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Development and Application of a New Method To Extract Bacterial DNA from Soil Based on Separation of Bacteria from Soil with Cation-Exchange Resin

CARSTEN S. JACOBSEN[1]†* AND OLE F. RASMUSSEN[2]‡

Department of Marine Ecology and Microbiology, National Environmental Research Institute, Frederiksborgvej 399, DK-4000 Roskilde,[1] and Department of Molecular Food Technology, Biotechnical Institute, Lundtoftevej 100, DK-2800 Lyngby,[2] Denmark

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A new method for the extraction of bacterial DNA from soil has been developed. Soil samples of 50 g were dispersed, and bacteria were released by use of a cation-exchange resin; subsequently, bacteria were separated from soil particles by low-speed centrifugation and lysed with lysozyme and ionic detergent, and the DNA was then purified by CsCl-ethidium bromide equilibrium density centrifugation. The extracted DNA was of high molecular weight and sufficiently pure for restriction enzyme digestion, DNA-DNA hybridization, and amplification by the polymerase chain reaction. The advantages of the new method are that the separation of bacteria from soil is considerably faster than by repeated blending, more samples can be handled, and furthermore no aerosols are formed during separation. Also, we investigated whether the CsCl-ethidium bromide equilibrium density centrifugation could be replaced by purification using Gene-Clean. However, this method produced DNAs which were insufficiently pure for several types of analysis. The new method was used to study survival of a 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading *Pseudomonas cepacia* DBO1 (pRO101) in unamended soil and in soil amended with 2,4-D. We found that the degrading strain, irrespective of inoculation level, was able to grow to the same high numbers in soil amended with 2,4-D, while the strain in nonamended soil were maintained at the inoculation level. Detection based on DNA extraction and subsequent dot blot DNA-DNA hybridization was in accordance with detection by plating on selective medium.

Direct counts of total bacteria in the environment are typically more than one order of magnitude higher than plate counts (4, 21). Consequently, methods that do not involve culturing of microorganisms are advantageous for many types of microbial ecological studies. Several detection and enumeration methods based on DNA techniques have been developed for monitoring specific microbial genotypes in environmental samples and for studying microbial community structures (11, 23). Two approaches for DNA isolation have been applied: (i) direct lysis and (ii) cell extraction.

Direct lysis involves treatment of a sample with hot sodium dodecyl sulfate (SDS) and mechanical disruption of cells by shaking with glass beads (19). The DNA is purified by using ethanol precipitation, CsCl-ethidium bromide (EtBr) density gradient centrifugation, and/or a hydroxyapatite column. Recovered DNA is sufficiently pure for dot blot DNA-DNA hybridization; however, it is sheared to fragment sizes smaller than 10 kb, excluding the application of Southern blot analysis.

Cell extraction involves separation of bacterial cells from soil particles by blending in a washing buffer followed by differential centrifugation (4). Cells are lysed with lysozyme and hot SDS, and the DNA is purified by using a hydroxyapatite column (27). Holben et al. (10) improved the lysis method and included polyvinylpolypyrollidone (PVPP) to reduce humic contamination of the extracted DNA. With this method, both the purity and the molecular weight of recovered DNA are high, making detection by Southern blot DNA-DNA hybridization possible.

The two approaches were evaluated by Steffan et al. (25). Although the direct-extraction method resulted in the highest yield, the cell extraction method was recommended if the extracted amount of DNA was sufficient since contamination with eucaryotic or extracellular DNA probably is eliminated.

A major limitation of the cell extraction method is that it is time-consuming. Another drawback is that spreading of organisms by aerosolization and other spills is practically unavoidable during the repeated blending and centrifugation steps.

Dispersion of soil particles by cation-exchange resins has been the basis for development of several methods to extract microorganisms from soils (8, 12, 17). Cation-exchange resins replace polyvalent cations with monovalent cations. Since polyvalent cations play a major role in the formation of complexes between clay particles and organic matter (3), cations are responsible for the formation of soil micropores in which a major part of the indigenous soil bacteria may be present (7). Bacteria are believed to be adsorbed to soil particles by the same types of bindings (26) and thus would be expected to be released by cation exchange. A single-step method using cation-exchange resin was developed by Macdonald (17) and modified by Herron and Wellington (8). Neither of these procedures, however, has been applied to extraction of bacterial DNA.

In this paper, we describe a cell extraction method for recovering bacterial DNA from soil samples. The method is based on a single-step soil dispersion procedure using a cation-exchange resin. The method was used to study the survival of a *Pseudomonas cepacia* strain in soil.

* Corresponding author.
† Present address: Microbiology Section, Department of Ecology and Molecular Biology, Royal Veterinary and Agricultural University, Rolighedsvej 21, DK-1958 Frederiksberg C, Denmark.
‡ Present address: Radiometer A/S, Emdrupvej 72, DK-2400 Copenhagen, Denmark.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. cepacia* DBO1(pRO101) (6) was kindly provided by R. H. Olsen. *Alcaligenes eutrophus* JMP134(pJP4) (2) was obtained from Deutche Sammlung für Mikroorganisme. *Escherichia coli* DH5-α was used as a host for recombinant plasmids. Plasmid pCJ17 was constructed by cloning a 5.5-kb *Eco*RI fragment of pRO101 harboring the transposon Tn*1721* in pUC9.

Bacterial growth media. *E. coli* and *P. cepacia* DBO1 (pRO101) were routinely grown in LB (18) supplemented with 30 μg of tetracycline per ml. Prior to isolation of plasmids, *P. cepacia* DBO1(pRO101) and *A. eutrophus* JMP134(pJP4) were grown in MMO medium (24) supplemented with 0.3% Casamino Acids and 500 μg of 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma Chemical Co., St. Louis, Mo.) per ml. *P. cepacia* DBO1(pRO101) was enumerated from soil on PTYG agar (0.25 g of tryptone, 0.25 g of peptone, 0.50 g of yeast extract, 0.50 g of glucose, 30 mg of $MgSO_4 \cdot 7H_2O$, 3.5 mg of $CaCl_2 \cdot 2H_2O$, 15 g of agar per liter) with 30 μg of tetracycline per ml and 25 μg of natamycin (Delvocid, Gist Brocades, Delft, Holland) per ml as selective agents.

Soil. A sandy loam was collected on an experimental farm in Roskilde, Denmark, at a site not sprayed with 2,4-D or other pesticides for at least 25 years. Characteristics of the soil were as follows: pH of soil H_2O, 6.5; cation-exchange capacity, 8.4 meq/100 g of dry soil; organic matter, 2.4%; clay, 10%; silt, 30.7%; fine sand, 31.9%; coarse sand, 24.1%. The soil was passed through a 4-mm (pore size) sieve on the day of collection and stored refrigerated in the dark for a maximum of 2 months.

Amendment of soil with 2,4-D and inoculation of bacteria. A 10% fraction of the soil was air dried overnight to allow the addition of 20 mg of 2,4-D per ml in 0.1 M $Na_2H_2PO_4$. In controls, only 0.1 M $Na_2H_2PO_4$ was added. The treated fraction was thoroughly mixed with the rest of the soil (1:9) to obtain a final concentration of 2,4-D of 500 ppm (wet weight), and the mixture was allowed to equilibrate at 15°C for 3 days before inoculation with bacteria. In previous experiments it was shown that less than 1% of the added 2,4-D was mineralized by the indigenous soil flora in three days (14). *P. cepacia* DBO1(pRO101) was harvested from late-log-phase cultures, washed two times in Winogradsky salt solution (4), and sprayed with a scent spray bottle onto the soil surface. After inoculation, the soil was carefully mixed. *P. cepacia* DBO1(pRO101) was added to the soil at an inoculation level of 2.5×10^7 CFU g of soil^{-1}. At 2 h and at 7 days after inoculation, the bacterial fraction was extracted as described below and plated in triplicate on PTYG agar supplemented with tetracycline.

Blending method. Bacteria were extracted from 50 g of soil by the procedure of Fægri et al. (4) with the modifications of Holben et al. (10).

Cation-exchange resin method. Bacteria were extracted from soil by a procedure similar to the method described by Herron and Wellington (8). Fifty grams of soil was transferred to a 250-ml centrifuge bottle with 10 g of Chelex 100 (Bio-Rad, Copenhagen, Denmark) and 100 ml of 0.1% Na-deoxycholate (Sigma)–2.5% polyethylene glycol 6000 (Sigma). The samples were shaken for 1 h at 100 rpm on an orbital shaker at 4°C with occasional (eight times) fast agitation by hand. Soil particles were pelleted by a 15-min centrifugation at 960 × *g*. The supernatant was filtered through a sterile gauze bandage to remove Chelex 100, and the bacterial fraction was harvested by a 20-min centrifugation at 22,100 × *g*. In cases of two rounds of extraction, pellets were resuspended in 100 ml of 0.1% Na-deoxycholate–2.5% polyethylene glycol 6000 and shaken and spun as described above.

Cell lysis and nucleic acid extraction. After being washed in 200 ml of TE (10) (pH 7), the bacterial fraction was resuspended in 20 ml of TE (pH 7) and 5 ml of 5 M NaCl was added. After a 10-min incubation at room temperature, samples were centrifuged at 22,100 × *g* at 4°C for 15 min. The pellet was resuspended in 5.5 ml of TE, and 500 μl of freshly prepared lysozyme (Sigma) (10 mg/ml in distilled H_2O) was added. After incubation at 37°C for 60 min, 500 μl of pronase (Sigma) (30 mg/ml in distilled H_2O, predigested for 30 min at 37°C) was added and the mixture was incubated 30 min at 37°C. Samples were then heated to 65°C. After 10 min of incubation at this temperature, 1.6 ml of preheated 10% SDS was added and the incubation was continued for another 10 min. Finally, samples were centrifuged at 43,700 × *g* at 4°C for 1 h and the supernatant was collected for DNA purification.

Purification of nucleic acids. (i) CsCl-EtBr equilibrium density centrifugation. CsCl-EtBr equilibrium density centrifugation was performed as described by Sambrook et al. (22) in 13.1-ml Beckman Quick-Seal tubes.

(ii) Gene-Clean. A 500-μl portion of the cleared lysate was added to a 2.2-ml Eppendorf microfuge tube together with 1 ml of 3 M NaI solution and 15 μl of Gene-Clean glass beads (BIO 101 Inc., La Jolla, Calif.). Diffusion washes were done instead of resuspensions according to the manufacturer's instruction to avoid shearing of large fragments.

Tests for purity of environmental DNA samples. When the purpose was to evaluate the purity of the extracted DNA samples, a pUC18 plasmid tracer was added. Successful digestion with selected restriction enzymes would result in the appearance of a clearly visible single band of plasmid DNA.

General DNA manipulations. Plasmids pRO101 and pJP4 were isolated with SDS and alkali lysis as described by Hirsch et al. (9). Plasmid preparation from *E. coli* and cloning in plasmid pUC18 were done according to standard procedures (22). Digestion with restriction enzymes was performed as recommended by the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany).

Preparation and validation of probe. Plasmid pCJ17 was digested with *Sma*I and run on a 1% agarose gel. The 1-kb fragment was quickly excised under UV light and purified by using GeneClean according to the manufacturer's recommendations. The fragment was labeled with ^{32}P by using a random priming kit (Boehringer Mannheim GmbH) and purified from unincorporated nucleotides by ethanol precipitation. Other regions of the 5.5-kb *Eco*RI fragment of pCJ17 were also screened for specificity. The 1-kb *Sma*I fragment, however, showed the lowest degree of hybridization to DNA from an indigenous soil.

Dot blotting, Southern blotting, and hybridization. Dot blotting was carried out with repetitive loading of 1-μl aliquots of DNA directly onto Hybond N filter paper. Denaturation and neutralization was done as described by the manufacturer (1). Southern blot analysis was performed by using either the capillary technique (22) or the Pharmacia Vacuum Blotting System (manufacturer's procedure).

Nucleic acids were linked to filter paper by UV exposure for 5 min. Prehybridization, hybridization, and washes were done at 65°C. Filters were placed on X-ray film for 2 h to 2 days at −80°C.

Analysis of data and statistics. The developed X-ray film

TABLE 1. Recovery of *P. cepacia* DBO1(pRO101) from soil by the cation-exchange method and the blending method

Method	Recovery[a] at:	
	2 h	7 days
Cation exchange	36.8 ± 2.6	35.4 ± 2.1
Blending		
1st round	26.9 ± 2.5	22.9 ± 1.4
2nd round	10.1 ± 0.9	13.1 ± 0.5
3rd round	4.2 ± 0.7	5.8 ± 1.1
Total	41.2 ± 4.4	41.8 ± 2.1

[a] The strain was introduced at ca. 10^7 CFU/g of soil (wet weight). The recoveries were determined by direct triplicate plating from triplicate experiments and are given with as a percentage (± standard error of the mean) of the total amount of tracer present.

image was analyzed by the Cream program (5). The light (in pixels) passing through the dots from the samples was measured and subtracted from the light passing through background dots. Plate counts were converted to CFU per gram of soil and analyzed by *t* test using SAS/STAT version 6.03 (Statistical Analysis Systems, SAS Institute, Cary, N.C.). All platings were done in triplicate, the bacterial extraction efficiency experiments were done in triplicate, the microcosm experiment was run without replication, and finally all hybridization and digestion experiments were run at least two times and a representative figure is shown.

RESULTS

Efficiency of recovery of the bacterial fraction from soil. The efficacy of the cation-exchange resin procedure for extracting bacteria from soil was compared with that of the method based on repetitive blending. The recovery of the added bacterial tracer was slightly higher (significant at the 5% level but not at the 1% level) with the blending method than with the cation-exchange method both after 2 h and after 7 days (Table 1). Addition of an extra identical extraction step in the cation-exchange method gave a higher recovery than with the blending method (data not shown); however, it also gave an increase in soil debris.

The bacterial fraction from the cation-exchange method contained some soil debris which could possibly interfere with the lysis. In an attempt to remove soil debris, we tried to flocculate clay particles with 0.1, 1, or 10 mM $CaCl_2$. The flocculation, however, resulted in a considerable decrease in recovery of the bacterial tracer, and flocculation was consequently not included in the final protocol. Washings of the pellet in TE removed part of the humic contamination, as judged by the color of the suspension after washings.

Yield, quality, and purity of the bacterial DNA. The DNA yield was approximately 1 μg/g of soil, and after CsCl-EtBr density gradient centrifugation the DNA was of high molecular weight and sufficiently pure to be digested with common restriction enzymes (Fig. 1). The environmental DNA was spiked with pUC18 plasmid DNA to validate digestion efficiency. The appearance of single-plasmid bands clearly indicates complete digestion with selected restriction enzymes. The DNA could also be used in hybridization experiments (see Fig. 3) and could serve as a template for the polymerase chain reaction (data not shown).

DNA is traditionally purified on a CsCl-EtBr density gradient. This method is, however, time-consuming. We have investigated the possibility of replacing the ultracentrifugation step with alternative purification methods, in particular purification by using Gene-Clean. These experiments were performed on extracts prepared by the blending method by splitting samples in two fractions, one for gradient purification and one for purification by Gene-Clean. After purification, the environmental DNA samples were spiked with pUC18 plasmid DNA to validate digestion efficiency. Only gradient-purified DNA was reproduciblly digested with *Hin*dIII, *Bam*HI, and *Eco*RI; digestion with *Eco*RI is shown in Fig. 2. Prolonged incubation and increased concentrations of enzymes did not result in reproducible digestion of Gene-Clean-purified DNA. Also, only gradient-purified DNA was sufficiently pure to serve as a template for polymerase chain reaction (data not shown).

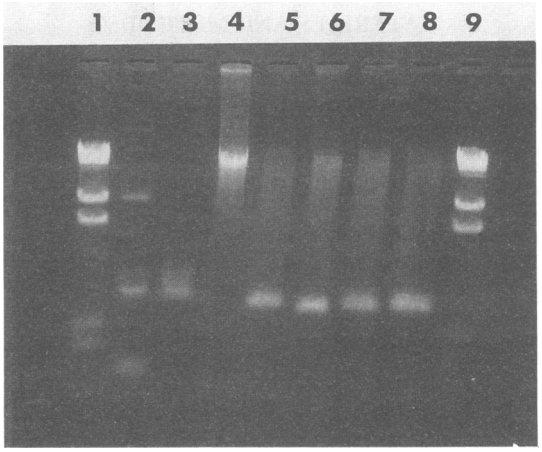

FIG. 1. Restriction enzyme digests of soil DNA and pUC18 plasmid. DNA from soil was extracted by using the cation-exchange method and purified by CsCl-gradient. The extracted DNA was spiked with pUC18 plasmid DNA in order to check for digestion efficiency. Digestion was carried out for 2 h in a total volume of 50 μl and with 50 U of enzyme. Lanes: 1 and 9, lambda DNA digested with *Hin*dIII; 2, 100 ng of nondigested pUC18; 3, 100 ng of pUC18 digested with *Eco*RI; 4, 4 μl of nondigested soil DNA; 5 to 8, 4 μl of soil DNA and 100 ng of pUC18 digested with *Eco*RI, *Hin*dIII, *Bam*HI, and *Sma*I, respectively.

Detection of *P. cepacia* DBO1(pRO101). The numbers of *P. cepacia* DBO1(pRO101) in soil amended with 500 ppm of 2,4-D increased to approximately 2×10^8 CFU/g (wet weight) of soil in 6 days irrespective of inoculation level, while no increase in numbers were observed in unamended soil (Fig. 3). From day 6 to day 13 the numbers of *P. cepacia* DBO1(pRO101) declined to 2×10^7 CFU/g of soil for the two lowest inoculation levels. Survival, estimated on the basis of probing DNA extracted by the new method with the 1-kb *Sma*I fragment of pCJ17, corresponded to survival estimated on the basis of plating on selective media (Fig. 3).

DISCUSSION

The need for methods to detect bacteria in soil without culturing has been the basis for several studies (10, 19, 25, 28); however, a broader application of the developed methods to ecological studies still needs to be demonstrated. In this study, we have successfully applied the gene probe approach to an ecological experiment as discussed below, and further we have demonstrated that high-quality DNA can be reproducibly extracted from the bacterial fraction of soil without the need for blending the samples. The blending steps take, in our hands, approximately 3 h of continuous work for processing a maximum of six soil samples, whereas

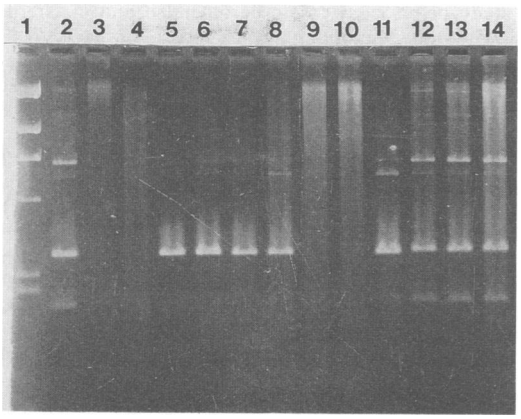

FIG. 2. Restriction enzyme digests of soil DNA and pUC18 plasmid. DNA from soil was extracted by using the blending method and purified by either CsCl-gradient (lanes 3 to 8) or Gene-Clean (lanes 9 to 14). The extracted DNA was spiked with pUC18 DNA in order to check for digestion efficiency. Digestion was carried out for 2 h in a total volume of 35 μl and with 20 U of enzyme. Lanes: 1, lambda DNA digested with *Hin*dIII; 2, nondigested pUC18; 3 and 9, 30 μl of nondigested soil DNA; 4 and 10, 30 μl of soil DNA digested with *Eco*RI; 5 and 11, 1 μl of soil DNA and 250 ng of pUC18 digested with *Eco*RI; 6 and 12, 5 μl of soil DNA and 250 ng of pUC18 digested with *Eco*RI; 7 and 13, 10 μl of soil DNA and 250 ng of pUC18 digested with *Eco*RI; lane 8 and 14, 30 μl of soil DNA and 250 ng of pUC18 digested with *Eco*RI.

the cation-exchange resin extraction takes approximately 1 h for 12 samples.

The new method is based on a single-step extraction using cation-exchange resins. Hopkins et al. (12) found that the single-step cation-exchange resin method (17) was the most effective of several single-step methods evaluated to disperse soil.

The initial separation of the bacterial fraction from soil debris and eucaryotic material ensures that extracted DNA is of bacterial origin and reduces problems associated with absorption of free DNA to soil particles (16). Cation-exchange resins have been shown to disperse soil effectively and facilitate representative separation of bacteria (12, 17). Since polyvalent cations on the soil particles are replaced with monovalent cations by the resin, the DNA-binding capacity of the clay particles which are extracted together with the bacteria are reduced (20). Hereby loss of DNA during the 43,700 × *g* spin following lysis is considerably reduced.

Soil humic compounds have an inhibitory effect on the activity of the proteolytic enzyme pronase (15) and possibly also on the lysozyme activity. By concentrating the bacterial fraction from the soil, this inhibitory effect is reduced, making the DNA extraction more efficient. However, if the concentration of the bacteria also concentrates humic acids and clay particles, the lysis procedure is less efficient. We found that two rounds of the cation-exchange extraction gave a higher cell yield; however, the overall recovery of DNA was reduced by a factor of 10. A likely reason is that more soil debris was present after two rounds of extraction, causing an inhibitory effect on the enzymes used in the lysis procedure. Consequently, extraction of DNA from soil is a trade-off between high recovery of bacteria and contamination with soil debris.

Steffan et al. (25) reported that repeated blending and direct lysis methods were equally time-consuming. The

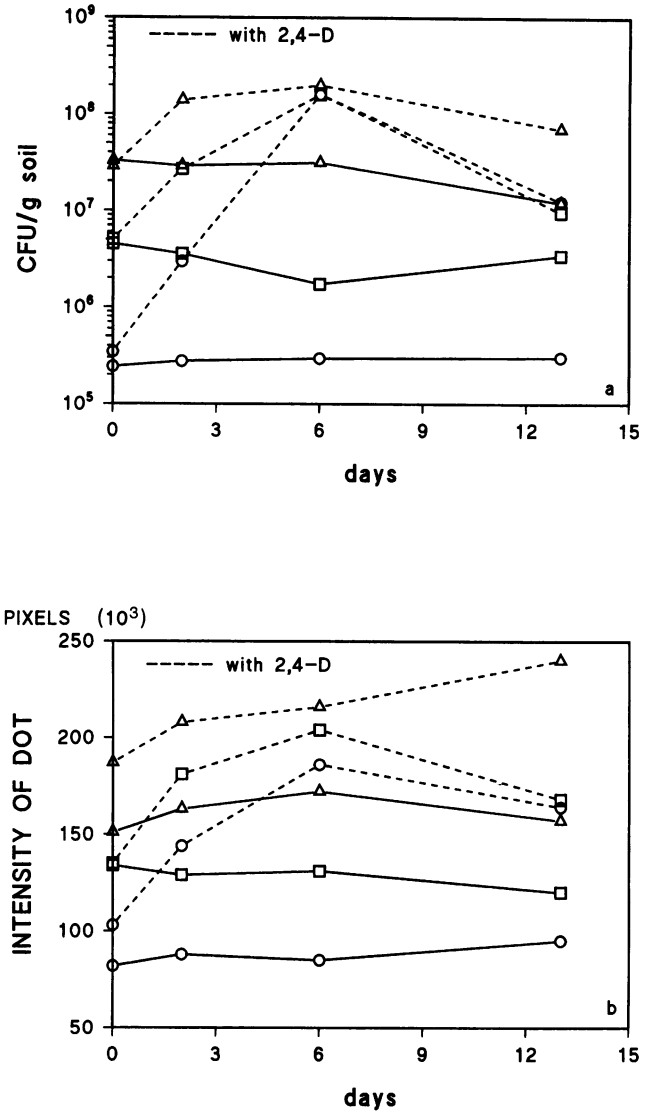

FIG. 3. Detection of *P. cepacia* DBO1(pRO101) by plating on PTYG agar with tetracycline (a) and by digitalized dot blot from soil DNA hybridized with the 1-kb *Sma*I fragment of pCJ17 (b). Solid lines, unamended soil; dashed lines, amendment with 500 ppm of 2,4-D. The strain was inoculated at 4×10^5 (○), 4×10^6 (△) or 4×10^7 (□) CFU per g of wet soil.

present procedure is considerably faster than both of these methods, making it possible to process more samples in one working day. Furthermore, formation of aerosols and other spills during repeated blending is eliminated with the cation-exchange method. Formation of aerosols and other spills may cause severe experimental problems by cross-contaminating samples, particularly if the extracted DNA is used for polymerase chain reaction.

The DNA extracted by the cation-exchange method is of high molecular weight and of a purity sufficiently high for several molecular applications. The quality is comparable to the quality of DNA obtained by the blending method (10) and superior to DNA obtained by various direct-extraction methods (19, 28).

Attempts to replace the CsCl gradient with easier and

quicker methods was not successful. For several applications it seems to be necessary to use time-consuming purification methods, such as CsCl density gradient or hydroxyapatite purification.

Application of the method. The cation-exchange method was used to monitor survival of *P. cepacia* DBO1 in unamended soil and soil treated with 2,4-D. We found that the carrying capacity for the 2,4-D-degrading strain was dictated by the presence of the substrate and that the same carrying capacity was reached regardless of the initial inoculation level. In the absence of selection by substrate, the degrading organisms were maintained at the level of inoculation. It was previously shown (13) that the growth of *P. cepacia* DBO1 in 48 h was significantly influenced by the concentration of 2,4-D in soil.

It was further found that detection by digitalization of dot blots from hybridization to extracted soil DNA was in accordance with detection by plating on selective media. The gene probe detection approach and the selective plating approach give in this case comparable results.

In conclusion, the cation-exchange resin method developed in this work has several advantages: it is fast, yields high-quality DNA, and does not cause spills to the surroundings.

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