because of saprophytic growth of the actinomycete. Our PCR-based detection protocol using *Frankia*-specific primers and DNA extracted from soil samples allowed us to estimate that the level of the *Frankia* population was about $0.2 \times 10^5$ genomes (i.e., amplifiable

target sequences) per g of soil, although a precise count of mycelial organisms is difficult to obtain. This is in agreement with results of Smolander (26) and Van Dijk (32), who respectively estimated *Frankia* spp. in soil to be $10^3$ or $10^5$ nodulating units per g of soil.

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