Use of Polymerase Chain Reaction and Electroporation of *Escherichia coli* To Monitor the Persistence of Extracellular Plasmid DNA Introduced into Natural Soils

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A modified protocol for DNA amplification by polymerase chain reaction (PCR) coupled with laser densitometric determination of the amount of PCR products, which allowed quantitation of target sequence numbers in soil extracts, was developed. The method was applied to monitor target loss during incubation of purified plasmid DNA in natural nonsterile soils. It revealed soil-specific kinetics of target loss. After 60 days, 0.2, 0.05, and 0.01% of the initially added *nahA* genes on plasmids were detectable by PCR in a loamy sand soil, a clay soil, and a silty clay soil, respectively. Electroporation of *Escherichia coli* was used in parallel to quantitate plasmid molecules in soil extracts by their transforming activity. It was found that transformation by electroporation was about 20 times more efficient and much less inhibited by constituents of soil extracts than transformation of Ca^{2+}-treated cells (G. Romanowski, M. G. Lorenz, G. Sayler, and W. Wackernagel, Appl. Environ. Microbiol. 58:3012–3019, 1992). By electroporation, greater than 10,000-fold plasmid loss was monitored in nonsterile soils. Transforming activity was found up to 60 days after inoculation of the soils. The studies indicate that PCR and electroporation are sensitive methods for monitoring the persistence of extracellular plasmid DNA in soil. It is proposed that plasmid transformation by electroporation can be used for the monitoring in soil and other environments of genetically engineered organisms with recombinant plasmids. The data suggest that genetic material may persist in soil for weeks and even for months after its release from cells.

Microorganisms release genetic material into their environment, as documented by a variety of studies. High-M_r chromosomal as well as plasmid DNA appears in cultures of bacteria (2, 4, 17, 46). The DNA originates from living as well as dead cells (17). Extracellular microbial DNA has also been found in marine waters and sediments (6, 25, 29, 31). Recently, it was shown that DNA is released from a genetically engineered strain in an aquatic microcosm (28). Recent data indicate that predation of bacteria by nanoflagellates may be the cause for the production of dissolved bacterial DNA in the marine environment (45). Extracellular DNA may enter the nutrient cycle following enzymatic degradation (5, 28, 29, 31, 45). As an alternative, it has been discussed, on the grounds of data on the fate of DNA in soil and sediment microcosms (13, 17, 19, 22, 27, 32–34), that free DNA may serve as a source of genetic information through its uptake into transformation-competent bacterial cells (3, 14–16, 18, 20, 21, 23, 30, 35, 40–42). Measuring the degradation kinetics of DNA introduced into soil provides data on the persistence of extracellular DNA.

Plasmid DNA persisted for several days when introduced into three different natural soils (33). This was demonstrated by the use of several monitoring procedures adapted to the detection of plasmid DNA in soil. They included (i) measurement of acid-soluble products formed from [^{3}H]thymidine-labeled plasmid DNA, (ii) detection of genes by slot blot, (iii) detection of plasmid molecules by Southern transfer hybridizations, and (iv) determination of the transforming activity of plasmid DNA with CaCl$_2$-treated *Escherichia*

coli. In the present study, these investigations were extended by the use of even more sensitive monitoring methods. A polymerase chain reaction (PCR) protocol for the reliable and quantitative determination of target nucleotide sequences in soil is described. Furthermore, electroporation of *E. coli* was used to detect small amounts of transforming plasmid DNA molecules in environmental DNA extracted from soil. These procedures were used to monitor the degradation of free plasmid DNA introduced into three natural soils. We were able to quantify the number of the 1,029-bp *nahA* target sequence of the plasmid pUC8-ISP (33) by PCR even 60 days after deposition of the plasmid DNA in three nonsterile soils, and intact plasmid molecules were still detectable by electroporation after this period in one nonsterile soil.

MATERIALS AND METHODS

Inoculation with plasmid DNA and extraction of soils. The soils used in this study (a loamy sand, a clay, and a silty clay soil) have been described elsewhere (33). The DNA for inoculation consisted of purified plasmid dimers of pUC8-ISP (provided courtesy of B. Ensley, Envirogen Inc., Lawrenceville, N.J.), which is pUC8 (Ap^r, 2.5 kb [47]) containing a 2.7-kb *Pvu*II fragment of the *nahA* gene of the natural plasmid NAH7, which determines enzymes for naphthalene degradation (8). A 1,029-bp sequence within the *nahA* gene (coding for the iron-sulfur protein ISP) served as the target for PCR and for hybridization. Inoculation and incubation of soil samples with pUC8-ISP DNA and extraction of the plasmid DNA from soil were done as described previously (33). Briefly, 10-g samples of nonsterile and ethylene oxide-treated soils were inoculated with 2 μg of plasmid DNA. The soil samples were incubated for 1 h to 60

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days at 23°C in glass vials. Another set of ethylene oxide-treated soil samples were inoculated with pUC8-ISP DNA (1 fg to 10 ng) and incubated for 1 h at 23°C.

The DNA was extracted by a standard DNA extraction procedure (25) which includes hot alkaline phosphate buffer (pH 8.6) extraction with sodium dodecyl sulfate. The DNA was further purified by polyvinylpyrrolidone treatment and phenol extraction (33). The preparation was dialyzed against 10 mM Tris-HCl (pH 7.0)–1 mM EDTA, precipitated with ethanol, washed with 70% ethanol, and dissolved in 0.7 ml of TE buffer (1 mM Tris-HCl [pH 7.0], 0.1 mM EDTA). The net DNA extraction efficiencies, determined with [^{3}H]thymidine-labeled plasmid DNA, were 17% (loamy sand soil), 8% (clay soil), and 15% (silty clay soil) for nonsterile soils and 14, 11, and 14%, respectively, for ethylene oxide-treated soil samples. The values are means of two independent determinations. The separately determined values did not deviate by more than 11% from the means.

Bacterial platings. Viable soil bacteria were enumerated by plating on 0.25× YEPG agar medium (36) and incubation for 3 days at 23°C. Platings of *E. coli* after electroporation were done on Luria broth agar as described before (33).

PCR in the absence and presence of soil extracts. All DNA amplifications were performed with a Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, Conn.) with PCR reagents purchased from the same manufacturer except when stated otherwise.

PCR was performed in 20 μl of reaction buffer containing deoxynucleoside triphosphates (dNTPs) (160 μM each), the two primers (35 nM each), 1.5 mM MgCl$_2$, 3 mg of bovine serum albumin (BSA; Bering, Marburg, Germany) per ml, pUC8-ISP DNA, and 0.2 U of AmpliTaq DNA polymerase. When the influence of soil extracts on PCR was studied, pUC8-ISP DNA was dissolved in 1 μl of extract from uninoculated soil (undiluted or diluted in buffer) and added to the assay mixture (final volume, 20 μl). The primers for symmetric PCR were a 20-mer (5'-CCCTAGCGCGTAAC TACCCC-3') and a 19-mer (5'-GGTCCAGACCTCGGTG GTG-3') synthesized by Genosys Biotechnologies (The Woodlands, Tex.). Mixtures of all reaction components except for the DNA and the *Taq* polymerase were prepared. After addition of the DNA solution (in 1 μl of reaction buffer or in 1 μl of soil extract), the reaction tubes were degassed for 5 min at 20 mbar. Then, one drop of light mineral oil was added. The tubes were heated for 5 min at 94°C (which made the solution cloudy because of precipitated BSA), and the *Taq* polymerase (in 2 μl of reaction buffer) was added. Gas bubbles were removed by stirring the reaction mixture with a pipette tip. The amplification reaction consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 65°C for 2 min, and strand synthesis at 72°C for 4 min. Temperature changes were performed at maximum velocity (approximately 1°C s^{-1}). A final incubation for 12 min at 72°C served to complete strand synthesis.

After amplification, aliquots (6.7 μl) from the reaction mixture were subjected to electrophoresis (0.7% agarose containing 0.25 μg of ethidium bromide per ml). The UV-illuminated gels were photographed with Polaroid positive-negative film no. 665 (Polaroid, Cambridge, Mass.). The negatives were densitometrically analyzed (laser densitometer 300B equipped with Imagequant 1.15 software; Molecular Dynamics, Krefeld, Germany). *Hin*dIII-digested λ DNA (300 ng) run on the same gel was used to control for correct exposure of the photonegative.

For high-sensitivity detection of target sequences in soil extracts, two successive rounds of PCR were employed, in which part of the amplification product of reaction I was the DNA substrate for reaction II. In reaction I, the composition of the reaction mixture and reaction parameters were as described above except that the final concentration of the dNTPs was only 80 μM each. After amplification, a 1-μl portion of reaction I was used as the template in reaction II, which was performed as described in the preceding sections. When reactions with standard amounts of pUC8-ISP DNA were run, they contained 540 fg, 5.4 fg, 540 ag, or 54 ag of DNA, which were equivalent to 50,000, 500, 50, and 5 plasmid molecules (100,000, 1,000, 100, and 10 *nahA* targets), respectively. The products of reactions I and II were electrophoretically separated, photographed, and analyzed by densitometry as described above.

Electroporation. *E. coli* SF8 (K-12 strain; *recB21 recC22 sbcB thr-1 leuB6 thi-1 lacY1 lop-11 tonA1 supE44* r$_K$⁻ m$_K$⁻; provided by R. Eichenlaub, Universität Bielefeld, Bielefeld, Germany) was used throughout the studies. Cell preparations and electroporation were done essentially as described by Dower et al. (7). SF8 was grown to 1.6 × 10^8 cells per ml at 37°C in SOB medium (11) and harvested by centrifugation (10 min at 5,000 × *g*, 4°C). All subsequent manipulations were done at 4°C. After two washes with sterile deionized water, the cells were resuspended in 0.05 volume of 10% (vol/vol) glycerol. After centrifugation (6 min at 6,000 × *g*), the cells were finally resuspended in 0.002 times the initial volume of 10% glycerol and shock-frozen in liquid nitrogen. The cells were stored at −80°C.

Electroporation was done with 40 μl of cell suspension (8 × 10^{10} ml^{-1}). After thawing in an ice bath, the cells were carefully mixed with 1 or 5 μl of DNA solution or soil extract and transferred to chilled electroporation cuvettes. Electroporation was performed with a 12.5-kV cm^{-1} pulse (200 Ω, 25 μF; time constant, 4.6 to 4.8 ms) in a Gene Pulser apparatus equipped with a pulse controller (Bio-Rad, Richmond, Calif.). Immediately after the pulse, the cells were diluted with 960 μl of SOC medium (11), transferred to culture tubes, and incubated for 1.5 h at 37°C for marker gene expression. Transformants were selected on LB agar medium containing 40 μg of ampicillin per ml. Following incubation overnight, ampicillin-resistant (Ap^r) colonies were counted. The presence of pUC8-ISP in the transformants was verified by colony hybridization with a digoxigenin-labeled *nahA*-specific gene probe (33) or by isolation of the plasmid from clones (12).

RESULTS

Quantitation of DNA target numbers by PCR amplification. The use of high concentrations of BSA in the hot-start PCR (see Materials and Methods) decreased the sensitivity of the reaction against components in the soil extracts (see next section) and made PCR product formation reproducible. Figure 1 shows that sets of PCR assays, each done with a freshly prepared master mix of nucleotides, MgCl$_2$, reaction buffer, BSA, and primers and samples of a set of standard target DNA solutions for each experiment, resulted in reproducible, target number-dependent PCR product formation. The only small variation was apparently introduced by the individual master mixes (Fig. 1). The direct relationship between the initial number of the target sequence and the densitometer reading allowed the determination of the number of *nahA* target sequences of pUC8-ISP DNA molecules in the range of 1.9 × 10^3 to 1.9 × 10^5 *nahA* targets in the assay, which is equivalent to 10 fg to 1 pg of pUC8-ISP DNA. Lower numbers of targets, down to as few as 10 per
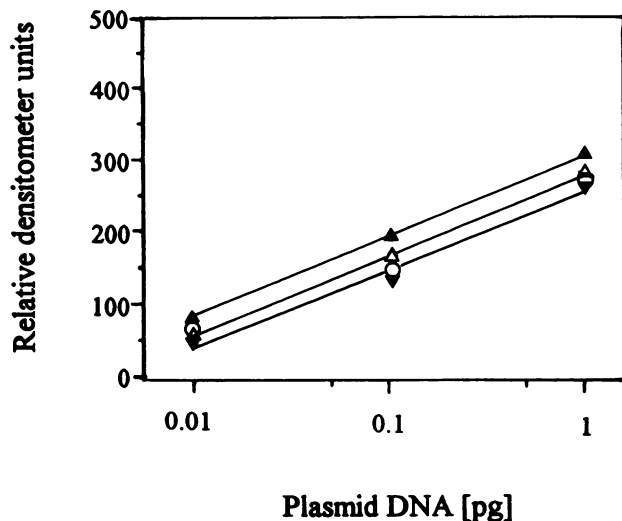
Plasmid DNA [pg]

FIG. 1. Densitometric quantitation of PCR products formed from various amounts of *nahA* targets. A set of pUC8-ISP DNA solutions, containing 10 fg, 100 fg, or 1 pg of DNA in 1 μl of TE buffer (corresponding to 1.9×10^3, 1.9×10^4, and 1.9×10^5 *nahA* targets, respectively) was used for all amplifications (20-μl reaction mixture; 30 amplification cycles). Fresh master mixes (containing BSA at 3 mg/ml) were prepared for each set of PCR assays. Same symbols indicate one experiment. The mean densitometer readings (± standard deviation) for six determinations (four of them are shown) were 300 ± 22 (1.9×10^5 targets), 179 ± 31 (1.9×10^4), and 73 ± 11 (1.9×10^3).

assay, were detectable by PCR amplification (see below) but were not in the range of the direct proportionality between the target number and densitometer reading shown in Fig. 1. At target numbers higher than 1.9×10^5, the amount of PCR products approximated a plateau (at a densitometer reading of about 500; data not shown). In further experiments with PCR used for quantitation of target numbers, the three standard DNA solutions shown in Fig. 1 were always run in

parallel PCR assays in order to calibrate for minor variations in the master mixes.

Effect of soil extracts on PCR. Portions (1 μl) of undiluted extracts from uninoculated nonsterile soils (loamy sand, clay, and silty clay) and serial decimal dilutions (in buffer) were mixed with various amounts of pUC8-ISP DNA and added to the PCR amplification mixtures (20 μl). Preliminary experiments with an extract from the loamy sand soil showed that PCR was inhibited by the undiluted extract and extracts diluted 10^{-1}, 10^{-2}, and 10^{-3}. PCR was possible with the 10^{-4} and 10^{-5} dilutions, but amplification was inefficient and unreproducible. Among several substances added (dimethyl sulfoxide [10% (vol/vol), final concentration], glycerol [5%], β-mercaptoethanol [10 mM], and BSA [various concentrations]), only BSA (3 mg ml^{-1}, final concentration) enhanced PCR in the presence of soil extracts. Figure 2A shows that, with BSA, the undiluted extract of the loamy sand soil still completely inhibited amplification even at a high target number (1.9×10^5 per assay). A 10^{-1} dilution of the extract gave only a small inhibition, resulting in 25 to 52% of the products obtained by PCR in buffer with BSA with the corresponding target number (Fig. 2B). A 10^{-2} and 10^{-3} dilution gave no inhibition.

In the presence of BSA, undiluted extracts of the clay and silty clay soils also completely inhibited PCR (data not shown). With 10^{-1} dilutions, inhibition was absent, and the products were proportional to the target number (data not shown). Thus, for determination of target numbers by PCR, 1-μl portions of at least 10^{-1}-diluted soil extracts per 20 μl of PCR assay volume were acceptable.

Sensitivity of detection of *nahA* targets in soil extracts. In the following experiments, the minimum number of plasmid DNA molecules which, after addition to soil, could be quantitatively assessed by PCR was determined. Samples of the three ethylene oxide-treated soils were extracted 1 h after inoculation with various amounts of pUC8-ISP DNA (100 ag to 1 ng per g of soil). Portions (1 μl) of 10-times-diluted extracts were included in the PCR assays. Densitometric analysis (Fig. 3B, panel I) of the products formed after one round of PCR (30 amplifications; Fig. 3A, panel I)
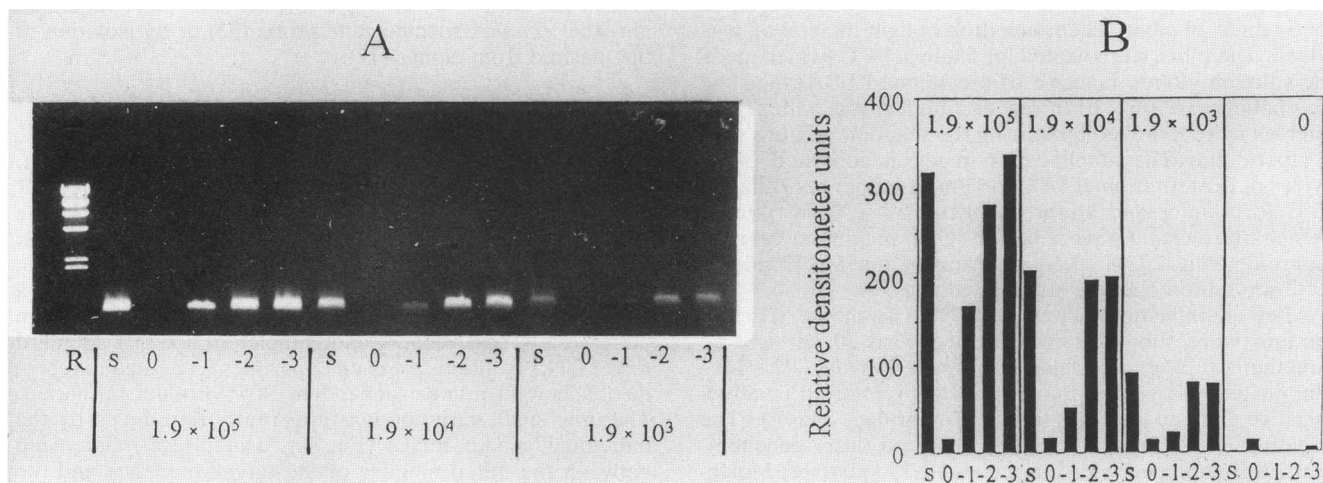

FIG. 2. Effect of extract from uninoculated loamy sand soil on PCR (master mixes contained BSA at 3 mg/ml). Amplification was done with various amounts of pUC8-ISP DNA (1.9×10^3 to 1.9×10^5 *nahA* targets, as indicated) either in buffer (assay s), in the presence of 1 μl of undiluted extract (assay 0), or in buffer-diluted 10^{-1}- to 10^{-3}-fold (assays −1 to −3) soil extract. (A) Electrophoretically separated PCR products (a PCR assay without pUC8-ISP DNA did not result in a band; data not shown). Lane R, *Hind*III-digested phage λ DNA. (B) Densitometry of PCR products.
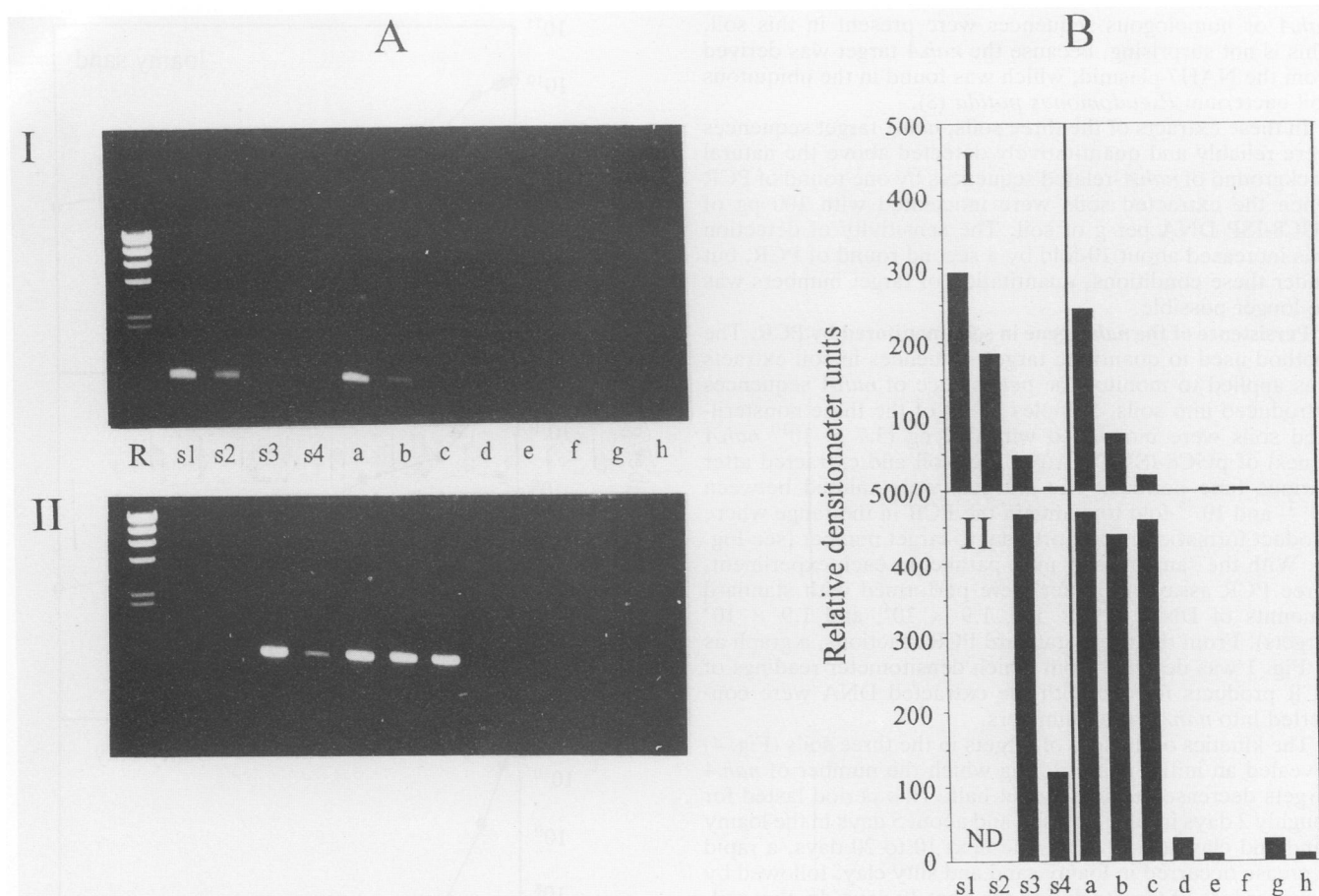
FIG. 3. Detection and quantitation of *nahA* targets in extracts of ethylene oxide-treated loamy sand soil 1 h after inoculation with pUC8-ISP DNA. Total DNA was extracted and used in two successive rounds of PCR (30 cycles each). The amplification products were separated in agarose gels, photographed (A), and densitometrically analyzed (B). In PCR assays without soil extract, plasmid DNA was applied in 1 μl of TE. s1, 540 fg (10^5 *nahA* targets); s2, 5.4 fg (10^3 *nahA*); s3, 540 ag (10^2 *nahA*); s4, 54 ag (10 *nahA*). In PCR assays with soil extract, 1-μl samples of 10-times-diluted extract were added to the amplification mixture. Soil samples were inoculated with: a, 1 ng of pUC8-ISP DNA per g of soil (1.9×10^8 *nahA* targets); b, 100 pg (1.9×10^7 *nahA*); c, 10 pg (1.9×10^6 *nahA*); d, 1 pg (1.9×10^5 *nahA*); e, 100 fg (1.9×10^4 *nahA*); f, 10 fg (1.9×10^3 *nahA*); g, 1 fg (1.9×10^2 *nahA*); h, 100 ag (1.9×10^1 *nahA*). I, first round of PCR; II, successive round of PCR. Lane R, *Hind*III-restricted λ DNA. The figure shows that, in PCR without soil extract (assays s1 to s4), substantial readings were obtained in densitometry of products from 10^5, 10^3, and 10^2 *nahA* targets after one round of PCR (BI, s1 to s3) and from 10 *nahA* targets after two successive rounds of PCR (s4).

showed that extracts from loamy sand soil samples which were inoculated with 1 ng, 100 pg, or 10 pg of pUC8-ISP DNA per g of soil gave substantial but decreasing amounts of PCR products (Fig. 3B, bars a to c). The assay with the smallest amount of DNA was at the limit of detection. Assuming that no target DNA degradation occurred during 1 h of incubation of DNA in the ethylene oxide-treated soil and considering the efficiency of DNA extraction (see Materials and Methods) plus the actual volumes of extract added to the PCR mixtures, the three PCR assays giving substantial amounts of products contained 3.7×10^4, 3.7×10^3, and 3.7×10^2 *nahA* target sequences. In other words, when at least 10 pg (corresponding to 3.7×10^2 targets per PCR assay) of plasmid DNA was added per g of soil, quantitative determination of the target number by one round of PCR was possible.

In a second round of PCR (30 amplifications; Fig. 3, panel II) with the products of the first round as the substrate, the assay which initially contained 370 *nahA* targets now gave a strong signal (assay c). Lower numbers of the target were not detectable (Fig. 3, panels II, assays d to h), although as few as 10 *nahA* copies (54 ag of pUC8-ISP DNA) were amplified to substantial signals when added to assays e, f, g, and h, containing 1 μl of 10^{-1}-diluted extracts of inoculated soils (data not shown). Presumably, the amount of *nahA* targets extracted from soil which was inoculated with less than 10 pg of pUC8-ISP DNA per g of soil was too small for PCR amplification, or the targets were no longer a substrate for PCR (see Discussion).

With 10^{-1}-diluted extracts of the silty clay soil and the clay soil, PCR products were obtained from soil samples inoculated with 100 pg or more of pUC8-ISP DNA per g of soil (data not shown), corresponding to $\geq 3.7 \times 10^3$ *nahA* targets present in the PCR assay. With two rounds of PCR, products were formed from silty clay soil inoculated with only 1 pg of pUC8-ISP DNA per g of soil (37 *nahA* targets per assay). From the clay soil, products (about 1 kb) were formed even from uninoculated samples, suggesting that

nahA or homologous sequences were present in this soil. This is not surprising, because the *nahA* target was derived from the NAH7 plasmid, which was found in the ubiquitous soil bacterium *Pseudomonas putida* (8).

In these extracts of the three soils, *nahA* target sequences were reliably and quantitatively detected above the natural background of *nahA*-related sequences by one round of PCR when the extracted soils were inoculated with 100 pg of pUC8-ISP DNA per g of soil. The sensitivity of detection was increased about 10-fold by a second round of PCR, but under these conditions, quantitation of target numbers was no longer possible.

Persistence of the *nahA* gene in soil, monitored by PCR. The method used to quantitate target sequences in soil extracts was applied to monitor the persistence of *nahA* sequences introduced into soils. Samples (10 g) of the three nonsterilized soils were inoculated with 200 ng (3.7 × 10^{10} *nahA* genes) of pUC8-ISP DNA per g of soil and extracted after various time periods. The extracts were diluted between 10^{-1}- and 10^{-5}-fold to maintain the PCR in the range where product formation is proportional to target number (see Fig. 1). With the same master mix, parallel to each experiment, three PCR assays in buffer were performed with standard amounts of DNA (1.9 × 10^3, 1.9 × 10^4, and 1.9 × 10^5 targets). From the three standard PCR reactions, a graph as in Fig. 1 was derived, from which densitometer readings of PCR products formed with the extracted DNA were converted into *nahA* target numbers.

The kinetics of the loss of targets in the three soils (Fig. 4) revealed an initial phase during which the number of *nahA* targets decreased to only about half. This period lasted for roughly 2 days in silty clay soil and about 5 days in the loamy sand and clay soils. During the next 10 to 20 days, a rapid decrease occurred in loamy sand and silty clay, followed by a slower loss of targets during the next 40 days. In clay soil, there was no such clear change in the decay rate. After 60 days in soil, the portions of *nahA* targets still detectable by PCR were 0.2% in loamy sand, 0.05% in clay, and 0.01% in silty clay soil (Fig. 4).

Influence of soil extracts on electroporation of *E. coli*. In order to monitor the presence of transforming plasmid DNA molecules in soil inoculated with pUC8-ISP DNA, electroporation of *E. coli* was used, with the Ap^r marker of the plasmid used for transformant selection. Soil extracts for electroporation experiments were taken from the stocks also used for PCR studies (see preceding sections). *E. coli* SF8 was electroporated in the presence of pUC8-ISP DNA (0.3 ng per assay) dissolved in buffer or in extracts of the three uninoculated soils. Figure 5 shows that 1 or 5 μl of extracts of the nonsterile or ethylene oxide-treated soils (added to 40 μl of cell suspension) did not or only weakly reduced transformation of *E. coli*. The reference transformation without extract gave 10^9 transformants per μg of pUC8-ISP DNA, i.e., formation of 1 transformant required about 100 plasmid molecules. A direct increase in the number of transformants with an increase in the amount of pUC8-ISP DNA (0.1 to 300 pg) per assay was obtained regardless of whether electroporation was performed in the presence or absence of undiluted extracts of nonsterile, uninoculated soils (Fig. 6).

Sensitivity of detection of transforming plasmid DNA in soils. The minimum number of pUC8-ISP DNA molecules in soil extracts which was detectable by electroporation was determined as follows. Ethylene oxide-treated soils (10-g samples) were inoculated with various amounts of pUC8-ISP DNA (1 pg to 1 ng per g of soil) and extracted after 1 h of
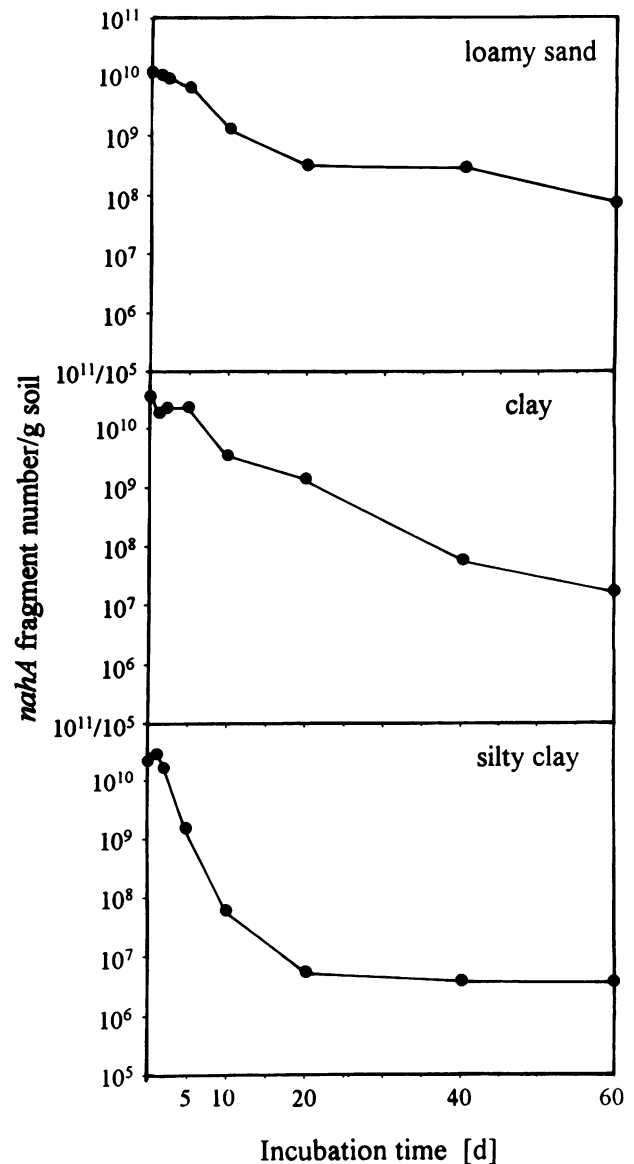

FIG. 4. Decrease in the number of *nahA* targets during incubation of pUC8-ISP (3.7 × 10^{10} *nahA* copies per g of soil) in three nonsterile soils. After various periods, total DNA was extracted from soil, and the number of *nahA* targets was quantified by PCR. The data shown are from a single series of determinations. Selected values (including those for 1 h and 60 days) were verified by independent PCR assays.

incubation as described in Materials and Methods. Figure 7 shows that electroporation with 5 μl of these soil extracts (the same as used for PCR) gave transformants when loamy sand or clay soil was inoculated with at least 10 pg (9 × 10^5 molecules) of plasmid DNA per g of soil. In the silty clay soil, the detection was 10 times less sensitive.

Measurement of persistence of plasmid DNA molecules in soils. Nonsterile and ethylene oxide-treated soils inoculated with pUC8-ISP DNA (200 ng/g of soil) were extracted after various periods of time (1 h to 60 days; same stocks as for PCR experiments). In nonsterile loamy sand and silty clay soil, slow inactivation of the transforming activity was
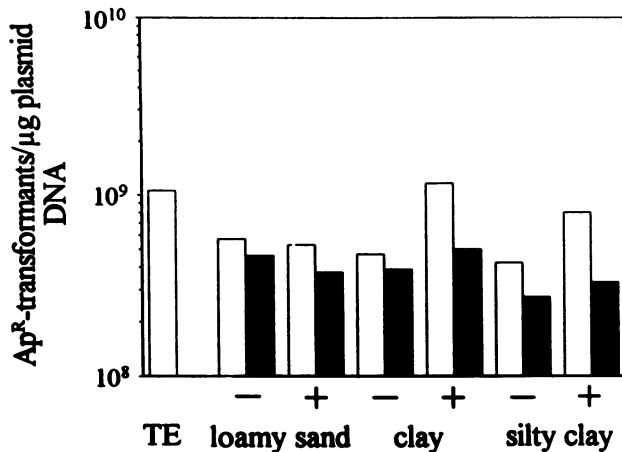
FIG. 5. Effect of soil extracts on electroporation-effected transformation of *E. coli* SF8. Electroporation was done with 0.3 ng of pUC8-ISP DNA dissolved in TE (1 μl) or in extracts (open columns, 1 μl; solid columns, 5 μl) of uninoculated soils. –, nonsterile; +, ethylene oxide treated. For details, see Materials and Methods.

observed during an initial phase of 1 to 2 days. Within the first 10 days, transforming activity declined approximately 1,000-fold (loamy sand and clay soil) to almost 100,000-fold (silty clay soil). In the loamy sand soil, 0.011% of the initial transforming activity was still present after 60 days, 0.004% was present after 40 days in clay, and 0.002% was present after 10 days in silty clay soil (Fig. 8). When low numbers of transformants were obtained, the presence of the plasmid in transformants was verified by clone analysis and colony blot hybridization (with a *nahA* gene probe) (33).

Ethylene oxide treatment did not prove successful as a sterilization procedure for the clay soil and the silty clay soil (33). This is probably why similar plasmid inactivation kinetics were observed in the ethylene oxide-treated and
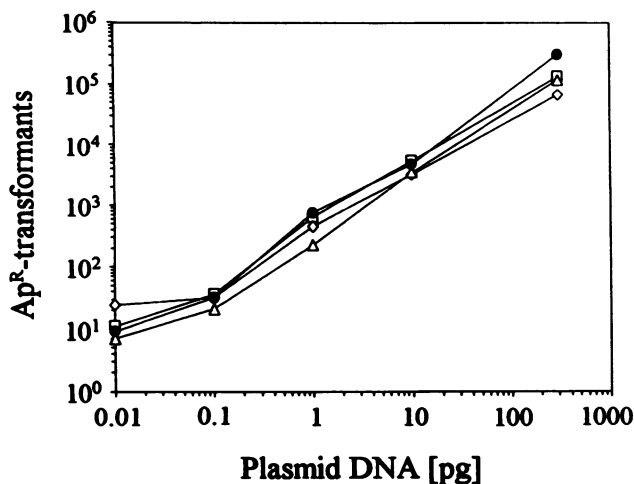

FIG. 6. Number of ampicillin-resistant transformants obtained by electroporation with various amounts of pUC8-ISP DNA. Electroporation was performed with plasmid DNA in 5 μl of TE (□) or 5 μl of extract from nonsterile, uninoculated loamy sand soil (●), clay soil (◇), and silty clay soil (△). Numbers of transformants and amounts of plasmid DNA are given per assay volume (45 μl). For experimental details, see Materials and Methods.

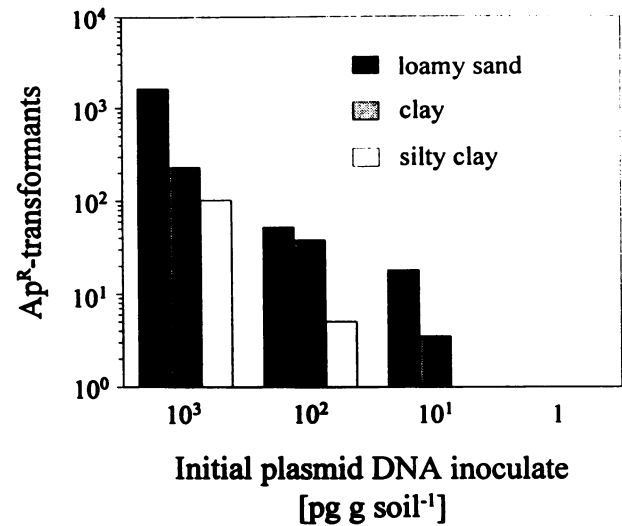## Initial plasmid DNA inoculate [pg g soil⁻¹]

FIG. 7. Quantitation of plasmid DNA in soils by electroporation. Ethylene oxide-treated soils were incubated with 1 pg to 1 ng of pUC8-ISP DNA per g of soil for 1 h and then extracted. The extracts (5 μl) were used for electroporation, and ampicillin-resistant colonies were scored. The number of transformants is expressed per assay volume (45 μl). For details, see Materials and Methods.

nonsterile clay soils (Fig. 8). In the loamy sand soil, however, the initial high rate of loss of transforming activity was substantially reduced by ethylene oxide treatment, suggesting that soil-borne DNases and/or nuclease-producing microorganisms were largely inactivated by the treatment.

DISCUSSION

Laser densitometry is a technique for the quantitation of PCR products (for a review, see reference 39) and proved to be instrumental for the determination of the number of target DNA molecules by PCR amplification assays in accordance with the protocol described here. Amounts of plasmid DNA from 10 fg to 1 pg (corresponding to 1.9 × 10³ to 1.9 × 10⁵ targets) per assay were quantitated by one round of PCR and were highly reproducible in independent experiments. With two rounds of PCR (30 cycles each), even 10 *nahA* copies were detectable, but quantitation was not possible. This sensitivity of detection of the *nahA* fragment is close to that obtained by Tsai and Olson (43), who were able to amplify a 371-bp DNA segment of the *E. coli* 16S rRNA gene (seven copies per chromosome) from fewer than 5 cells, using DNA extracted from late-log-phase cells, by 40 PCR cycles. The reproducible quantitation of the number of target sequences as described here is in contrast to other studies (9), in which the amount of the PCR products varied considerably within replicates at the same DNA dilution, which made determination of the target number in a given sample ambiguous and precluded target concentration determinations over logarithmic ranges of dilutions.

The reason for the higher reproducibility of the PCR assays in this work than in the studies described by Gilliland et al. (9) is not clear. In both studies, *Taq* polymerase from the same manufacturer was used, although we included a twofold-higher concentration in the assay. However, our hot-start PCR assay contained BSA at a high concentration (3 mg/ml). Furthermore, we used a primer concentration about 6 times lower than that in the other experiments (0.2
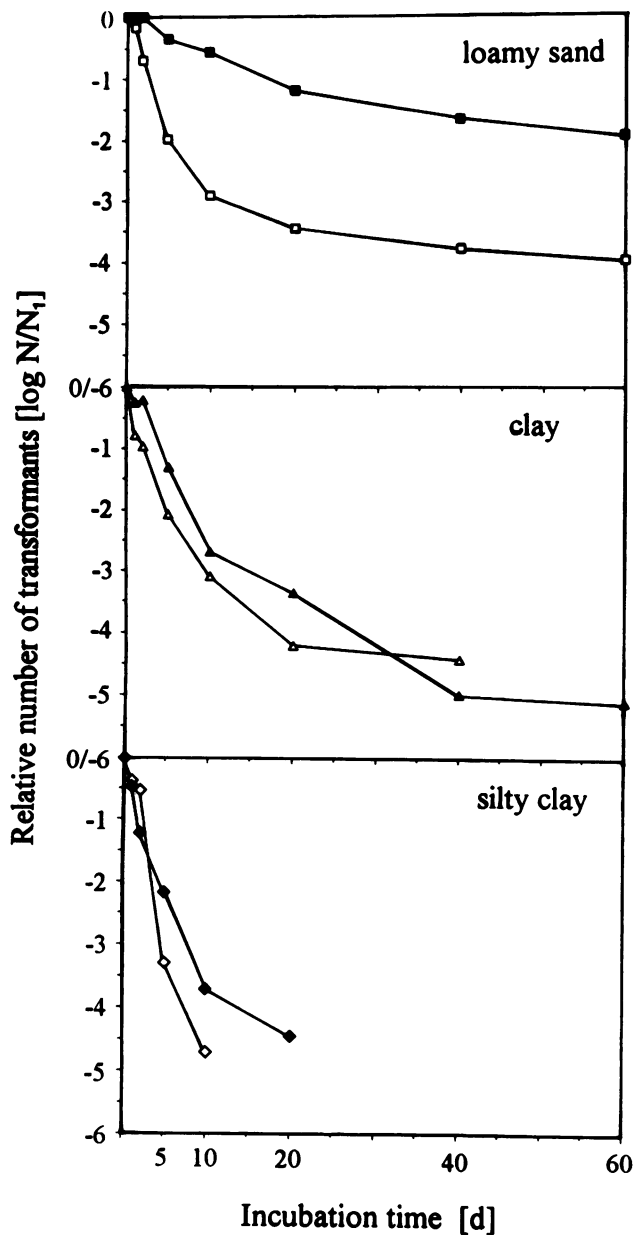
FIG. 8. Kinetics of loss of transforming plasmid DNA in non-sterile (open symbols) and ethylene oxide-treated (solid symbols) soils, determined by electroporation of *E. coli* SF8. Transformation is expressed as the log of the frequency of transformants at a given time point (*N*) relative to the frequency of transformants 1 h after inoculation (*N*₁). These frequencies were 2.8×10^5 (loamy sand soil), 1.6×10^5 (clay soil), and 1×10^5 (silty clay soil) for nonsterile soils and 2.3×10^5, 1.9×10^5, and 8.8×10^4, respectively, for ethylene oxide-treated soils. The data are means of two independent determinations.

μM [9]). Perhaps one or more of these alterations improved the amplification reaction and made it so consistent. In fact, calibration for minor variations in the performance of each master mix was possible with standard amounts of target DNA.

The strong inhibition of PCR by soil extracts seen in this study was also observed in previous experiments (43). The

researchers concluded that humic substances interfere with *Taq* polymerase activity in a way that prevents amplification of the template DNA. The yellow to brown color of the soil extracts used here indicated the presence of substances which could be humic acids or tannic or fulvic substances. Nanogram quantities of these contaminants can impair PCR (44). Addition of BSA to the assay and the use of 10-fold-diluted extracts was a way to avoid the inhibition and did not lead to the production of unspecific amplification products.

PCR signals from the *nahA* target DNA were obtained after inoculation with at least 1.9×10^5 target sequences per g of soil in the form of naked DNA. Since the volume of our PCR assay was only 20 μl, 5 times fewer targets would be detectable with 100-μl assays (4×10^4). When DNA inside of cells was added to soil and subsequently extracted (43), approximately 3.5×10^3 copies (500 cells with 7 copies of 16S rRNA genes) of the target sequence (371 bp) per g of soil were detectable by PCR (100-μl assay volume).

Steffan and Atlas (38) reported the detection of 15 to 20 copies (one cell of *Pseudomonas cepacia*) of the target (a chromosomal repetitive sequence) per g of sediment by a combination of PCR with DNA extracted from seeded sediment and dot blot hybridization analysis of the amplification products. A probable reason for the seemingly lower sensitivity of detection in this study may be the use of extracellular DNA, which, during its incubation in soil, may have been subject to enzymatic attack and also to chemical and physical alterations caused by abiotic factors (33), as the template for PCR. This may have excluded a portion of DNA molecules as amplification targets during the 1-h incubation period in nonsterile soil. The approximately three times larger target sequence (and hence higher vulnerability during incubation in soil and during extraction) used in this study than in the work of Tsai and Olson (43) may also have contributed to the apparently lower sensitivity of detection.

The loss of *nahA* targets as quantitated by PCR accelerated after 2 to 5 days in the soil and decreased beyond day 20. These kinetics may be related to the activity of soil microbiota in the microcosm. During the first 2 days after DNA inoculation, growth of bacteria (initial concentration, 10^6 CFU/g of soil) was exponential and reached a plateau after 5 days (approximately 2×10^7 CFU/g of soil [33]). It is possible that the bacterial growth was coupled with de novo synthesis of extracellular nucleases. The retarded loss of *nahA* genes may then result from nucleases produced mainly after growth of cells had ceased (as known, e.g., for nutrient depletion-induced production of extracellular enzymes, including nucleases in *Bacillus subtilis*; for a review, see reference 24). Alternatively, some time may have been required for the translocation of nucleases to the sites where the introduced *nahA* DNA was located in the soil matrix.

Here we show that electroporation of *E. coli* is superior to the transformation of CaCl₂-treated *E. coli* cells with soil DNA (33) for two reasons. First, with the same plasmid DNA used previously, transformation efficiency was ca. 20 times higher (10^9 transformants per μg of DNA in the absence of soil extract). Second, whereas transformation of CaCl₂-treated cells was inhibited 20- to 670-fold by soil extracts (33), the efficiency of transformation by electroporation was only weakly reduced (Fig. 5). This, together with the facts that the number of transformants followed the amount of plasmid DNA over about a 100,000-fold range and that as little as 10 to 100 pg of plasmid DNA per g of soil was detectable (Fig. 7), strongly recommends electroporation as a powerful biological assay for the detection of plasmid DNA molecules in environmental samples. We suggest that this

method can be adopted for the monitoring, in soil and other environments, of genetically engineered organisms which carry recombinant plasmids. In that case, the transformation assay for plasmids in total DNA extracts would not be confined to culturable cells or to the species that was released. Rather, the plasmids may remain detectable in viable but nonculturable cells and also in cells of the ambient microbial population to which the plasmids may have been transferred.

Similar to the initial slow loss of nahA targets in soils as quantitated by PCR, a period (1 h to 2 days) of slow inactivation of the transforming activity of plasmid DNA was noticed in the loamy sand and silty clay soils (compare Fig. 4 and 8). Because a single double-strand break inactivates the transforming activity of a plasmid (1), it is concluded that, for at least 1 or 2 days in the soils, a major portion of the introduced plasmid molecules stayed intact (i.e., circular). This finding is in accord with the results of Southern transfer hybridizations of extracts from soils inoculated with pUC8-ISP DNA, which showed a strong signal at the positions of open circular and supercoiled pUC8-ISP DNAs after 1 day in the silty clay soil and after 2 days in the loamy sand soil, but only a faint signal in the clay soil after 1 day (33). In the electroporation assay, the clay soil also did not show a lag of plasmid inactivation during the first few days (Fig. 8). However, despite the large decrease in the number of transforming plasmid molecules until day 10 of the incubation, transforming activity was still present after 40 days in the clay soil and even after 60 days in the loamy sand soil. Hence, it can be assumed that a considerable portion of the nahA copies detected by PCR were associated with the transforming activity in the soils. It seems that niches are present in soil where genes or plasmid molecules may persist. It is known that DNA adsorbs to soil minerals, including quartz, feldspar, and various clay minerals (3, 14, 19, 26, 27, 34). DNA thereby becomes resistant to enzymatic degradation (10, 14, 19, 22, 27, 34, 35). DNA adsorption and DNase I resistance have also recently been demonstrated with nonpurified mineral samples taken from a groundwater aquifer (35).

The data presented here indicate that, at the level of molecule integrity or genetic information content, free DNA is not immediately destroyed in soil but can be found weeks or months after introduction. The kinetics of the degradation of introduced DNA imply that free DNA may be present in soil. Extracellular DNA has been extracted from sediment (25), and recent data show that the diversity of rRNA genes retrieved from sediment exceeded the number of observed bacterial morphotypes present (37). The researchers proposed that part of the recovered sequences originated from extracellular DNA. It has been suggested that, because of its persistence, DNA released from living or dead organisms may constitute an extracellular gene pool in soil (15).

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