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Kinetics of the Persistence of Chromosomal DNA from Genetically Engineered *Escherichia coli* Introduced into Soil†

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Investigations to quantify bacterial survival and DNA persistence of a genetically engineered population of *Escherichia coli* introduced into soil microcosms were carried out. The survival of *E. coli* was monitored by plate counting and immunofluorescence methods, whereas the persistence of the DNA was evaluated by using a most-probable-number–polymerase chain reaction method. Whereas the *E. coli* population density declined below the plate-counting-technique detection threshold (10^2 CFU · g^{-1}) after 15 days, 10^3 extracellular and 5×10^5 total DNA target sequences were still detected after 40 days. Additionally, the *E. coli* cell counts fell below the detection limit of the immunofluorescence method (10^5 cells · g^{-1}) before the end of the experiment. Colony hybridizations did not reveal gene transfer to the indigenous microflora. These results confirm the persistence of residual *E. coli* target sequences that could not be detected by the classical cell counting method and offer promising applications for the environmental detection of microorganisms, either engineered, pathogenic, or released for beneficial effects.

Among modified organisms resulting from the advent of recombinant DNA techniques, *Escherichia coli* K-12 remains the most commonly used of microorganisms engineered for commercial production of desired proteins or other products. The large-scale use of production strains in fermentation processes has raised questions about the consequences of an industrial accident which could result in the unwanted release into the environment of up to 10^{17} recombinant cells. This prospect has increased the interest in detecting genetic interactions between soil-introduced bacteria and the indigenous microflora. While genetic exchange in soil by transformation, transduction, or conjugation has already been reported for introduced strains, evidence for the occurrence of gene transfer from inoculated microorganisms to the resident soil bacteria has been obtained only for conjugation (11, 29, 39). This has been made possible by the use of specific gene markers located on wide-host-range plasmids and by the availability of appropriate methods to counterselect the introduced bacterial population. In contrast, conditions which could lead to natural transformation have been investigated. These include numerous studies of the release of DNA from microbial cells, its persistence in natural environments by adsorption onto soil particles, and its ability to transform previously inoculated bacteria (24, 30, 31).

The aim of the present study was to investigate the correlation between the survival of the *E. coli* bacterial population inoculated into the soil and the persistence of its DNA. The most commonly used methods to enumerate soil-introduced bacteria include direct plating, MPN (most probable number), and serology; however, they are dependent for the most part on the organism's culturability (3, 5, 6, 9, 21). Moreover, the specificities and sensitivities of such techniques remain quite low, indicating a need for improved methods to detect and enumerate microorganisms in the environment.

Current research has focused on extraction and purification of soil microbial DNA for molecular analyses (10, 12, 13, 14, 23, 25, 26, 37, 38, 41). The MPN-polymerase chain reaction (PCR) method, recently described by Picard et al. (25), appears to be the method of choice not only for detecting but also for enumerating specific gene sequences. Moreover, this PCR method can be applied to quantify total (intra- and extracellular) DNA extracted according to the complete protocol from Picard et al. (25) or only extracellular DNA according to the method described by Ogram et al. (23). For this purpose, the efficiency of the soil DNA extraction procedure was investigated and improved. This permitted bacterial survival and DNA persistence of soil-introduced *E. coli* to be monitored by plate counting, immunofluorescence, and counts of total and extracellular DNA. Comparison of the persistence of a specific bacterial gene with the corresponding population survival represents an attempt to investigate die-out versus die-back (20) and may provide additional knowledge about the fate of soil-introduced genetically engineered microorganisms and specific genes.

MATERIALS AND METHODS

Soil and microcosms. The microcosms used in this study consisted of 15-ml polypropylene tubes (Falcon) filled with 5 g (dry weight) of a silt loam soil (27, 28). After sampling, moist field soil was sieved at 2 mm and kept at 4°C. Nonsterile soil was preincubated at 28°C for 1 week before use to allow the indigenous microflora to reach an equilibrium state avoiding a thermic artifact at time zero of the experiment. The air-dried soil was sterilized by exposure to 25 kGy of gamma irradiation with a ^{60}Co radiation source. Soil samples were assayed for sterility by plating on nutrient agar (Biomérieux, Charbonnières, France). Incubation at

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† This work is dedicated to the memory of Geneviève Faurie.

28°C took place in a moisture-tight chamber, and soil moisture was controlled by regular weighing.

Bacterial strain and soil inoculation. *E. coli* K-12 EL1003, which is resistant to kanamycin (500 μg · ml^{-1}) and neomycin (500 μg · ml^{-1}), is a genetically engineered strain designed for industrial production (16). Its chromosome contains a 10-fold-repeated 7.3-kb sequence, designated MNT, which includes the *nptII* gene. Prior to soil inoculation, *E. coli* was grown at 37°C in Luria broth (LB) medium (19) supplemented with appropriate antibiotics (Sigma Chemical Co., St. Louis, Mo.), washed twice, and resuspended in saline solution (NaCl 0.9% [wt/vol]) to the desired cell concentration. Soil was inoculated with a suspension volume adjusted to establish final moisture content to field capacity. Plate counts on LB agar were performed on the bacterial inoculum to determine the *E. coli* density actually added to each sample. In control experiments, the bacterial suspension was replaced by the same volume of sterile distilled water.

Enumeration of bacterial populations. Microorganisms were extracted by blending the 4.9 g (dry weight) of the soil sample with 50 ml of sterile water for 2 min in a mixer (Waring Blender Eberbach Corporation). The soil suspension was then serially diluted 10-fold in the saline solution. The indigenous microflora was evaluated by plating the dilutions (three plates per dilution) onto nutrient agar supplied with cycloheximide (200 μg · ml^{-1}). Selective enumerations of *E. coli* cells from natural soil (up to 10^2 CFU · g of dry $soil^{-1}$) were performed on LB agar supplemented with cycloheximide (200 μg · ml^{-1}), kanamycin (500 μg · ml^{-1}), and neomycin (500 μg · ml^{-1}), while LB agar without antibiotics was used to count *E. coli* cells from sterile soil. As control, similar experiments were performed on unseeded microcosms. Each experiment was performed in duplicate.

DNA extraction from soil. To recover total (i.e., intracellular and extracellular) DNA, DNA was directly extracted from soil on the basis of the method described by Picard et al. (25); a range of extraction buffers ranging from pH 5 to 8 was tested to optimize DNA recovery.

Extracellular DNA was extracted from soil on the basis of the method described by Ogram et al. (23). Five grams of soil was sequentially extracted four times with 2 ml of sodium phosphate buffer (0.12 M [pH 7]). This procedure has been shown to remove 99.9% of radiolabeled DNA added to sediment (23). The pooled supernatants were purified on an Elutip d column (Schleicher & Schuell, Dassel, Germany). DNA precipitation with ethyl alcohol was performed on the final recovered solution (400 μl), and the pellet was resuspended in 10 μl of pure water.

Whatever the extraction method used, an aliquot (2/10 μl) of each diluted or undiluted DNA solution was loaded on an agarose gel (0.8%) containing 1.5 μl of ethidium bromide · ml^{-1} and electrophoresed in Tris-borate-EDTA buffer (19) at 10 V · cm^{-1} for 30 min. The gels were destained in distilled water and photographed with a 312-nm UV source.

Oligonucleotides and primers. According to the specifications from Picard et al. (25), PCR primers (114 and 115) were designed to amplify a relatively short fragment (113 bp) in the 10-fold-repeated MNT region specific for the *E. coli* strain EL1003 used in this study. The sequences of primers 114 and 115 are 5′-CCATGCCCCCGAACTGCAGG-3′ and 5′-GCAAACGGTGCAACATTGCC-3′, respectively. Another set of 20-mer primers, UAR-1939 (5′-TATGGAATT TCGCCGATTTT-3′) and UAR-2105 (5′-TGTTTGCCTC CCTGCTGCGG-3′), was used to amplify a 166-bp region of the *uidA* gene coding for β-glucuronidase and specific for *E. coli* and *Shigella* species (2). Primers 68 (5′-GTTTCGCAT GATCAAACAAGAT-3′) and 69 (5′-GCTCAGAAGAGC TCGTCAA-3′) were used to amplify an 804-bp fragment of the *nptII* gene (28).

PCR amplifications. PCR amplifications were performed in a total volume of 50 μl in 0.5-ml Eppendorf tubes, under a layer of paraffin and in 1× buffer (10× buffer contains 100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl2, and 0.1% [wt/vol] gelatin), with 200 μM each deoxynucleoside triphosphate, 0.1 μM each primer, 1 μl of template DNA, and 2.5 U of *Taq* DNA polymerase (GIBCO-BRL, Gaithersburg, Md.). With a DNA thermal cycler (Dri-BlocR PHC-3; Techne Inc., Princeton, N.J.), template DNA was initially denatured at 95°C for 3 min and the amplification reactions were carried out for 40 cycles under the following conditions: DNA denaturation at 95°C for 1 min, annealing of primers at 55°C for 1 min, and extension at 72°C for 1 min. At the end of the cycles, an additional incubation at 72°C was run for 3 min to ensure complete extension. In negative- and positive-control samples, either no DNA or 1 ng of total genomic *E. coli* EL1003 DNA was used as template. For each PCR experiment, 10 μl of the reaction solution was loaded on a 2% agarose gel to check for a positive amplification.

DNA target sequence quantification with MPN-PCR. To estimate the MPN of DNA targets in a solution, the DNA samples were serially diluted 10-fold in pure water, and three samples of 1 μl for each dilution were tested for amplification. The detection threshold of the PCR protocol was established by amplification of a range of known quantities of DNA isolated from *E. coli* EL1003 as well as serially diluted DNA samples extracted from soil microcosms to which known concentrations of *E. coli* EL1003 cells had been added. The MPN of DNA targets was calculated by using the tables of Fisher and Yates (7).

RESULTS AND DISCUSSION

The first part of this study was aimed at determining the optimal conditions for DNA extraction with regard to the physical and chemical characteristics of the soil tested. By comparing the patterns obtained when DNase I (Sigma; 0.5 μg · ml^{-1})- and RNase A (Sigma; 0.2 μg · ml^{-1})-treated soil solutions and untreated ones were electrophoresed, it was possible to confirm that the signals visualized on ethidium bromide-stained gels corresponded in fact to DNA (RNase and DNase activities have previously been reported to be unaffected by impurities such as humic materials [41]) (Fig. 1A). Adsorption of DNA to sand and clay is pH dependent and may affect the efficiency of the recovery of the DNA. Evidence for increased adsorption of DNA to sand (32) or to clay (22) at low to neutral pH has been reported. Therefore, a set of TENP buffers (25) equilibrated to pHs ranging from 5 to 8 were checked for their efficiencies in recovering the DNA. The highest recovery of DNA was obtained with TENP at pH 7 (Fig. 1B). From these results, we infer that this parameter should be tested for each new soil source. Quantitation of the amount of DNA extracted when optimal conditions were applied (TENP at pH 7) was accomplished by comparing patterns of twofold serially diluted DNA solutions extracted from soil with standard amounts of herring sperm DNA (Sigma) on the same gel. As little as 20 μg of DNA recovered per g of soil (Fig. 1C) could be estimated by this procedure. This limit compared favorably with DNA yields ranging from 10 to 90 μg, which had been

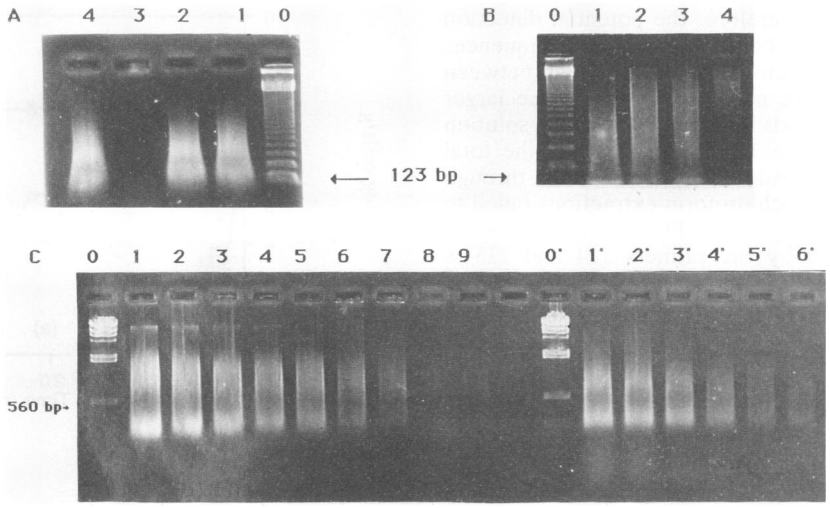

FIG. 1. Ethidium bromide-stained agarose gels of total DNA extracted from unseeded soil (1 μl of total extracts [10 μl] was loaded into each well). (A) Shown are a 123-bp ladder (GIBCO-BRL) DNA size marker (lane 0) and total soil DNA that was untreated (lane 1), incubated at 37°C (lane 2), DNase digested (lane 3), or RNase digested (lane 4). (B) Shown are a DNA size marker (lane 0) and total DNA extracted with TENP buffer at the following pHs: 8 (lane 1), 7 (lane 2), 6 (lane 3), and 5 (lane 4). (C) Shown are a *Hin*dIII-cut lambda bacteriophage DNA molecular size marker (lanes 0 and 0') standard amounts of herring sperm DNA (lanes 1 to 9, 1,750, 875, 437, 350, 175, 87, 44, 22, and 11 ng, respectively), and total soil DNA (lane 1', undiluted; lanes 2' to 6', 1/2-, 1/4-, 1/8-, 1/16-, and 1/32-fold diluted, respectively).

previously reported for a wide range of soils (25, 34, 37, 40, 41).

In a second part, the MPN-PCR method described by Picard et al. (25) was developed for monitoring the DNA persistence of the *E. coli* EL1003 population introduced into the soil. The two assumptions of the MPN concept (4) are easily applicable to PCR detection. These are (i) that target DNA sequences are distributed randomly throughout the solution and (ii) that the presence of at least one target DNA sequence is enough to generate a positive amplification which can be visualized on an agarose gel. Preliminary experiments established that PCR performed with 1 fg of pure genomic *E. coli* EL1003 DNA was able to generate an amplified product detectable on stained agarose gels (Fig. 2). Bej et al. (2) reported that 1 to 10 fg of target DNA, respectively, could be detected via PCR and gene probe analysis. If an average amount of 9×10^{-9} μg of DNA per *E. coli* cell is assumed (19), this can be expressed as a PCR detection threshold of one target sequence per reaction volume. To check the validity of the MPN-PCR protocol in enumerating soil microbial cells, MPN-PCR enumerations performed on DNA extracted from nonsterile soil previously seeded with $10^5 \pm 10^4$) and 10^3 ($\pm 10^2$) *E. coli* EL1003 cells per g of dry soil (as determined by CFU counts) yielded an estimate of $2.3 \times 10^6 \pm 0.3 \times 10^6$ and $2.3 \times 10^4 \pm 0.3 \times 10^4$ target sequences per g of dry soil, respectively. The 113-bp PCR products generated with DNA template extracted from seeded soil and those obtained with DNA from the reference strain were found to have similar restriction patterns and positive hybridization signals with the 113-bp PCR fragment used as a probe, confirming the specificity of the PCR amplification (data not shown).

The inhibitory effect of the soil components remaining after DNA purification on the *Taq* DNA polymerase activity was estimated. PCRs were performed with 3 ng of chromosomal *E. coli* EL1003 DNA and 1 μl of a serially diluted DNA solution extracted from soil as template. In agreement with data reported by Tsai and Olson (42) and Picard et al. (25), a 10^2-fold (for total DNA) or a 10-fold (for extracellular DNA) dilution of the soil solution was necessary for a

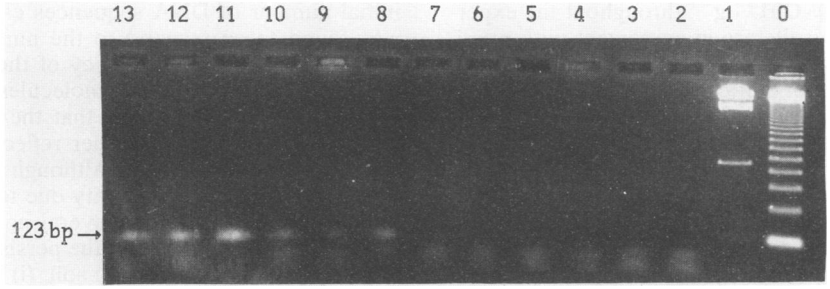

FIG. 2. Ethidium bromide-stained agarose gel of PCR products with the paired primers 114 and 115 specific for *E. coli* EL1003. Lanes: 0, 123-bp ladder DNA size marker; 1, *Hin*dIII-cut lambda bacteriophage DNA molecular size marker; 2 to 5, PCR products which were used as templates [2, no DNA; 3, *E. coli* S17-1(pMH1801) DNA (3 ng); 4 and 5, 10^2- (1 μl) and 10^3) (1 μl)-fold diluted DNA solutions extracted from unseeded soil]; 6 to 13, PCR products which were used as template DNA (0.01, 0.1, 1, 10, and 100 fg and 1, 10, and 100 pg of genomic *E. coli* EL1003 DNA, respectively).

successful amplification. Therefore, the potential detection threshold was determined to be 10^3 and 10^2 target sequences per 100 mg of dry soil, respectively. The differences between the two methods that were noted were due to the larger amounts of humic compounds released in the DNA solution during the sonication treatment which is part of the total DNA extraction protocol. Additional purifications through Elutip d columns or phenol-chloroform extractions failed to reduce the MIC for the soil lysate on PCR (data not shown).

The sequence targeted by the primers 114 and 115 is located on a DNA region close to the *nptII* gene. The applicability of the *nptII* gene as a reliable biomarker for soil-introduced bacteria had been reported earlier (13, 26) and was confirmed in the present study. Colonies extracted from the soil never hybridized to the *nptII* gene. The absence of *E. coli* cells in the soil was directly confirmed by plating soil suspensions on the *E. coli*-selective PTX agar medium (8). Additionally, 1 μl of 10-, 10^2-, and 10^3-fold diluted DNA solutions extracted from nonsterile and unseeded soil and used as DNA template in PCRs failed to amplify with the paired primers 114 and 115, 68 and 69, or UAR-1939 and UAR-2105, indicating the absence of DNA sequences corresponding, respectively, to the strain EL1003, the *nptII* gene, and the bacterial species *E. coli* (results not shown).

The validity of detection protocols also depends on the stability of the chromosome-integrated MNT sequences (including that of the *nptII* gene) under nonselective environmental conditions. To assess their stability, *E. coli* EL1003 was inoculated for 15 days in the sterilized soil prior to plating the soil suspensions on media that were amended or unamended with antibiotics. Similar population densities were obtained on both media, indicating that the cells maintained and expressed the *nptII* marker under nonselective environmental conditions (results not shown). This was confirmed by the positive colony hybridization signals with the *nptII* probe observed for each of the isolates developed on the nonselective medium (results not shown).

Finally, the third part of the present work was aimed at monitoring the survival of *E. coli* cells and their DNA. To monitor bacterial survival and DNA persistence simultaneously, soil microcosms were seeded with 3×10^7 CFU of *E. coli* EL1003 as described above. Periodically, the soil from one microcosm was used to monitor the persistence of the *E. coli* extracellular DNA. From a second microcosm, 100 mg (dry weight) of soil was used for total-DNA extraction, and the remaining portion was used to enumerate *E. coli* cells and the indigenous bacteria.

The *E. coli* EL1003 population in the seeded natural soil was found to rapidly decline with a biphasic pattern, while the indigenous microflora remained nearly constant at the original density of 3×10^7 CFU · g^{-1} throughout the experiment (Fig. 3A). These results are in agreement with previous data on the survival of soil-introduced *E. coli* (5, 6) and can be related to the effect of biotic factors such as predation and competition (27, 39). Simultaneously, the *E. coli* population specifically counted by the indirect fluorescent-antibody technique (33) (with strain-specific fluorescent antibodies and Nucleopore black polycarbonate membranes [0.4-μm pore size]) indicated a decline from 10^7 to 10^5 cells per g after 10 days and later dropped below the detection threshold (10^5 cells · g^{-1}). Concomitantly, the number of total *E. coli* target DNA sequences decreased from 5×10^8 to 5×10^5 within the first 5 days and was maintained at this level throughout the experiment (Fig. 3B). Similarly, the extracellular DNA of *E. coli* decreased to 10^4 to 10^3 sequences per g after 8 days and persisted at this density (Fig. 3B).

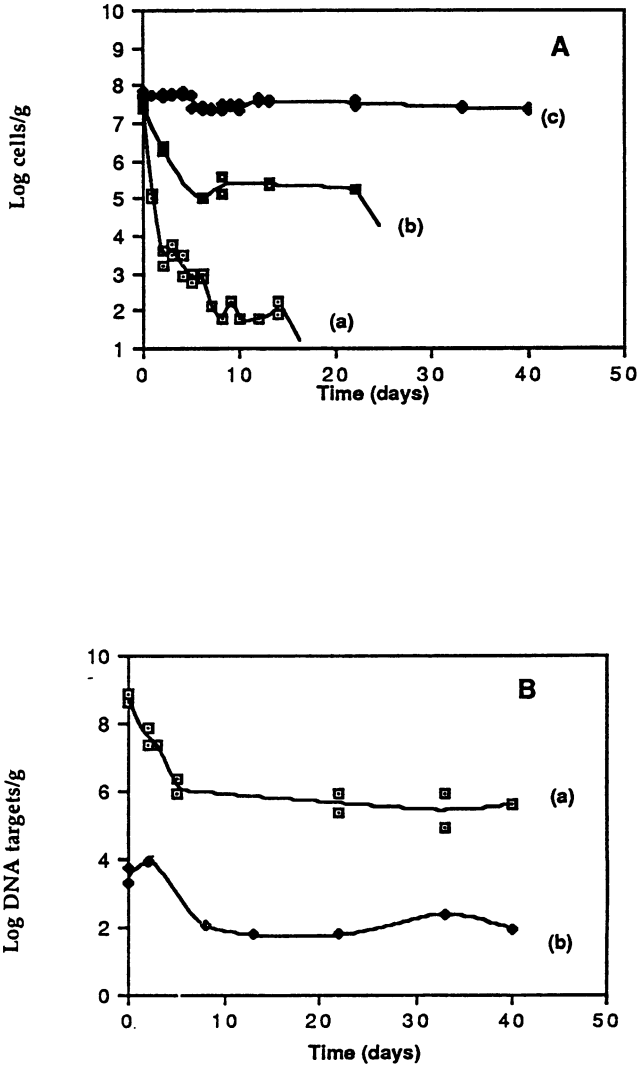

FIG. 3. (A) Persistence of *E. coli* EL1003 in nonsterile soil. Plotted are values for log of CFU · g^{-1} (a), log of cells · g^{-1} counted by immunofluorescence (b), and indigenous bacteria (log of CFU · g^{-1}) (c). (B) Persistence of *E. coli* EL1003 target DNA in nonsterile soil. Plotted are values for log of sequence number · g of dry $soil^{-1}$ for total (a) and extracellular (b) DNA.

If an average of 10 DNA targets per cell is assumed, the initial number of DNA sequences estimated by MPN-PCR was found to correspond to the number of the inoculated cells, confirming the efficiency of the method in extracting and enumerating soil DNA molecules. The analysis of cells and DNA kinetics suggests that the initial decrease of the target DNA sequence number reflects the predatory pressure on the *E. coli* cells. Although no conclusion can be drawn as to the DNA integrity due to the shearing effect of the extraction procedure, several nonexclusive hypotheses can be suggested to explain the persistence of 5×10^5 DNA target sequences per g of dry soil. (i) The maintenance of an *E. coli* population that has become nonculturable could account for the results. Roszack and Colwell (32) have provided evidence for the occurrence of a viable but nonculturable bacterial state in the environment. However, *E. coli* EL1003 cells could not be detected by immunofluores-

cence performed after day 14. (ii) The persistence of target sequences in the soil could also result from a genetic transfer to representatives of the indigenous microflora and multiplication of the recombinant strains. At the end of the experiment, the presence of the *nptII* marker was investigated by probing the soil bacterial colonies selected for kanamycin resistance (50 $\mu g \cdot ml^{-1}$) with the *nptII* probe. No positive hybridization signal was detected among the 100 randomly screened Km^r colonies isolated from the seeded soil (data not shown), making this explanation unlikely. However, the presence of the target sequences on the host chromosome did not provide conditions for the highest transfer rates, while the only reported transfer to the indigenous soil bacteria had been obtained for conjugative plasmids (11, 29, 36). (iii) In view of previous results, it is possible that a proportion of the *E. coli* DNA is released in the soil as the cells die and that it may still be detected. Clay and sand are among the soil components known to allow adsorption of large molecules such as nucleic acids or proteins and to contribute in protecting extracellular DNA against enzymatic degradation (1, 17, 18). Moreover, colloid-adsorbed DNA has been shown to remain an effective transforming agent for other bacteria (15, 24). A controversy has emerged from available data about the DNA persistence of soil-introduced bacteria. Whereas *nif* and Tn*5* sequences located in the genome of *Enterobacter agglomerans* have been detected in soil-extracted DNA 70 days after the strain inoculation (34, 35), Henschke et al. (10) reported no amplification of a plasmid target sequence in DNA extracted from soil 28 days after the introduction of *E. coli.*

This work confirms the persistence of the DNA in the soil for long periods of time. It also highlights the applicability of the MPN-PCR technique to monitor the fate of the DNA released in the environment following the deliberate or accidental inoculation of engineered or wild-type organisms.

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