Gene Probe Analysis of Soil Microbial Populations Selected by Amendment with 2,4-Dichlorophenoxyacetic Acid

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Soils with a history of 2,4-dichlorophenoxyacetic acid (2,4-D) treatment at field application rates and control soils with no prior exposure to 2,4-D were amended with 2,4-D in the laboratory. Before and during these treatments, the populations of 2,4-D-degrading bacteria were monitored by most-probable-number (MPN) enumeration and hybridization analyses, using probes for the *tfd* genes of plasmid pJP4, which encode enzymes for 2,4-D degradation. Data obtained by these alternate methods were compared. Several months after the most recent field application of 2,4-D (~1 ppm), soils with a 42-year history of 2,4-D treatment did not have significantly higher numbers of 2,4-D-degrading organisms than did control soils with no prior history of treatment. In response to laboratory amendments with 2,4-D, both the previously treated soils and those with no prior history of exposure exhibited a dramatic increase in the number of 2,4-D-metabolizing organisms. The MPN data indicate a 4- to 5-log population increase after one amendment with 250 ppm of 2,4-D and ultimately a 6- to 7-log increase after four additional amendments, each with 400 ppm of 2,4-D. Similarly, when total bacterial DNA from the soil microbial community of these samples was analyzed by using a probe for the *tfdA* gene (2,4-D monooxygenase) or the *tfdB* gene (2,4-dichlorophenol hydroxylase) a dramatic increase in the level of hybridization was observed in both soils. Probes to the *tfdC*, *-D*, *-E*, and *-F* genes did not hybridize to the bacterial community DNA to any significant extent before or after 2,4-D treatment, indicating that pathways different from the canonical pJP4-encoded pathway at the DNA sequence level, and possibly at the functional level, account for the degradative activity in these soils. Quantitative hybridization data and MPN values were in agreement, indicating that most of the 2,4-D-degrading populations were detected by the *tfdA* and *tfdB* gene probes. The hybridization patterns detected in Southern analyses of bacterial community DNA indicated that a dominant 2,4-D-degrading population was selected and maintained in these soils.

In recent years gene probe-based methods for the detection of populations and genotypes of interest in mixed microbial populations have been described (for reviews, see references 16, 22, and 30). Some of these methods are based on the use of probes in colony hybridization experiments to detect bacterial colonies that contain sequences homologous to a particular gene probe. This approach requires culturing of organisms, often under conditions that select for the phenotype of interest, and has been shown to be successful for the detection of certain catabolic phenotypes (17, 29, 31) and mercury resistance genes in bacterial populations (2, 3). Other methods are based on the extraction and purification of DNA from the microbial community present at the time of sampling and require no culturing (12, 14, 15, 25, 36). However, most experiments using these methods demonstrate the detection of populations that have been added to the microbial community rather than indigenous populations containing appropriate homologies to the probes employed. Also, studies of the application of gene probe methods to environmental samples have demonstrated the presence of a population(s) or DNA sequence in the sample at a single time point but have not monitored population dynamics in response to experimental treatment or to changing environ-

mental conditions. This paper reports the use of gene probes to monitor the response of an indigenous soil microbial community to selection caused by addition of a new substrate.

The results show the responses of soil microbial communities to repeated additions of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). This compound has been used extensively for more than 40 years in agricultural and turf grass applications, but it is not generally considered an important environmental toxicant because soil microbial populations completely degrade this compound in a relatively short time (13, 26, 27, 32). The gene probes constructed for use in these experiments comprise internal regions of the six structural genes for 2,4-D metabolism encoded on plasmid pJP4. In addition to monitoring 2,4-D-degrading populations by using these probes, we determined the numbers of 2,4-D-degrading organisms by the most-probable-number (MPN) method. We also evaluated whether any difference could be detected between populations in plots in which 2,4-D had been used for 42 years and those in control plots that had not received 2,4-D.

MATERIALS AND METHODS

Soil site. The soils used were from the Experimental Farm, Indian Head, Saskatchewan, Canada. The soil type is Indian

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Head clay, an Orthic Black Chernozem (Udic Boroll) which contains 25.3 g of organic carbon kg^{-1} in the top 2.5 cm (4). The effects of long-term (42 years) field application of 2,4-D on soil microbial and biochemical processes at this site have been studied (4, 8). Soil samples from three replicate plots (6R1, 6R2, and 6R4) that had been treated with 2,4-D at the rate of 1.68 kg ha^{-1} from 1947 to 1969 and at the rate of 1.12 kg ha^{-1} from 1969 to the time of sampling in 1989 are herein referred to as experienced soils. Soil samples were also taken from three replicate plots (5R1, 5R2, and 5R3) that had never received 2,4-D treatment and are adjacent to, but separated from, the treatment plots. These are referred to as no-history soils. All 2,4-D-treated and untreated plots are 3 by 21 m and are separated by pathways 0.9 m wide. A 3-year rotation of wheat-wheat-summer fallow is followed on all sites. Composite soil samples were taken for these experiments by carefully removing the top 2.5 cm of surface soil from five equidistant locations down the center of each plot and combining these subsamples. This experimental design, in conjunction with the soil sampling strategy, provided for soil samples with no history of 2,4-D treatment that were taken 4.95 m (of which 4.5 m had plant cover) from the closest 2,4-D application area. The composite soil samples were sieved (2 mm) and stored in polyethylene bags at field moisture content (20 to 25% [wt/wt]) at 4°C until used.

2,4-D amendment of soils. Subsamples (50 g) of the soils from each of the plots were dried overnight at 65°C and then amended with 6.25 ml of 2,4-D (20 mg/ml in 100 mM phosphate buffer [pH 7.0]) or 6.25 ml of buffer alone and mixed thoroughly. This 2,4-D in carrier soil was then added to 450 g (calculation based on dry weight) of the same soil (native and undried) in polyethylene bags and thoroughly mixed to give a final 2,4-D concentration of 250 ppm. Sterile distilled water was added to bring the moisture content to 25% (80% water-holding capacity). The soil samples were incubated at 25°C in the dark for the duration of the experiment. The concentration of 2,4-D in the soil samples was monitored by high-pressure liquid chromatography (HPLC) as described below. When the level of 2,4-D was less than 25 ppm, subsequent treatments with 2,4-D or phosphate buffer were made by adding the appropriate amount of the solution dropwise to the soil and then mixing thoroughly to give a final 2,4-D concentration of 400 ppm in the treated samples.

Quantitation of 2,4-D biodegradation. The biodegradation of 2,4-D was measured as the disappearance of the compound as determined by HPLC. At appropriate time points 1-g samples were taken from the 2,4-D-treated soils and combined with 1 ml of sterile distilled water in a microcentrifuge tube. This soil slurry was mixed vigorously for 1 min and then pelleted by centrifugation for 5 min in a microcentrifuge at 16,000 × g. The supernatant was filtered to remove particulates and then analyzed by HPLC on a Lichrosorb RP-18 column (Anspec Co., Ann Arbor, Mich.) with methanol–0.1% H_3PO_4 (60:40) as the eluant. Loss of A_{230} at an elution time of ~5.5 min indicated 2,4-D metabolism; no other UV-absorbing peaks were detected. This method recovers about 95% of the 2,4-D present in these soil samples.

Quantitation of 2,4-D-degrading bacterial populations. The enumeration of 2,4-D degrading bacteria by MPN determination was performed for each soil sample at time zero and after the first, third, and fifth treatment with 2,4-D or phosphate buffer. MPN analyses were performed by inoculating 1.8 ml of MMO basal salts medium (33) containing 500 ppm of 2,4-D and 0.3% Casamino Acids with 0.2 ml of

appropriately diluted soil suspensions from the treated and untreated soils. Five replicate sets of tubes were assayed for each soil sample at each time point. The inoculated tubes were incubated at 25°C with shaking for 1 week prior to analysis, after which 1 ml of the MPN medium from each tube was cleared of cells by centrifugation for 5 min in a microcentrifuge at 16,000 × g. HPLC analysis was performed on the supernatant, with positive tubes being scored as those with less than 100 ppm of 2,4-D remaining. The population density was calculated from MPN tables.

Purification of microbial community DNA from soil. Microbial community DNA was extracted and purified from 50 g of each of the soil samples as described previously (15), with the following modifications: (i) the bacterial pellet obtained by differential centrifugation was not washed with 2% sodium hexametaphosphate and was washed only once with TE (33 mM Tris, 1 mM EDTA [pH 8.0]) buffer prior to lysis, (ii) the NaCl and Sarkosyl pretreatment steps prior to lysis were combined into a single preincubation in 1 M NaCl–0.1% Sarkosyl for 10 min at room temperature, (iii) during lysis, the 60-min incubation at 37°C with 5 mg of lysozyme per ml was reduced to 30 min, (iv) the 60-min incubation at 37°C with 1 mg of type XIV pronase per ml was reduced to 30 min, and (v) the lysates were prepared for cesium chloride-ethidium bromide equilibrium density centrifugation after 1 to 3 h of incubation on ice. Approximate quantification of DNA was accomplished spectrophotometrically by measuring A_{260}. Because some humic contaminants from soil that copurify with DNA also absorb at this wavelength, precise quantitation of DNA was achieved by densitometric analysis of agarose gels containing DNA stained with ethidium bromide as described previously (14) since this dye binds only to the DNA (there is no RNA present). The average DNA yield for these samples was 0.88 μg/g of soil.

DNA probes for detection of 2,4-D-degrading populations. For analysis of the microbial community DNA isolated from the soil samples, we constructed a suite of probes directed against internal regions of each of the six structural genes for 2,4-D metabolism (*tfdA*, *-B*, *-C*, *-D*, *-E*, and *-F*) that are encoded on the pJP4 plasmid (10, 21, 28, 35). All restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were purchased commercially and used according to the manufacturers' specifications. The probe for the *tfdA* gene was constructed as an 801-bp *StyI* fragment cloned into the *SmaI* site of the pUC9 plasmid. The *tfdB* gene probe was cloned as a 1,416-bp *XhoI* fragment into the *SalI* site of pUC9. The probe for the *tfdC* gene was cloned as a 702-bp *PvuI-BanII* fragment which was treated with the Klenow fragment of DNA polymerase I to generate a blunt-ended fragment which was subsequently cloned into the *SmaI* site of pUC9. The *tfdD* gene probe was cloned as a 918-bp *BanII-PstI* fragment by first digesting pRO1946 (21) with *BanII*, creating a blunt end by using the Klenow fragment of DNA polymerase I, and then digesting with *PstI*. This fragment was cloned into pUC9 that had been digested with *HincII* and *PstI*. For the *tfdE* gene probe, a 623-bp *AvaI-MscI* fragment was treated with the Klenow fragment of DNA polymerase I to generate blunt ends and then cloned into the *SmaI* site of pUC9. The probe for the *tfdF* gene consists of a 934-bp *XmnI* fragment cloned into the *SmaI* site of pUC9. The entire sequence of each of these genes has been published (28, 35).

To eliminate considerations of hybridization signal arising from homology between microbial community DNA and the pUC9 vector used to construct the *tfd* gene probes, we used

the polymerase chain reaction (PCR) to selectively amplify the cloned inserts of *tfd* gene sequences but not the vector sequences. This was accomplished using a set of primers (synthesized locally) that correspond to regions of pUC9 just adjacent to the site(s) of insertion of the probe fragment to direct synthesis of new strands by PCR such that only the inserts are amplified. The sequences of the primers are as follows: M13F-CCCAGTCACGACGTTGTAAAACGAC and M13R-AGGAAACAGCTATGACCATGATTAC. The sequences of these primers are common to all of the pUC vectors as well as to the M13 mp single-stranded phage vectors; thus, the primers are suitable for amplifying any cloned insert in any of these vectors. PCR reactions were set up by using the GeneAmp PCR kit (Perkin Elmer Cetus, Norwalk, Conn.) according to the manufacturer's specifications, using 1.6 pg of the appropriate plasmid with the cloned probe sequence. To increase the efficiency of the PCR reaction, each plasmid was linearized by using an appropriate restriction enzyme with no recognition sites in the region to be amplified. The PCR protocol consisted of 25 cycles of 1 min at 94°C to denature the double-stranded DNA, 2 min at 50°C to anneal the primers, and 2 min at 72°C for strand synthesis with the Perkin Elmer Cetus Thermal Cycler. The PCR reaction products were passed through a spun column to remove unincorporated nucleotides and excess primers (1). One-tenth of the PCR reaction product (250 to 500 ng of amplified insert) was used in labeling reactions to generate a probe. This represents approximately a millionfold excess of insert to vector, which probably exceeds the purity obtained by gel purification of the insert.

Labeling of probes for the *tfd* genes was performed with a nick translation kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the manufacturer's specifications, using [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Boston, Mass.). Unincorporated nucleotides were removed by passage through a spun column prior to use.

DNA blot preparation, probe hybridization, and quantitation. Filters for slot blot analyses and Southern blot analyses were prepared as described previously (15) except that BA-S supported nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) was used to facilitate removal of the probe for subsequent rehybridization. For slot blots, community DNA samples (0.5 μg) were spotted on the filters by using a Bio-Dot SF manifold with a six-by-eight sample array (Bio-Rad Laboratories, Richmond, Calif.). For Southern blots, 2-μg DNA samples were digested with *Eco*RI and *Hin*dIII. The resulting fragments were size fractionated by electrophoresis on a 0.7% agarose gel and then subjected to capillary transfer to hybridization membranes as described previously (1). DNA was fixed to the hybridization membranes by using a UV cross-linker (Stratagene, La Jolla, Calif.) and the manufacturer's recommendations for exposure. Prehybridization and hybridization conditions were as described previously (15). Quantitative hybridization results were obtained by using a radioactive blot analyzer (Betascope; Betagen Corp., Waltham, Mass.) which generates quantitative data and an autoradiographic image from membranes hybridized with radioactive probes. Bound probe was stripped from the membranes prior to rehybridization by washing the membranes for 1 to 2 h at 65°C in 5 mM Tris (pH 8.0)–0.2 mM EDTA–0.05% sodium PP$_i$–0.1× Denhardt's solution (1).
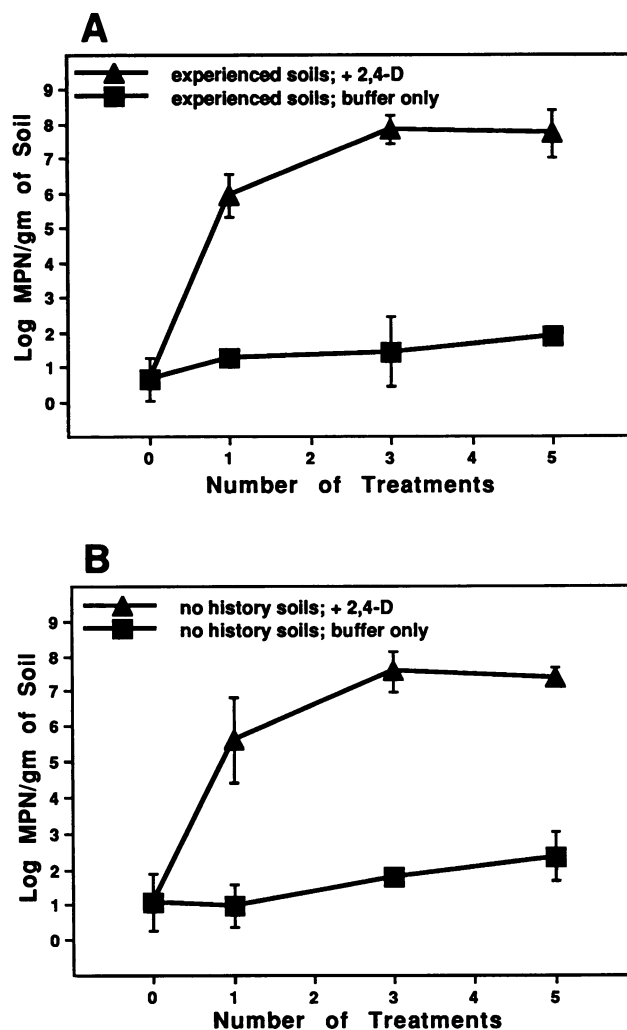

FIG. 1. MPN enumeration of 2,4-D-degrading populations in response to 2,4-D treatment of soils. The data are the means and standard deviations for three replicate plots of soils with a 42-year history of treatment with 2,4-D (A) and with no prior history of 2,4-D exposure (B). All soils were treated with either 2,4-D or buffer.

RESULTS

MPN enumeration of 2,4-D degraders. The numbers of 2,4-D-degrading organisms in both the experienced and the no-history soils exhibited similar patterns in response to 2,4-D treatment (Fig. 1). Both soils had similar low numbers of 2,4-D-degrading organisms at time zero (4.4 and 11.7 degraders per g of soil for three replicate experienced and no-history soils, respectively). After a single treatment with 250 ppm of 2,4-D the numbers of 2,4-D-degrading organisms increased to 8.9 × 10^5 and 4.1 × 10^5 degraders per g for the experienced and no-history soils, respectively. Maximum 2,4-D-degrading populations of 7.1 × 10^7 and 3.6 × 10^7 degraders per g of soil, respectively, were detected following two additional treatments, each with 400 ppm of 2,4-D, after which the populations remained at these levels for the duration of the experiment.

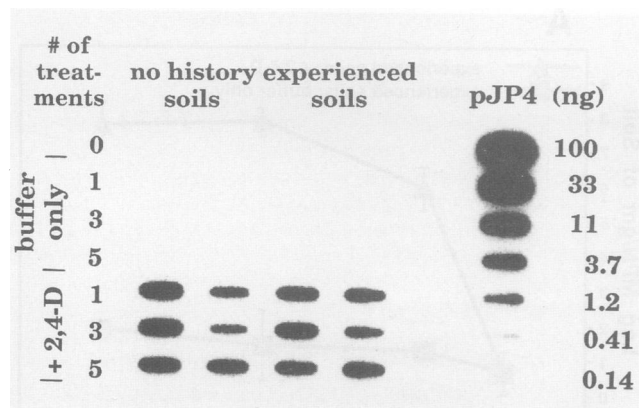**Detection of 2,4-D-degrading populations by using gene probes.** Total DNA from the bacterial community in soil was

FIG. 2. Detection of 2,4-D-degrading populations in the soil microbial community by using the tfdA gene probe. Total bacterial community DNA was extracted from replicate soil samples after zero, one, three, and five treatments with either 2,4-D or buffer. Each well contained 0.5 μg of bacterial community DNA such that the hybridization signal intensity corresponds to the ratio of target (tfdA-homologous) to nontarget DNA. Known amounts of plasmid pJP4 DNA were included as a positive control and for comparative quantitation.

TABLE 1. Quantitative hybridization data for soil bacterial community DNA obtained with the tfdA, -B, -C, -D, -E, and -F gene probes

| Gene probe | No. of treatments | Mean cpm[a] | | | |
| | | Experienced soils | | No-history soils | |
		Buffer only	+2,4-D	Buffer only	+2,4-D
tfdA	0	0	0	0	0
	1	1	260	0	300
	3	0	237	0	274
	5	3	245	2	267
tfdB	0	23	23	25	25
	1	22	192	48	318
	3	24	201	31	279
	5	27	234	32	266
tfdC	0	5	5	4	4
	1	6	4	7	7
	3	6	10	5	9
	5	5	14	5	8
tfdD	0	8	8	4	4
	1	5	6	8	9
	3	6	7	4	6
	5	7	11	4	5
tfdE	0	14	14	12	12
	1	16	16	25	27
	3	17	18	16	20
	5	17	26	16	17
tfdF	0	6	6	8	8
	1	5	7	10	15
	3	6	13	9	9
	5	9	14	12	9

[a] The values given represent the mean counts per minute obtained from 0.5 μg of bacterial community DNA from two replicate soil samples. Nonspecific background hybridization to the membrane was corrected for by subtracting the mean signal for six spots containing no DNA from all other signals.

isolated at each time point for use in hybridization experiments with probes for each of the six tfd genes (tfdA, -B, -C, -D, -E, and -F). DNA from both the experienced soils and the no-history soils had no significant hybridization to the tfdA gene probe prior to the application of 2,4-D in the laboratory (Fig. 2). Following a single treatment with 250 ppm of 2,4-D, the hybridization signal increased dramatically in both soil types and was maintained throughout the course of treatments with 2,4-D. By contrast, the hybridization signals from both soil types treated with phosphate buffer rather than 2,4-D showed no such increase (Fig. 2). A similar autoradiograph was obtained with the tfdB gene probe, but probes for the tfdC, -D, -E, and -F genes showed no significant hybridization to any of the DNA samples. Quantitation of hybridization signals on the filters for each of the six gene probes was obtained by using the Betascope radioactive blot analyzer (Table 1). We have previously cited the lower level of detection obtained with gene probes without target or signal amplification as 10^4 gene copies per g of soil for nonsterile soil (15). Therefore, the small hybridization signals observed prior to the first 2,4-D treatment and for the samples treated with buffer only (i.e., when the MPN data indicate fewer than 10^3 2,4-D-degrading organisms per g of soil) probably constitute nonspecific or heterologous background hybridization. For the tfdA and tfdB gene probes, the hybridization signal increased dramatically in both soils in response to the first 250-ppm addition. In contrast, no significant increase in hybridization was detected with the tfdC, -D, -E, or -F gene probe. Four additional treatments, each with 400 ppm of 2,4-D, did not further increase the hybridization signals for any of the tfd gene probes.

Comparison of the MPN data (Fig. 1) with a plot of tfdA gene probe hybridization signals versus the number of treatments with 2,4-D (Fig. 3) reveals that similar results were obtained by the two alternate methods. Each method detected the dramatic response of the microbial community to the initial 2,4-D treatment and the leveling off of this response during subsequent treatments, while background

levels remained relatively constant in the soils treated with buffer only. By comparing the hybridization signals obtained with known amounts of pJP4 plasmid with the hybridization signal for the community DNA we can estimate the number of gene copy equivalents present in the soil. For both the tfdA (Fig. 2) and the tfdB (data not shown) gene probes, the hybridization intensity for the community DNA samples was comparable to that obtained with 1.2 ng of pJP4 DNA, which corresponds to 1.4 × 10^7 copies of the pJP4 plasmid. Since the mean community DNA yield for these samples was 0.88 μg/g of soil and 0.5 μg of community DNA was used in these analyses, this corresponds to detection of about 3 × 10^7 tfdA or tfdB gene copy equivalents per g of soil. Assuming that pJP4 is a single-copy or low-copy-number plasmid, this value compares favorably with the population density of ~5 × 10^7 2,4-D-degrading organisms per g of soil determined by MPN enumeration in the later stages of the experiment (Fig. 1).

Southern analyses of the bacterial community DNA samples digested with EcoRI and HindIII were performed in an attempt to assess the diversity of organisms with homology to the probes used (Fig. 4). When DNA samples isolated after one, three, and five treatments with 2,4-D were hybridized with the tfdA or tfdB gene probe, most of the hybridization signal was localized in a single band (Fig. 4A, closed arrowhead, and 4B, open arrowhead, respectively); no specific hybridization signal was detected prior to 2,4-D treatment (Fig. 4, lanes 1 and 2). The tfdA and tfdB gene probes both appear to hybridize to the same DNA fragment (approximately 5.7 kb in size based on known, nonhybridizing
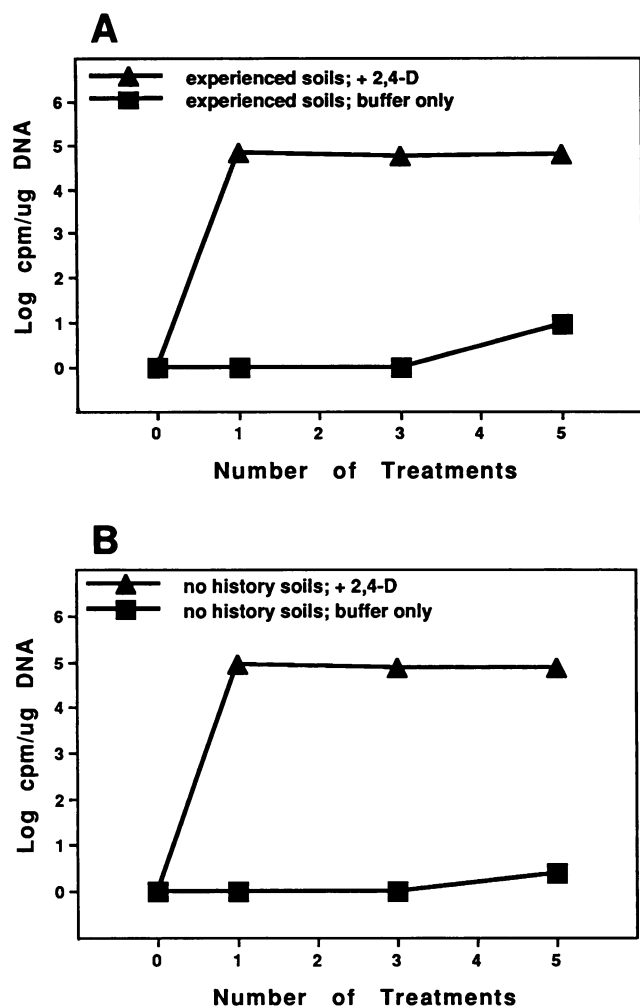
FIG. 3. tfdA probe hybridization signal versus the number of 2,4-D treatments. The hybridization signal is the mean value obtained from bacterial community DNA from two replicate plots of both experienced soils (A) and no-history soils (B).

size standards). This is in apparent contrast to the pJP4 plasmid, in which the tfdA and tfdB genes are separated by 13 kb (35) including three EcoRI sites (one of them within the tfdA gene) and three HindIII sites. It is possible that the data reveal two distinct but similar-size DNA fragments; however, Fig. 4A and B represent the same hybridization membrane, which was stripped and reprobed, and the hybridization bands for the tfdA and tfdB probes match exactly. Some minor bands of hybridization were also detected in these analyses, but these represent only a small fraction of the total hybridization signal. These data indicate that a single population of 2,4-D-utilizing bacteria (or possibly different populations carrying the same plasmid-borne 2,4-D catabolic pathway) experienced dramatic outgrowth in response to the addition of 2,4-D. If there were several different 2,4-D-degrading populations (or pathways) with homology to the probes, a more complex hybridization pattern would be expected. It is possible that other 2,4-D-degrading populations or pathways that have no homology to the probes used are also present since these would go undetected in this analysis. This, however, seems unlikely since the hybridiza-

tion signals detected with these probes indicate approximately the same numbers of 2,4-D-degrading organisms as those indicated by the MPN analysis.

Hybridization of the filter when the entire pJP4 plasmid is used as a probe generates a more complex autoradiogram (Fig. 4C). Again, a hybridization signal is detected only in the community DNA isolated from soils following treatment with 2,4-D, not in DNA isolated from soils prior to treatment. The band of hybridization in the community DNA which is homologous to the tfdA and tfdB gene probes is indicated by the closed circle in Fig. 4C. Note that this is a single discrete band even though both the tfdA and tfdB genes of pJP4 are present as labeled probes, again suggesting that the homologies to these genes in the soil community DNA are present on the same fragment. Only the two smallest (bottommost) bands of hybridization observed in the soil community DNA appear to correspond in size to bands observed in the control lanes containing plasmid pJP4, which was also digested with EcoRI and HindIII. Since the tfdA and -B genes, but not the tfd-C, -D, -E, and -F genes, of pJP4 have homology to the bacterial community DNA, there must be significant regions of non-tfd DNA sequences on pJP4 that are also homologous to DNA of the bacterial community selected by growth on 2,4-D. It is also significant that the pattern of the hybridization bands observed after the initial treatment with 2,4-D remained constant throughout the additional four treatments, indicating that the population that predominates after the first treatment was not succeeded or displaced during the course of the experiment. Thus, although there is clearly sequence relatedness between the 2,4-D-degrading population(s) in these soil samples and the pJP4 plasmid, there are also substantial differences, a notion also supported by the lack of significant hybridization to the tfdC, -D, -E, and -F genes.

DISCUSSION

We have successfully used gene probe and MPN methods to monitor the response of native soil microbial communities to the addition of 2,4-D as a growth substrate. The MPN values are lower than those reported by Cullimore (8) for the same soils, perhaps reflecting differences in method, incubation time, or sample storage times and conditions prior to enumeration. Indeed, our MPN cultures were incubated for 7 days, compared with 28-day incubations used by Cullimore, and extended incubation times result in MPN values about 1 log unit higher for soils with low numbers of 2,4-D-degrading organisms (18). Both methods detected a sharp increase in 2,4-D-metabolizing populations in response to the amendments.

After the first amendment, the hybridization data appear to overestimate the population, as determined by MPN (10^7 and 10^5 organisms, respectively). This is perhaps due to preferential recovery of 2,4-D-degrading bacteria at early times when they are rapidly growing and presumably less firmly attached to soil particles than are the remainder of the bacterial community (13, 14), effectively enriching for sequences homologous to the probes in the bacterial community DNA. After the initial treatment with 2,4-D the rate of increase in 2,4-D-degrading populations decreases, reaching a steady-state population (Fig. 1), possibly allowing for firmer attachment and thereby reducing or abolishing this phenomenon. Overall, however, the results obtained from hybridization experiments using probes for the tfdA and tfdB genes compare favorably with the MPN enumeration data.
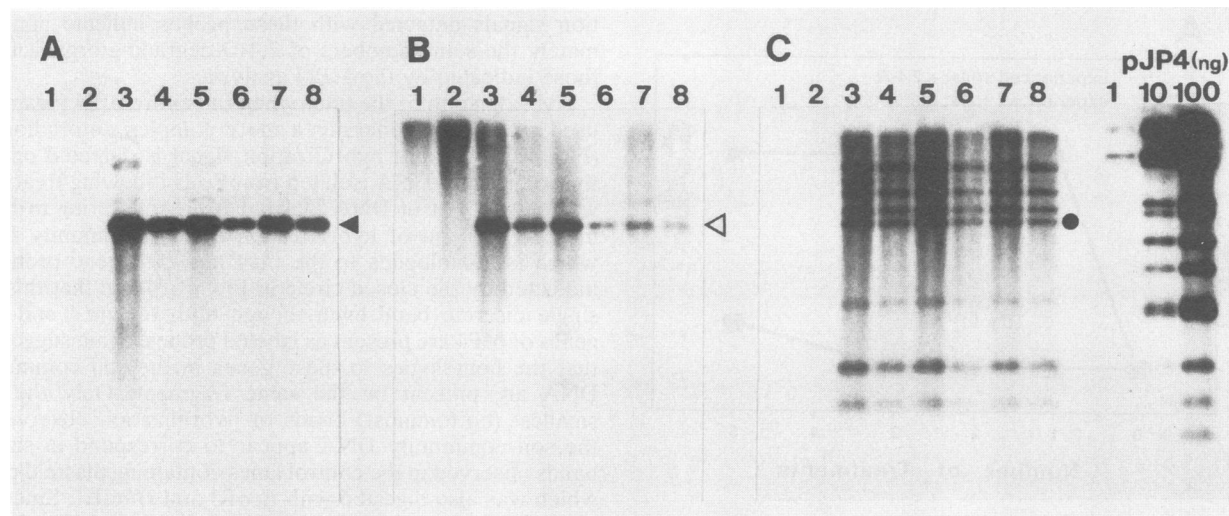
The prior history of the soils had no apparent effect on the

FIG. 4. Southern analyses of microbial community DNA from 2,4-D-experienced soils. Bacterial community DNA samples (2 μg) were digested with EcoRI and HindIII, size fractionated by electrophoresis, denatured, and then transferred to a hybridization membrane. The probes used were the *tfdA* gene probe (A), the *tfdB* gene probe (B), and the entire pJP4 plasmid (C). The lanes in each panel correspond to bacterial community DNA isolated from replicate soil samples after zero (lanes 1 and 2), one (lanes 3 and 4), three (lanes 5 and 6), and five (lanes 7 and 8) treatments with 2,4-D. Symbols: ◀, ◁, and ●, hybridization signals arising from homology to *tfdA*, *tfdB*, and both *tfdA* and *tfdB*, respectively.

response of the microbial communities to additions of 2,4-D in the laboratory. The MPN and gene probe data indicate similar low levels of 2,4-D-degrading organisms in both the soils that had a 42-year history of prior treatments with 2,4-D and control soils that had no prior exposure to this compound. The implications of these data are that 2,4-D field application rates (~1 ppm) and frequency are not sufficient to maintain 2,4-D-degrading populations at levels above those observed in soils not being treated with this compound. Thus, it seems likely that there were increases in the population levels of 2,4-D degraders in direct response to field application rates of 2,4-D, as shown previously (8), but that this population(s) subsequently decreased to initial levels during the period between the last field treatment and the onset of these experiments.

The site was originally chosen in the belief that 42 years of 2,4-D use may have selected variants in the native population that were more fit (better adapted) for use of 2,4-D as a substrate than organisms in the control soil, which had never been exposed to this compound. This result was not observed, possibly because the sample size was too small. The amount of 2,4-D used in the 42 years would theoretically have produced 2.5×10^{14} cells per plot (cell quota = 1.5 pg of substrate per cell) which, given the natural mutation rate (~10^{-8} per locus per generation), should have generated perhaps 10^6 variants in the rate-limiting step(s) per plot. Since there is a low probability of a given variant having an enhanced fitness coefficient and there is not a natural means of rapid microbial dissemination in soil, it is perhaps not surprising that no such variants were encountered in the small samples taken from the field (~$1/10^5$ of the 63-m^2 plot). Although further amendments with 2,4-D in the laboratory generated another 10^8 cells per sample, this number is too small to give a reasonable probability of an enhanced variant arising at this stage. Furthermore, no turnover of the 2,4-D-degrading populations occurred during the course of this experiment to allow selective enrichment of such a variant, if it did exist, to detectable proportions. This analysis also

points out the basic difficulty in measuring natural genetic change in soil populations, namely the physical inability to process large enough volumes of soil to detect rare events. This limitation might be minimized if dispersal of variants (mixing) could be greatly enhanced so that the more fit variant more widely colonizes and hence is more likely to be sampled.

While both MPN enumeration and gene probe analyses can be used to monitor the response of the microbial community to 2,4-D treatment, each method has inherent advantages and disadvantages. Each method generates different types of data that, taken together, provide a more comprehensive understanding of what is occurring in the soil microbial community. The MPN approach has both good low-end and good high-end detection capabilities. In our experiments we were capable of monitoring population changes of more than 6 orders of magnitude in a single analysis. While the MPN method was useful for determining the number of 2,4-D-degrading organisms present in our samples, it reveals nothing about the diversity of populations with this ability and nothing about how the genes encoding 2,4-D degradation in these samples are related to the genes of the canonical 2,4-D degradative pathway on pJP4.

Gene probe analyses, on the other hand, lack the low-end sensitivity of detection achieved by the MPN method but can reveal information on the diversity of 2,4-D-degrading populations, their relatedness to the probes employed, and the stability of these populations throughout the course of the experiment. In our previous work we have determined a low-end detection limit for gene probe analyses of microbial community DNA of about 10^4 copies of the target gene per g of nonsterile soil (15). Greater sensitivity of detection can be achieved by using target genes that exist in multiple copies per organism or by amplifying either the number of copies of the target gene or the hybridization signal (22, 30, 34). However, efforts to amplify target sequences in indigenous populations will likely be hampered by lack of strict conser-

vation of DNA sequences important to the amplification process.

It is interesting that only the *tfdA* and *tfdB* gene probes yielded hybridization data that correlate with the treatment regimen and MPN data. These two genes encode the enzymes for the first two steps in the pJP4-borne pathway for 2,4-D degradation. The product of the *tfdA* gene (2,4-D monooxygenase [etherase]) is not specific for chlorinated substrates (it will recognize phenoxyacetic acid [35]), yet aryl etherase activities, compared with other enzymatic activities, are probably not very common in soil microbial communities. This may explain why the *tfdA* gene probe hybridization signal correlated well with both the treatments and the MPN data and had a very low background hybridization signal. The *tfdB* gene product (2,4-dichlorophenol hydroxylase) recognizes only chlorinated substrates (24), yet phenol hydroxylases are rather common, being involved in a number of degradative pathways. Perhaps this explains why the *tfdB* gene probe hybridization signal correlated with 2,4-D treatment and the MPN data yet there was a significantly higher background hybridization signal. The *tfdC*, *-D*, *-E*, and *-F* genes encode chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, 4-carboxymethylene butenolide hydroxylase, and *trans*-chlorodiene-lactone isomerase, respectively (10). These enzymes constitute the lower *ortho*-cleavage pathway for the degradation of 2,4-D (except for maleylacetate reductase, which is chromosomally encoded [23]). These enzymes have broad substrate specificity, and the enzymatic functions are common to a number of *ortho*-cleavage pathways. It may be that the broad distribution of the presumably old and divergent genes for these enzymes explains why probes for these genes from pJP4 failed to yield data that correlated with the treatment regimen.

The detection of several bands of hybridization when the entire pJP4 plasmid was used as a probe in Southern analyses even though the *tfdC*, *-D*, *-E*, and *-F* genes themselves have no apparent homology to the bacterial community DNA is interesting. The source and significance of the non-*tfd* homology is not known, but it may represent common plasmid "backbone" features as described by Burlage et al. (5) or homology to the rather extensive cryptic region of pJP4. These data illustrate the importance of designing gene probes that contain only sequences related to the function of interest to avoid false-positive hybridization signals from unrelated sequences.

It should be noted that, although the *tfdA* and *tfdB* gene probes accounted for most of the 2,4-D-degrading populations in the soils used in these experiments, this may not be the case for all soils. It is likely that alternate, less-related pathways for 2,4-D degradation exist in some soils, as evidenced by the isolation of 2,4-D-degrading organisms whose DNA has no apparent homology to the *tfd* genes (6, 7, 19, 20). Such pathways would go undetected by the probes used here. Thus, in soil microbial community studies, a lack of correlation between probe hybridization data and enumeration data obtained through alternate analyses such as MPN is an indication that additional or different gene probes may be required (20). Such indications can be of use in efforts to identify or account for the diversity of populations present that have the function of interest.

The pattern of the hybridization bands observed in the Southern analysis with the entire pJP4 plasmid as a probe remained constant throughout the course of the experiment, indicating that the population that predominates after the first treatment is not succeeded or displaced during subsequent treatments and incubation. The Southern analyses using the *tfdA* and *tfdB* gene probes indicate that a single population of organisms predominates in these soils under these conditions. The possibility of a single plasmid-borne pathway being present in different populations of organisms cannot be excluded. It has been demonstrated, for example, that the pJP4 plasmid is self-transmissible to a variety of gram-negative organisms under laboratory conditions and that the 2,4-D-degradative genes on the plasmid can be expressed in many of these diverse genetic backgrounds (9, 11). Thus, the increase in 2,4-D-degrading populations as determined by MPN and gene probe analyses might result from rapid outgrowth of a single population carrying the pathway, from dissemination of a plasmid-borne pathway by horizontal gene transfer, or from both.

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