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William Holben
University of Montana - Missoula, Bill.Holben@mso.umt.edu

Janet K. Jansson
Michigan State University

Barry K. Chelm
Michigan State University

James M. Tiedje
Michigan State University

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DNA Probe Method for the Detection of Specific Microorganisms in the Soil Bacterial Community†

WILLIAM E. HOLBEN,[1,2] JANET K. JANSSON,[1] BARRY K. CHELM,[2,3] AND JAMES M. TIEDJE[1,3]*

Department of Crop and Soil Sciences,[1] Plant Research Laboratory,[2] and Department of Microbiology and Public Health,[3] Michigan State University, East Lansing Michigan 48824

We developed a protocol which yields purified bacterial DNA from the soil bacterial community. The bacteria were first dispersed and separated from soil particles in the presence of polyvinylpolypyrrolidone, which removes humic acid contaminants by adsorption to this insoluble polymer. The soil bacteria were then collected by centrifugation and lysed by using a comprehensive protocol designed to maximize disruption of the various types of bacteria present. Total bacterial DNA was purified from the cell lysate and remaining soil contaminants by using equilibrium density gradients. The isolated DNA was essentially pure as determined by UV spectral analysis, was at least 48 kilobases long, and was not subject to degradation, which indicated that there was no contaminating nuclease activity. The isolated DNA was readily digested by exogenously added restriction endonucleases and successfully analyzed by slot blot and Southern blot hybridizations. Using single-stranded, ^{32}P-labeled DNA probes, we could detect and quantitate the presence of a specific microbial population in the natural soil community on the basis of the presence of a DNA sequence unique to that organism. The sensitivity of our methodology was sufficient to detect *Bradyrhizobium japonicum* at densities as low as 4.3×10^4 cells per g (dry weight) of soil, which corresponds to about 0.2 pg of hybridizable DNA in a 1-μg DNA sample.

Methods for the detection of specific microorganisms in environmental samples have traditionally been the fluorescent-antibody and selective plating techniques, each of which is useful but limited in some aspects. The prospect for the intentional or accidental release of genetically engineered microorganisms into the environment has served to highlight the need for further methodologies for monitoring the fate of particular microorganisms in the environment, especially for those that become nonculturable (8, 22) or for the detection of gene transfer to new populations. The methods of molecular biology that allow detection of particular DNA sequences can be used to detect particular genes, and hence organisms containing these genes, in the environment. If the total DNA of soil microorganisms could be recovered directly from soil, then the fate of particular genes or organisms could be monitored directly without the requirement for successful culture of the recovered organism.

Previously used protocols for the isolation of bacterial DNA from soil involved trichloroacetic acid treatment and ethanol-ether extraction of soil bacterial fractions filtered through diatomaceous earth (24) or purification of DNA from detergent and high salt in cell lysates by hydroxyapatite column chromatography (J. Goksoyr, personal communication). These methods are tedious, allow processing of only a few samples per week, and have not been demonstrated to yield DNA of a purity sufficient for use in restriction digestion and hybridization reactions.

To adapt DNA probe methodology for use in soils, the following features of a protocol needed to be improved or developed. (i) A procedure was needed which would allow processing of more samples simultaneously and in a shorter period of time for analysis of the number of treatments and replicates needed for ecological studies. (ii) The isolated DNA had to be of sufficient purity and size for use in

experiments involving digestion with restriction endonucleases, transfer to cellulose nitrate membranes, and hybridization to DNA probes. Humic acids and other contaminants, if not removed, could reduce the efficiency of digestion by restriction endonucleases and the specificity of hybridization. (iii) It was also necessary to develop probes both sensitive and specific enough to detect the presence of a particular sequence of low frequency in the complex mixture of DNAs isolated from the soil bacterial community. The standard method of labeling probes by nick translation (16) did not appear to be sensitive or specific enough for probing natural populations.

The objective of this work was to develop a protocol for the use of sequence-specific DNA probes to detect specific genes and microorganisms in soil. We successfully used both slot blotting and Southern transfer to fix DNA to cellulose nitrate filters which were subsequently used in hybridization experiments to detect *Bradyrhizobium* strains in soil. Both a naturally occurring sequence, the *rbcL* gene, and a sequence engineered into *Bradyrhizobium* strains, the *nptII* gene, were used as probes.

MATERIALS AND METHODS

Bacterial strains and phages. *Bradyrhizobium japonicum* BJ110 is a stable, small-colony derivative of *B. japonicum* USDA 3I1b110 (18). *B. japonicum* CRM52 is a ribulose-1,5-bisphosphate carboxylase-oxygenase-deficient, phosphoribulokinase-deficient, derivative of *B. japonicum* BJ110 obtained by gene replacement with the *nptII* (kanamycin resistance) gene (18). *Escherichia coli* JM103 and the M13 mp phage vectors are described elsewhere (4).

Culture media. *B. japonicum* BJ110 and CRM52 were cultured at 30°C on YEMN medium, which contains 0.04% yeast extract, 1.0% mannitol, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl, and 10 mM KNO_3. All other strains were cultured at the appropriate temperature (30 or 37°C) in Luria broth, which contains (per liter): 10 g of tryptone

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* Corresponding author.
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(Difco Laboratories, Detroit, Mich.), 5 g of yeast extract, and 5 g of NaCl. Enumeration of B. japonicum CRM52 in soil was accomplished by plating of appropriate dilutions of soil suspensions onto YEM (same as YEMN except containing no KNO_3) agar containing 30 μg of chloramphenicol (to which B. japonicum BJ110 and CRM52 are naturally resistant) per ml, 50 μg of kanamycin (the nptII gene confers kanamycin resistance) per ml, and 300 μg of cycloheximide (to control fungal growth) per ml. The plates were incubated for 7 to 10 days at 30°C before counting.

Inoculation and sampling of bacteria in soil. Bacterial cultures of B. japonicum BJ110 and CRM52 were grown to mid-log phase (optical density at 600 nm, 0.5) in YEMN broth. The cultures were harvested by centrifugation (23,000 × g for 20 min at 4°C), washed once with an equal volume of sodium phosphate buffer (15 mM, pH 7.0), and again collected by centrifugation. The cell pellets were suspended in 1/20 volume of sodium phosphate buffer and starved for 48 h by incubation at room temperature. Appropriate dilutions of the bacterial suspensions were made with 15 mM sodium phosphate buffer such that, regardless of cell density, 50 ml of total inoculum was added to 10 g of vermiculite (W. R. Grace and Co., Cambridge, Mass.) and thoroughly mixed. The vermiculite-inoculum mixture was then thoroughly mixed with 700 g (dry weight) of Capac loam soil (a fine-loamy, mixed, mesic, aeric Ochraqualf) which had been sifted through a 2-mm (square aperture) sieve. The inoculated soil was transferred to plastic pots with slotted bottoms and hydrated to approximately 80% of field capacity by placement in 5 to 6 cm of sterile, distilled water until moisture visibly reached the soil surface. At appropriate time intervals, minicore samples were taken from the soil pots by using copper pipes, (28-mm diameter by 170-mm length) with the resulting hole being filled by a polypropylene test tube of the same dimensions. This nondestructive sampling method allowed multiple samples to be taken from a single soil pot. A 10-g subsample of the minicore was used for enumeration of B. japonicum by selective plating, and a 50-g subsample was used for isolation of total bacterial DNA from soil.

Isolation of the bacterial fraction from soil. The bacterial fraction was isolated from soil by using a modification of the fractionated centrifugation technique described by Goksoyr and co-workers (11). Soil samples (50 g) were combined with 200 ml of homogenization solution (described below) and 10 g of acid-washed polyvinylpolypyrrolidone (PVPP) and then homogenized in a Waring blender for three 1-min intervals, with cooling in an ice water bath for 1 min between intervals. The homogenate was transferred into 250-ml centrifuge bottles, and the fungal biomass and soil debris were pelleted by centrifugation at 1,000 × g for 15 min at 4°C. The supernatant (SN1) was transferred to a fresh 250-ml centrifuge bottle and subjected to centrifugation at 23,000 × g for 20 min at 4°C to collect the bacterial fraction while the soil pellet (P1) was subjected to further (usually two more) rounds of homogenization and centrifugation. The combined bacterial pellets for all rounds of homogenization were suspended in 200 ml of 2% (wt/vol) sodium hexametaphosphate, pH 8.5 (Pfaltz and Bauer, Inc., Waterbury, Conn.), and then collected by centrifugation at 23,000 × g for 20 min at 4°C. The bacterial pellet was then washed twice by suspension in 200 ml of TE buffer, which contains 33 mM Tris (pH 8.0) and 1 mM EDTA. The bacteria were collected by centrifugation and suspended in 100 ml of TE buffer to yield the bacterial suspension for lysis.

The homogenization solution consisted of Winogradsky

salt solution (20), diluted 1:20 [1.43 mM K_2HPO_4, 1.01 mM $MgSO_4 \cdot 7H_2O$, 2.14 mM NaCl, 4.75 μM $Fe_2(SO_4)_3 \cdot 7H_2O$, 14.8 μM $MnSO_4 \cdot 4H_2O$], to which 0.2 M sodium ascorbate was added just before use to achieve a final concentration of 0.2 M. The acid-washed PVPP was prepared as described by Evans et al. (10). Insoluble PVPP (Sigma Chemical Co., St. Louis, Mo.) was suspended in 3 M HCl (typically 300 g of PVPP was suspended in 4 liters of 3 M HCl) for 12 to 16 h at room temperature. The suspension was then filtered through MIRACLOTH (Chicopee Mills, Inc., Milltown, N.J.), and the PVPP was suspended in 4 liters of 20 mM potassium phosphate (pH 7.4) and mixed by stirring for 1 to 2 h. This process was repeated until the suspension reached pH 7.0, after which it was filtered through MIRACLOTH and the PVPP was air dried overnight.

Lysis of the bacterial fraction. The bacterial suspension (in 100 ml of TE buffer) was brought to 1 M NaCl by addition of 25 ml of 5 M NaCl, incubated at room temperature for 10 min, and then collected by centrifugation at 23,000 × g for 20 min at 4°C. The pellet was suspended in 10 ml of TS buffer (50 mM Tris [pH 8.0]; 50 mM NaCl), transferred to a 50-ml polycarbonate centrifuge tube, and then brought to 0.1% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.) by addition of 50 μl of 20% Sarkosyl. This mixture was incubated at room temperature for 10 min, after which the bacteria were again collected by centrifugation. The bacterial pellet was drained well and suspended in 3.5 ml of Tris-sucrose-EDTA, which contains 0.75 M sucrose, 50 mM Tris (pH 8.0), and 10 mM EDTA. Lysozyme was added to a final concentration of 5 mg/ml by addition of 0.5 ml of a 40-mg/ml solution of lysozyme in TS buffer, and the samples were incubated at 37°C for 60 min. Pronase (0.5 ml of a 10-mg/ml solution in TS of type XIV pronase from Streptomyces griseus [Sigma]) that had been predigested by incubation for 30 min at 37°C was added to the bacterial cell-lysozyme mixture, mixed by vortexing, and then incubated at 37°C for 60 min. The mixture was brought to 65°C, followed by addition of 0.25 ml of 20% Sarkosyl, and then further incubated for 10 min at 65°C, after which the lysates were stored on ice overnight. Other methods of bacterial lysis used for comparison were the method of Doi (9) and extensive sonication (3 min on ice) with a Branson Sonic Power Co. (Danbury, Conn.) Sonifier, model S-125.

CsCl-EtBr equilibrium density centrifugation. Bacterial lysates were cleared by centrifugation at 40,000 × g for 1 h at 4°C, and the supernatant was transferred to a clean 50-ml polycarbonate centrifuge tube. The volume was adjusted to 25 ml by using distilled water, and 27 g of finely ground cesium chloride (CsCl) was added and mixed by gentle inversion. After the CsCl was fully dissolved, 2.5 ml of 10-mg/ml ethidium bromide (EtBr) was added and the refractive index was adjusted to 1.3865 by addition of distilled water (if >1.3865) or CsCl (if <1.3865). The DNA was banded by equilibrium density centrifugation at 18°C for 16 to 20 h in a VTi 50 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 45,000 rpm. The DNA band was made visible by illumination with long-wave UV light, which caused the EtBr-DNA complex to fluoresce, and the DNA was removed from the gradient with a 16-gauge needle and a 5-ml syringe. The DNA, still in CsCl-EtBr, was again banded by ultracentrifugation under the same conditions for increased purity. After the second banding, the EtBr was removed from the DNA by multiple extractions with isopropanol (stored over a saturated NaCl solution) until the aqueous (lower) phase was colorless and then extracted once more. The DNA sample was diluted with 2 volumes of distilled water, followed by
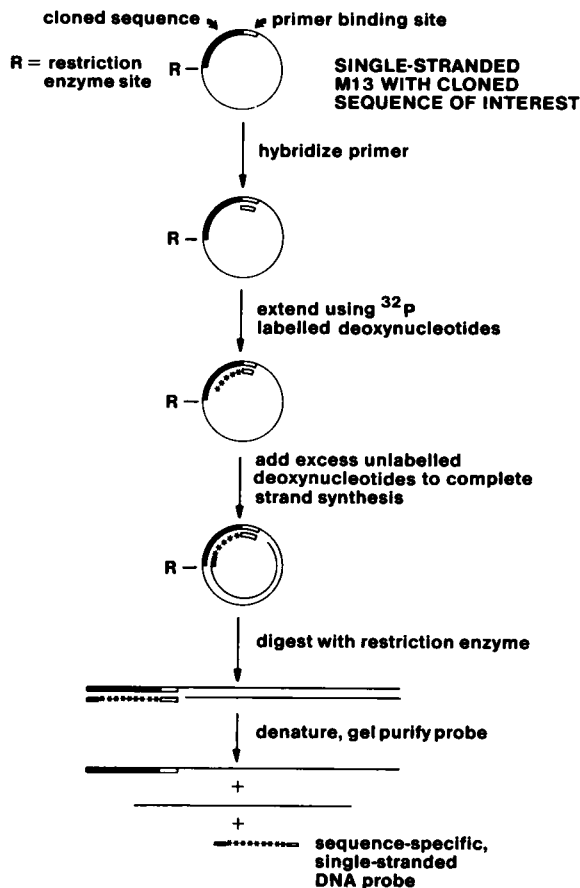
cloned sequence ↘ ↙ primer binding site

R = restriction
enzyme site

R —⟨ ⟩ SINGLE-STRANDED
M13 WITH CLONED
SEQUENCE OF INTEREST

hybridize primer

R —⟨ ⟩

extend using ³²P
labelled deoxynucleotides

R —⟨ ⟩

add excess unlabelled
deoxynucleotides to complete
strand synthesis

R —⟨ ⟩

digest with restriction enzyme

denature, gel purify probe

+

+

sequence-specific,
single-stranded
DNA probe

FIG. 1. Synthesis of an α-³²P-labeled, single-stranded DNA probe. Probes were labeled by a primer extension reaction from a single-stranded DNA template isolated from recombinant M13 phage. Asterisks indicate the ³²P-labeled region.

addition of twice the diluted volume of cold ($-20°C$) 100% ethanol, and the DNA was precipitated at $-20°C$ overnight. The DNA was collected by centrifugation at 7,000 × g for 30 min at 4°C, dried in vacuo, and then suspended in 400 μl of distilled water. The DNA solution was transferred to a 1.5-ml microcentrifuge tube to which 40 μl of 3 M sodium acetate (pH 5.2) was added, and the DNA was reprecipitated by addition of 840 μl of cold ethanol, followed by incubation overnight at $-20°C$. The DNA pellet was collected by centrifugation for 15 min at 4°C in a microcentrifuge, dried in vacuo, and suspended in a small volume (usually 100 μl) of distilled water. The concentrations of the purified DNA solutions obtained were determined by A_{260} (ϵ_{260} = 6,500).

Restriction endonuclease digestion of DNA. Restriction endonuclease digestions of DNA were performed according to manufacturer specifications.

Synthesis of single-stranded DNA probes. Figure 1 provides a graphic representation of the protocol for the synthesis of single-stranded DNA probes. Single-stranded DNA probes specific for the *nptII* gene were synthesized by primer extension by using the universal M13 sequencing primer and M13 mp19 phage (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). To maximize the specific activity of the probes, all four radiolabeled deoxynucleotides (i.e., [α-³²P]dATP, [α-³²P]dTTP, [α-³²P]dCTP, and [α-³²P]dGTP) were used in the primer extension reaction. The *nptII* gene

was cloned as a 1.1-kilobase (kb) *Hin*dIII-*Sma*I fragment into the double-stranded replicative form of M13 mp19 by using standard recombinant DNA techniques (4, 16). Recombinant phage were transformed into *E. coli* JM103, and single-stranded phage DNA was isolated from the mature phage as previously described (4). The universal primer was hybridized to the single-stranded DNA by incubation of a mixture of 0.5 μg of DNA, 3.4 pmol of universal primer, and 0.2 μl of 10× Klenow buffer (70 mM Tris [pH 7.5], 70 mM MgCl₂, 500 mM NaCl) in a 2-μl total volume at 95°C for 5 min, followed by slow cooling to room temperature. For the primer extension reaction, the above-described mixture was combined with 0.8 μl of 10× Klenow buffer, 1.7 μl of each of the four α-³²P-radiolabeled deoxynucleotides (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), and 0.5 U of the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories) in a total volume of 10 μl and incubated at room temperature for 60 min. The reaction was completed by addition of 2 μl of an aqueous solution containing each of the four deoxynucleotides (unlabeled) at a concentration of 3 mM (pH 7.0) and incubation at room temperature for an additional 60 min. The DNA (now double stranded) was digested with the restriction endonuclease *Hin*dIII in a 50-μl total volume for 60 min.

The digested sample was combined with 150 μl of single-stranded DNA loading buffer (89 mM Tris [pH 8.0], 89 mM boric acid, 2 mM EDTA, 0.1% [wt/vol] xylene cyanole, 0.1% [wt/vol] bromophenol blue, 80% formamide), denatured by immersion in boiling water for 10 min, and size fractionated by electrophoretic separation in a denaturing gel (1.5 mm × 22 cm × 26 cm) consisting of 4% polyacrylamide and 50% (wt/vol) urea in 1× TBE buffer (89 mM Tris [pH 8.0], 89 mM boric acid, 2 mM EDTA) for 10 h at 400 V. The labeled probe fragment was localized in the gel by autoradiography (30 s to 1 min of exposure time was generally sufficient), excised, and then eluted from the polyacrylamide, which was macerated in 400 μl of elution buffer (0.3 M LiCl, 10 mM Tris [pH 7.5], 0.05% [wt/vol] sodium dodecyl sulfate [SDS], 0.1 mM EDTA) and incubated at 37°C for at least 6 h. The eluant was filtered through a QUIK-SEP column (Isolab, Inc., Akron, Ohio) to yield the purified probe.

DNA hybridization. Cellulose nitrate filters for slot blot analyses were prepared essentially as described by Palva (19). Typically, 1 μg of sample DNA was denatured in 0.3 M NaOH for 5 min at 100°C, chilled on ice, and then neutralized by addition of an equal volume of cold 2 M ammonium acetate. The denatured DNA samples were spotted onto a cellulose nitrate filter that had been prewetted with 1 M ammonium acetate by using a slot blot manifold (Schleicher & Schuell, Inc., Keene, N.H.). For Southern blot analyses, DNA was size fractionated by electrophoresis in 0.7% agarose gels and transferred to cellulose nitrate filters as previously described (7, 23). All cellulose nitrate filters were baked in vacuo at 80°C for 2 h to fix the DNA samples.

The solutions used for DNA hybridization analyses have been described elsewhere (16). The filters were prehybridized for at least 6 h at 42°C in heat-sealed pouches containing 100 μl of prehybridization fluid per cm² of filter. Prehybridization fluid contains 5× Denhardt solution, 5× SSPE, 50% formamide, and 200 μg of denatured (by immersion for 10 min in boiling water) salmon sperm DNA per ml. Denhardt solution (1×) consists of Ficoll, polyvinylpyrrolidone (molecular weight, 40,000), and bovine serum albumin, each at 200 μg/ml. SSPE (1×) contains 0.18 M NaCl, 10 mM NaH₂PO₄ (pH 7.4), and 1 mM EDTA. Hybridization was performed at 42°C for 24 to 30 h by using 100 μl of

hybridization fluid per cm² of filter. Hybridization fluid consists of 5× Denhardt solution, 5× SSPE, 50% formamide, 10% (wt/vol) dextran sulfate, and 200 μg denatured salmon sperm DNA per ml. After hybridization, the filters were washed once for 20 min with agitation at room temperature with each of the following solutions: 2× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate)–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS, and then once at 50°C with 0.1× SSC–1.0% SDS. After washing, filters were exposed to X-ray film (Kodak X-Omat AR) at −70°C with a Quanta III intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). Exposure times were based on the anticipated intensity of the radioactive signal (generally, 2 to 10 days).

Efficiency of recovery of bacterial fractions. Soil prepared as for inoculation experiments, but not inoculated, was used to determine the efficiency of recovery of the bacterial fraction. Viable cell counts of the soil and separated bacterial fractions were performed by plating appropriate dilutions (in duplicate) onto PTYG medium (6), which contains the following ingredients per liter of distilled water: peptone, 0.25 g; tryptone, 0.25 g; yeast extract, 0.5 g; glucose, 0.5 g; MgSO₄ · 7H₂O, 30 mg; CaCl₂ · 2H₂O, 3.5 mg; Bacto-Agar (Difco), 15.0 g, and in addition, 300 μg of cycloheximide per ml. The plates were incubated aerobically for 1 week at 30°C, after which the total number of colonies were counted.

Enumeration of bacteria by acridine orange direct counts was performed essentially as described by Ramsay and Bawden (21). The soil pellets obtained after 1, 2, or 3 rounds of homogenization-centrifugation (P1, P2, and P3) were suspended in 200 ml of homogenization solution and then homogenized in a Waring blender for 1 min. These soil suspensions and the bacterial cell suspensions (supernatants SN1, SN2, and SN3) were serially diluted into 50 mM Tris buffer (pH 7.5). Acridine orange was added to a final concentration of 5 μg/ml. After staining, 10-ml samples were filtered onto Nuclepore 0.2-μm (pore size) polycarbonate filters which were previously stained with Irgalan black. Enumerations were made with an Olympus AHBS microscope equipped with a mercury lamp and filters appropriate for epifluorescence microscopy.

RESULTS AND DISCUSSION

Efficiency of recovery of the bacterial fraction from soil. The DNA isolation protocol involves initial separation of the soil bacterial fraction from fungal mycelia and soil debris. By first isolating the bacterial fraction from soil, we ensured that the DNA obtained was of bacterial origin and not of fungal or other origin. We also sought to protect the DNA from direct contact with soil, since nucleic acids in soil are readily degraded by nuclease-producing microorganisms (13) and because the high binding capacity of soil for phosphate and DNA is well known (B. G. Ellis, personal communication; 13). It is also likely that methods for direct extraction of DNA from soil involving alkaline hydrolysis would result in contamination of the DNA by humic and fulvic acids, since alkali extraction is the most common method for their recovery from soil (14).

Separation of the bacterial fraction from soil was accomplished by using a modification of the fractionated-centrifugation technique first described by Faegri et al. (11). Fractionated centrifugation involves homogenization of a soil sample into a buffered salt solution, followed by a low-speed centrifugation step which pellets the soil debris and fungal mycelia while leaving the bacterial cells in suspension. We

TABLE 1. Efficiency of recovery of the bacterial fraction from soil

Sample or parameter	No. of organisms[a]	
	Direct counts	Viable counts
SN1	1.7×10^6	6.2×10^6
SN2	5.0×10^8	6.1×10^6
SN3	1.1×10^9	4.8×10^6
Total recovered [SN1 + SN2 + SN3]	3.3×10^9	1.7×10^7
No. remaining [P3]	6.3×10^9	3.4×10^7
% Recovered $\left[\frac{SN1 + SN2 + SN3}{SN1 + SN2 + SN3 + P3} \right]$	34.9	33.3

[a] Acridine orange direct count data were obtained by averaging the numbers of organisms obtained from at least 10 microscopic fields per sample. Viable count data were obtained by averaging the numbers of organisms on replicate plates for each sample.

used Waring blenders for the homogenization steps, as did Faegri et al., since the use of blenders has been shown to be superior to mechanical shaking for the release of bacteria from soil (15). This cell suspension was then subjected to high-speed centrifugation to collect the bacterial fraction.

The efficiency of recovery of the soil bacterial fraction was determined by comparing the number of bacteria recovered with the number of bacteria remaining in the soil. Since accurate enumeration of bacteria in soil is problematic (1, 11), we used two widely used techniques for counting bacteria, namely, enumeration of viable counts by plating on nonselective medium and acridine orange direct counts. For each method, enumerations of bacteria were performed on the supernatants (SN1, SN2, and SN3, which contain bacteria in suspension) for each of the low-speed centrifugation steps through three rounds of homogenization-centrifugation and the final pellet (P3, which contains the soil, fungal biomass, and unreleased bacteria). The results obtained by using each method, expressed as percent recovered, are given in Table 1. The efficiencies of recovery were similar whether determined by viable counts or acridine orange direct counts, although the actual numbers of cells enumerated by each method differed by 2 orders of magnitude. The disparity in the number of cells enumerated when the two methods are compared is a well-documented phenomenon (1, 11) and is based on the inability to culture a large percentage of organisms directly from soil. The inability of most soil bacteria to survive transfer from soil directly onto culture medium seems not to affect determinations of the efficiency of extraction made by the viable-count method, probably because culturable and nonculturable cells are released from soil with the same efficiency.

Approximately 10% of the total bacteria present in the soil used were released per round of homogenization-centrifugation (Table 1). Others have shown, on the basis of bacterial cell size distribution, that the fractionated-centrifugation technique releases all bacterial types from soil with equal efficiency through up to eight rounds of homogenization-centrifugation (1, 2). Thus, the bacterial fraction released after a single round of homogenization-centrifugation is apparently as representative of the total bacterial population as the bacterial fraction collected after multiple rounds of extraction. The number of rounds of soil extraction is thus determined by the sensitivity needed for the experiment rather than by the need to obtain most of the population to ensure recovery of all types of bacteria. We found that three rounds of homogenization-centrifugation were sufficient when the organism of interest was present at levels of 10⁴ or

more per g of soil. It is likely that actual recoveries of organisms depend on the type of soil being tested. It has been shown, for example, that the clay content of soil affects the dispersion of microorganisms by homogenization (1, 15).

Lysis of the bacterial fraction and DNA purification. The isolated bacterial fraction was lysed by using a protocol that includes the salient features of several individual lysis protocols designed for use with different types of bacteria. We developed this comprehensive lysis protocol in an effort to ensure maximal disruption of the various types of bacteria present in the natural soil population. Bacterial cell lysates were then subjected to equilibrium density centrifugation on CsCl-EtBr gradients to purify the total bacterial DNA from proteins, RNA, and other contaminants.

The ability of our protocol to lyse the bacterial fraction was assessed by comparing it to the method of Doi (9) and to extensive sonication. Pure cultures of *S. coelicolor*, *E. coli* ED 8654, *B. japonicum* CRM52, *Bacillus subtilis* 168, *Corynebacterium floccumfaciens*, *Agrobacterium tumefaciens* A6, *Pseudomonas putida*, and *P. cepacia* PCO1224 were grown to mid-log phase under appropriate culture and temperature conditions. Each culture was divided into three aliquots, and each aliquot was lysed by one of the methods described above. All three lysis methods resulted in total clearing of the cell suspensions, indicating complete lysis. However, lysis by sonication resulted in DNA of very small size (approximately 500 base pairs), whereas the Doi method and our own lysis protocol yielded DNA in the 50- to 150-kb range, as indicated by agarose gel electrophoresis (some data not shown). However, the Doi method was inferior to our own method of lysis when the purity of DNA isolated from soil was compared. The DNA recovered from soil by the Doi method had a brownish tint, was substantially contaminated, as indicated by UV spectral analysis, and was refractory to digestion by restriction endonucleases (data not shown).

Sensitivity and specificity of the single-stranded DNA probes. Since it was important to be able to detect organisms present at low population densities (by detecting DNA sequences present at very low levels in a complex mixture of DNA), we devised a probe system that would maximize both specificity and sensitivity. We developed a strategy (Fig. 1) by which a sequence-specific, single-stranded DNA probe was synthesized from a single-stranded DNA template. Each of the four deoxynucleotides in the initial reaction was labeled to maximize the amount of radioactive label incorporated. DNA probes synthesized in this fashion offer several advantages over probes synthesized by nick translation of double-stranded DNA. Namely, (i) very high specific activities are obtained; e.g., nick translation reactions typically yield specific activities of 10^7 to 10^8 dpm/μg of DNA (this includes nonprobe vector sequences which are also labeled by this process), whereas probes synthesized by our primer extension reaction typically have specific activities of 1×10^{10} to 2×10^{10} dpm/μg of DNA (which is essentially all probe sequence). (ii) Background hybridization resulting from nonspecific binding, cross-homology to other sequences, or hybridization of labeled vector sequences is eliminated or dramatically reduced since the probe product is a single strand of DNA with complete homology to the sequence of interest (except for about 20 base pairs of upstream primer). This feature is likely to be important, since it has been shown that the genomes of many organisms contain homology to common, broad host range plasmids and that many plasmids have homology to other plasmids (3, 12). (iii) Concerns of probe sequences rehybridizing to their complement in solution, as can happen when denatured
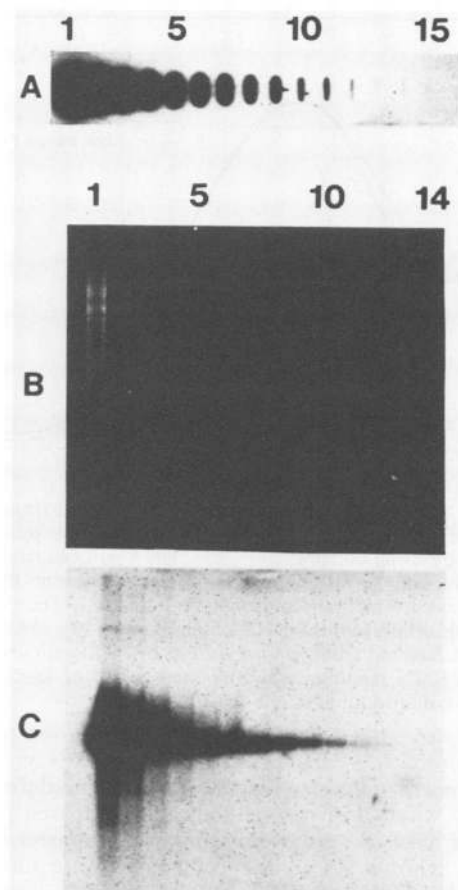

FIG. 2. Single-stranded DNA probe sensitivity. The sensitivity of an α-^{32}P-labeled, single-stranded DNA probe for the *nptII* gene was determined by hybridization to cellulose nitrate filters to which serial dilutions of known amounts of genomic DNA isolated from a pure culture of *B. japonicum* CRM52 were fixed. (A) Autoradiogram obtained after hybridization of the *nptII* gene probe to undigested *B. japonicum* CRM52 genomic DNA fixed to cellulose nitrate. Lanes 1 through 15 contained, respectively, 1.00, 0.50, 0.25, 0.13, 0.062, 0.031, 0.016, 0.0078, 0.0039, 0.0019, 0.00098, 0.00049, 0.00024, 0.00012, and 0.000063 μg of total genomic DNA. (B) EtBr-stained 0.7% agarose gel containing *B. japonicum* CRM52 genomic DNA digested with *Eco*RI. (C) Autoradiogram obtained after transfer of DNA from the agarose gel to cellulose nitrate and hybridization with the *nptII* gene probe. Lanes 1 through 14 of panels B and C contained, respectively, 2.00, 1.00, 0.50, 0.25, 0.13, 0.063, 0.031, 0.016, 0.0078, 0.0039, 0.0019, 0.00098, 0.00049, and 0.00024 μg of *Eco*RI-digested total genomic DNA.

double-stranded probes are used, are obviated since the probe is single stranded. (iv) The ability to carefully control the specific activity of the synthesized probe DNAs by controlling the reaction conditions makes it possible to synthesize different probes with the same specific activity so that results obtained with various probes can be directly compared.

The results of experiments to determine the sensitivity of the single-stranded probes in both slot blot and Southern blot analyses are shown in Fig. 2. In the slot blot experiment, the single-stranded probe was capable of detecting as little as 0.02 pg of the sequence of interest (Fig. 2A). This assumes that the 1.2-kb fragment containing the sequence of interest comprises about 1/5,000 of the genome of *B. japonicum*. Similarly, in the Southern blot experiment, the single-
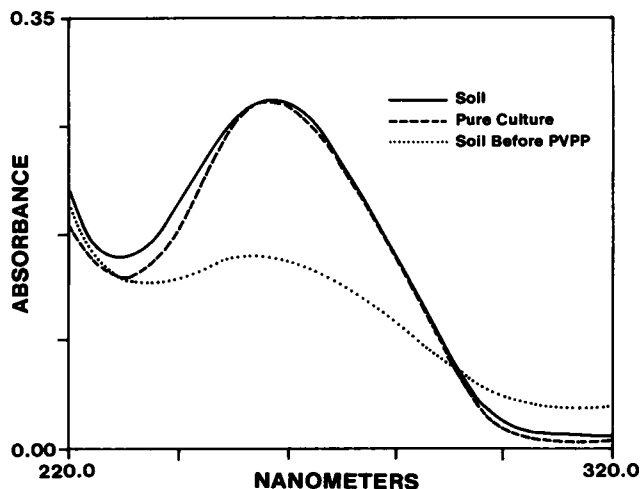
FIG. 3. UV absorption spectra of DNAs isolated from soil and from a pure culture. ————, DNA isolated from the soil bacterial population by using our protocol. ......, DNA isolated from the soil bacterial fraction by using our protocol but without PVPP and sodium ascorbate in the homogenization solution. These two soil DNA samples represent the DNA obtained from 10 g of Capac soil. ------, DNA isolated from a pure culture of B. japonicum CRM52 grown in YEMN medium, lysed by the method of Doi, and then purified by equilibrium gradient centrifugation.

stranded probe was capable of detecting as little as 0.1 pg of the sequence of interest (Fig. 2B and C). The difference in sensitivity when the two methods are compared probably results, at least in part, from incomplete transfer of DNA from the agarose gel to the cellulose nitrate filter when Southern transfer is used.

The 1-μg DNA samples used in the slot blot experiments and the 2-μg DNA samples used in the Southern blot experiments do not exceed the DNA-binding capacity of the cellulose nitrate filters (data not shown). An increase in sensitivity could therefore be gained by increasing the amount of DNA in the sample. However, when binding large amounts of DNA to cellulose nitrate filters, control experiments must be performed to show that the DNA-binding capacity of the filter has not been exceeded. The preliminary results of more recent experiments indicate that we can successfully detect as few as 10^3 organisms per g of soil. Whereas this quantity can clearly be detected, the results currently available are not in a presentable format. The results from our slot blot and the Southern blot analyses using the M13-derived, single-stranded DNA probes represent a substantial improvement in sensitivity and specificity over DNA probes labeled with either radioactive or biotinylated residues by nick translation (25).

Comparison of DNA isolated from soil to DNA isolated from pure culture. A major consideration in the isolation of bacterial DNA from soil was to purify the DNA from soil contaminants, especially humic acids, which make DNA refractory to complete restriction endonuclease digestion and hybridization analyses (unpublished data). This was facilitated by including insoluble PVPP and sodium ascorbate in the initial homogenization steps of our protocol. The sodium ascorbate serves as a reducing agent to prevent oxidation of phenols while phenolic compounds such as humic acids are removed by adsorption to the insoluble polymer PVPP (10). Inclusion of sodium ascorbate and PVPP in the DNA isolation protocol resulted in a twofold increase in DNA yield as measured by A_{260} and a significant

increase in the purity of bacterial DNA obtained (Fig. 3). The UV spectral profiles of total genomic DNA isolated from a pure culture and total bacterial DNA isolated from soil by using our technique were also compared. The results obtained for each DNA compared favorably, indicating that DNA isolated from soil by our protocol was of a purity comparable to that of DNA isolated from a pure culture (Fig. 3).

The results of experiments designed to compare total bacterial DNA isolated from nonsterile soil inoculated with B. japonicum CRM52 (hereafter designated soil DNA) with DNA isolated from a pure culture of B. japonicum CRM52 (designated culture DNA) are shown in Fig. 4. The experiments were designed to assess (i) the average size of the DNA obtained from each source, (ii) the presence of contaminating (endogenous) nucleases, (iii) the ability of the DNAs to be readily digested to completion by exogenously added restriction endonucleases, and (iv) the quality of data obtained from DNA-DNA hybridization analyses by using radioactively labeled single-stranded probes.

Untreated soil DNA was found to be of a size (~50 kb) similar to that of untreated culture DNA (Fig. 4A). Both DNAs are considered to be free of contaminating nucleases, since there was no decrease in apparent size upon incubation for 4 h under conditions favorable for nuclease digestion (Fig. 4A). When soil DNA and culture DNA were incubated with 5 U of EcoRI per μg of DNA, both appeared to be readily digested to completion (Fig. 4A). The characteristic
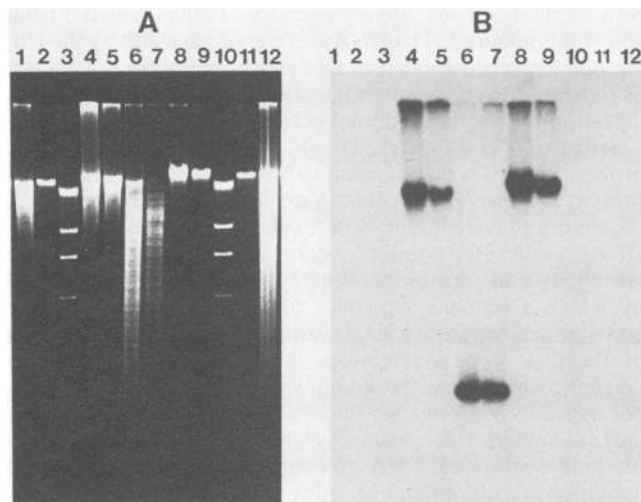

FIG. 4. Comparison by restriction endonuclease digestion and DNA probe hybridization of DNAs isolated from soil and from pure culture. (A) EtBr-stained 0.7% agarose gel. (B) Autoradiogram obtained after Southern transfer from the gel to cellulose nitrate and hybridization with the single-stranded nptII gene probe. Lanes: 1, EcoRI-digested DNA isolated from nonsterile soil inoculated with 10^9 B. japonicum BJ110 organisms per g (dry weight); 2 and 11, linearized DNA from phage lambda (~48 kb); 3 and 10, HindIII-digested DNA from phage lambda (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56, and 0.12 kb); 4, 5, and 6, respectively, unincubated, incubated (for 4 h at 37°C in 1× React 2 buffer [Bethesda Research Laboratories]) with no exogenous nuclease added, and EcoRI-digested DNA isolated from nonsterile soil inoculated with 10^9 B. japonicum CRM52 per g (dry weight); 7, 8, and 9, respectively, EcoRI digested, incubated (as described above) with no exogenous nuclease added, and unincubated DNA isolated from a pure culture in YEMN medium of B. japonicum CRM52; 12, EcoRI-digested DNA isolated from uninoculated, nonsterile soil.
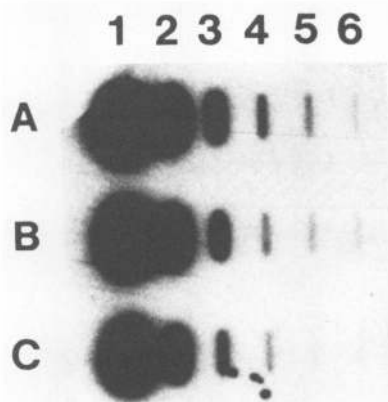
FIG. 5. Detection of *B. japonicum* CRM52 in soil by slot blot analysis. Each sample applied to the filter contained 1 μg (total) of undigested DNA isolated from soil inoculated with *B. japonicum* CRM52. Capac soil was inoculated with (lanes): 1, 4.6 × 10^8; 2, 4.6 × 10^7; 3, 5.4 × 10^6; 4, 3.6 × 10^5; 5, 4.3 × 10^4; or 6, no *B. japonicum* CRM52 organisms per gram (dry weight) at 0 weeks and incubated in covered pots at room temperature for the specified interval. Soil bacterial DNA was isolated at: 0 (A), 1 (B), or 2 (C) weeks after inoculation.

ladder of DNA fragments obtained when *B. japonicum* DNA is digested with *Eco*RI was apparent in DNA obtained from pure culture. This ladder was not as apparent in DNA obtained from inoculated nonsterile soil, since it is a complex mixture of DNA from the soil bacterial community. However, because *B. japonicum* cells added at 10^9 per g of soil comprised approximately half of all the bacteria in the soil sample, elements of the ladder could still be seen. Overall, the DNA isolated from soil performed essentially the same as that obtained from a pure culture, as indicated by both the EtBr-stained agarose gel (Fig. 4A) and the autoradiogram obtained after hybridization with the probe for the *nptII* gene (Fig. 4B). In particular, lanes 6 and 7 of Fig. 4B demonstrate that we are capable of specifically detecting the sequence of interest (representing the organism of interest) as a fragment of predicted size in DNA isolated from nonsterile soil. The specificity of the *nptII* gene probe is also demonstrated in Fig. 4B, since there was a lack of any detectable hybridization signal to total bacterial DNA isolated from nonsterile soil inoculated with *B. japonicum* BJ110 (the parental strain of CRM52 which does not contain the *nptII* gene) and DNA from uninoculated, nonsterile soil.

Results of slot blot experiments using soil bacterial DNA. The slot blot apparatus allows DNA obtained from soil samples to be fixed onto cellulose nitrate filters in discrete, slot-shaped areas. The filters were subsequently used in hybridization experiments to detect the DNA sequence of interest. The results of an experiment in which the fate of *B. japonicum* CRM52 inoculated into nonsterile soil was monitored for 2 weeks after inoculation are given in Fig. 5. Since each DNA sample applied to the cellulose nitrate filter contained the same total amount of DNA (1 μg), the intensity of the hybridization signal at each position indicates the number of copies of the *nptII* gene present in the DNA sample. *B. japonicum* CRM52 contains one stably integrated copy of the *nptII* gene in its chromosome; therefore, the intensity of hybridization reflects the number of *B. japonicum* CRM52 organisms present in the soil. We were able to detect this strain in soil throughout the range of 10^8 to 10^4 per g (dry weight) of soil on the basis of the presence of this single-copy sequence. Thus, we were able to detect the

microorganism of interest in soil, even though it made up only a very small fraction (10^{-5}) of the total bacterial population. The results obtained by using the DNA probe methodology indicate that the numbers of *B. japonicum* CRM52 organisms in the soil did not change significantly during the course of the experiment, regardless of the initial inoculum density, and are in agreement with results obtained by selective plating (Fig. 5; some data not shown).

Some low-level background hybridization was observed in the uninoculated control (Fig. 5). It is not clear whether this resulted from hybridization to *nptII* homology in the DNA obtained from indigenous microorganisms, or simply from nonspecific hybridization. As shown previously, there was no background hybridization apparent when DNAs isolated from soil were analyzed by Southern transfer (Fig. 4). The lack of detectable background hybridization with this technique probably derives from low levels of background hybridization being dispersed throughout the entire lane when the DNA samples are size fractionated by agarose gel electrophoresis. This is in contrast to the slot blot format, in which the entire DNA sample is localized to a very small area of the filter, which effectively concentrates very low levels of background hybridization, resulting in a detectable signal. If background hybridization obscures the results of slot blot analyses, the Southern blot technique can be used to reduce or eliminate this problem.

Detection of parental and engineered derivative strains in a single soil sample. A probe was constructed for the *B. japonicum rbcL* gene which encodes the ribulose-1,5-bisphosphate carboxylase-oxygenase large subunit. The *rbcL* probe was constructed by cloning a 0.95-kb *Eco*RI-*Bam*HI fragment which contains the 3' two-thirds of the *rbcL* gene (T. Cotter, personal communication) into M13 mp9. This probe flanks the site of insertion of the *nptII* gene in CRM52 (Fig. 6). When genomic DNA from *B. japonicum* BJ110 and CRM52 was digested with the restriction endonuclease *Sma*I (which cuts the DNA outside of the region used as the probe), the fragment containing the 3' end of the *rbcL* gene was of a different size for each organism (Fig. 6). This forms the basis for the identification of both strains in a single soil sample with a single probe. *B. japonicum* BJ110 and CRM52 were readily identified, either singly or together, in the soil sample by virtue of the *rbcL* hybridization signal of the predicted size (Fig. 7). By comparison to known standards, or by scanning densitometry, quantitation of the numbers of organisms should be possible. Note that in this case, the probe used hybridized to a wild-type genomic sequence rather than to an engineered DNA sequence and that by using this probe we were able to detect and differentiate between wild-type *B. japonicum* BJ110 and an engineered derivative of this strain.

The same principle, that of restriction fragment length polymorphism, which allowed us to detect a parental strain and its engineered derivative in this experiment can also be used to detect more than one species (or genus) in a single soil sample, as we have done for two different species of pseudomonads (manuscript in preparation). It should also be possible to detect multiple organisms in soil by combining probes (for wild-type or engineered DNA sequences) unique to each organism for hybridization to total bacterial DNA isolated from soil containing these organisms.

Using our method, we detected not just an organism of interest but a particular gene or DNA sequence of interest in an entire community of soil bacteria. This technique, in conjunction with Southern transfer, has the potential to detect genetic rearrangements, as well as horizontal gene
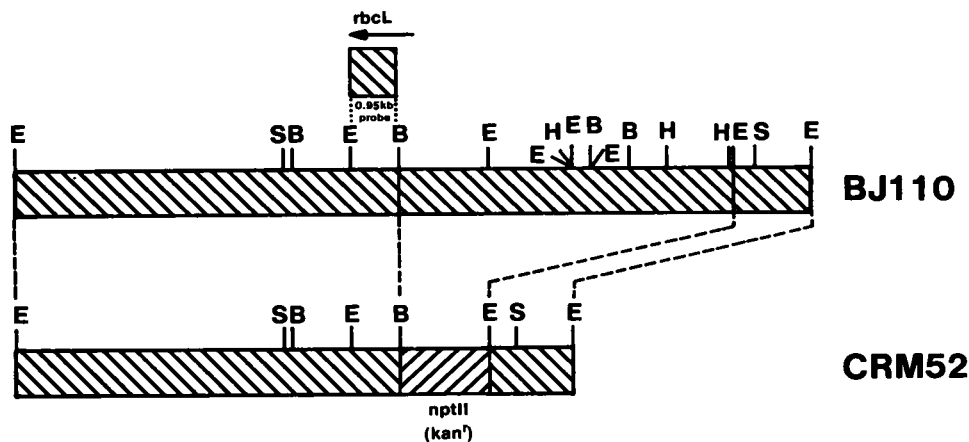
FIG. 6. Restriction map of the *rbcL* region of the chromosomes of *B. japonicum* BJ110 and CRM52. The location and orientation of the *rbcL* gene are designated by the arrow. *B. japonicum* CRM52 was constructed (18) by using site-directed mutagenesis by deleting the 8.6-kb *Bam*HI-*Eco*RI fragment of the BJ110 chromosome and replacing it with a 1.8-kb *Bam*HI-*Eco*RI fragment containing the *nptII* gene, but CRM52 is otherwise isogenic to strain BJ110. Abbreviations: E, *Eco*RI; S, *Sma*I; B, *Bam*HI; H, *Hin*dIII.

transfer to other species. The basis for detecting such phenomena is that if a gene or DNA sequence of interest were involved in a genetic rearrangement within the original organism or incorporated into the DNA of a different organism (e.g., as the result of a horizontal gene transfer event), the DNA flanking the sequence of interest would be different. The result of this would be that the DNA sequence of interest would reside on either a larger or a smaller DNA fragment, when digested with an appropriate restriction enzyme, than in the original organism. This would be detected as a band of a different size during subsequent hybridization and autoradiography.

Some advantages of the described methodology are as follows. (i) The bacterial fraction, and hence the DNA, is isolated in situ and therefore requires no culturing of soil microorganisms. The importance of this distinction is that most microorganisms in environmental samples cannot readily be cultured under laboratory conditions (8, 17, 22). In fact, when transfer of genetic information to other organisms is of concern, the inability to culture all types of soil microorganisms would be a major consideration. Isolation of DNA in situ also allows direct quantitations and comparisons to be made. (ii) A specific gene or DNA sequence particular to a microorganism is detected so that gene expression is not required. This is important because bacteria in the environment often exist under conditions of nutrient limitation and therefore may not actively express a gene product required for detection (5). (iii) Multiple organisms can be monitored in a single sample. (iv) The potential exists to detect genetic rearrangements and horizontal gene transfer. (v) Reporter genes and phenotypes are not required since the probe directly detects the sequence of interest. These methods, therefore, are useful not only with mutant or genetically engineered microorganisms but with natural isolates as well.

The use of this probe methodology in conjunction with Southern transfer is analytically powerful, being able to detect multiple organisms, genetic rearrangements, and possibly also gene transfer. However, when simply monitoring the presence of an organism in soil using DNA probes, the slot blot approach is more appropriate, since it allows for rapid processing of large numbers of DNA samples in a simpler format. The protocols described were devised to maximize the likelihood of the successful application of
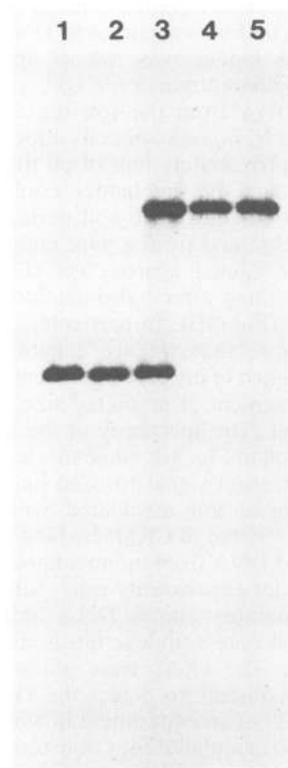

FIG. 7. Detection of parental and engineered derivative strains in a single soil sample. Autoradiogram obtained after hybridization of the single-stranded *rbcL* gene probe to DNA fixed to cellulose nitrate by Southern transfer from a 0.7% agarose gel. The lanes contained *Sma*I-digested DNA as follows: 1, *B. japonicum* CRM52 genomic DNA isolated from pure culture; 2, soil bacterial DNA isolated from Capac soil inoculated with ~10^7 *B. japonicum* CRM52 organisms per g (dry weight); 3, soil bacterial DNA isolated from Capac soil inoculated with ~10^7 *B. japonicum* CRM52 and BJ110 per g (dry weight); 4, soil bacterial DNA isolated from Capac soil inoculated with ~10^7 *B. japonicum* BJ110 per g (dry weight); 5, *B. japonicum* BJ110 genomic DNA isolated from pure culture.

DNA probe methodology to environmental microbiology. It is likely that, with continued use and improvements in methodology, some of the steps involved may be either combined or deleted and the sensitivity of detection may be improved. Efforts toward these goals are under way.

LITERATURE CITED

1. **Bakken, L. R.** 1985. Separation and purification of bacteria from soil. Appl. Environ. Bacteriol. **49:**1482–1487.
2. **Balkwill, D. L., D. P. Labeda, and L. E. Casida, Jr.** 1975. Simplified procedures for releasing and concentrating microorganisms from soil for transmission electron microscopy viewing as thin-sectioned and frozen-etched preparations. Can. J. Microbiol. **21:**252–262.
3. **Bender, C. L., and D. A. Cooksey.** 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. **165:**534–541.
4. **Bethesda Research Laboratories.** 1985. BRL M13 cloning/dideoxy sequencing instruction manual. Bethesda Research Laboratories, Gaithersburg, Md.
5. **Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart.** 1975. Influence of environmental stress on enumeration of indicator bacteria from natural waters. Appl. Microbiol. **29:**186–194.
6. **Bone, T. L., and D. L. Balkwill.** 1986. Improved flotation technique for microscopy of in situ soil and sediment microorganisms. Appl. Environ. Microbiol. **51:**462–468.
7. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9:**269–278.
8. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Biotechnology **3:**817–820.
9. **Doi, R. H.** 1983. Isolation of *Bacillus subtilis* chromosomal DNA, p. 162–163. *In* R. L. Rodriquez and R. C. Tait (ed.), Recombinant DNA techniques: an introduction. Addison-Wesley Publishing Co., Readney, Mass.
10. **Evans, H. J., B. Koch, and R. Klucas.** 1972. Preparation of nitrogenase from nodules and separation into components. Methods Enzymol. **24:**470–476.
11. **Faegri, A., V. L. Torsvik, and J. Goksoyr.** 1977. Bacterial and fungal activities in soil: separation of bacteria by a rapid fractionated centrifugation technique. Soil Biol. Biochem. **9:**105–112.
12. **Golub, E. I., and K. B. Low.** 1986. Unrelated conjugative plasmids have sequences which are homologous to the leading region of the F factor. J. Bacteriol. **166:**670–672.
13. **Greaves, M. P., and M. J. Wilson.** 1970. The degradation of nucleic acids and montmorillonite-nucleic acid complexes by soil microorganisms. Soil Biol. Biochem. **2:**257–268.
14. **Hayes, M. H. B., and F. L. Hines.** 1986. Nature and properties of humus-mineral complexes, p. 103–158. *In* P. M. Huang and M. Schnitzer (ed.), Interactions of soil minerals with natural organics and microbes. SSSA special publication no. 17. Soil Science Society of America, Inc., Madison, Wis.
15. **Kanazawa, S., S. Takeshima, and K. Ohta.** 1986. Effect of Waring blender treatment on the counts of soil microorganisms. Soil Sci. Plant Nutr. **32:**81–89.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Martin, J. K.** 1975. Comparison of agar media for counts of viable soil bacteria. Soil Biol. Biochem. **7:**401–402.
18. **McClung, C. R., and B. K. Chelm.** 1987. Genetic locus essential for formate-dependent growth of *Bradyrhizobium japonicum*. J. Bacteriol. **169:**3260–3267.
19. **Palva, A.** 1985. Nucleic acid spot hybridization for detection of *Chlamydia trachomatis*. FEMS Microbiol. Lett. **28:**85–91.
20. **Pochon, J.** 1954. Manuel technique d'analyse microbiologique du sol. Masson et Cie., Paris.
21. **Ramsay, A. J., and A. D. Bawden.** 1983. Effects of sterilization and storage on respiration, nitrogen status and direct counts of soil bacteria using acridine orange. Soil Biol. Biochem. **15:**262–268.
22. **Roszak, D. B., D. J. Grimes, and R. R. Colwell.** 1984. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. **30:**334–338.
23. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
24. **Torsvik, V. L., and J. Goksoyr.** 1978. Determination of bacterial DNA in soil. Soil Biol. Biochem. **10:**7–12.
25. **Zwadyk, P., Jr., R. C. Cooksey, and C. Thornsberry.** 1986. Commercial detection methods for biotinylated gene probes: comparison with [^{32}P]-labelled DNA probes. Curr. Microbiol. **14:**95–100.