# Use of Gene Probes To Aid in Recovery and Identification of Functionally Dominant 2,4-Dichlorophenoxyacetic Acid-Degrading Populations in Soil

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The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was applied to soils in microcosms, and degradation was monitored after each of five repeated additions. Total DNAs were isolated from soil bacterial communities after each 2,4-D treatment. The DNA samples were analyzed on slot blots and Southern blots by using a *tfdA* gene probe subcloned from plasmid pJP4 and a *Spa* probe derived from a different 2,4-D-degrading isolate, a *Sphingomonas paucimobilis* strain. 2,4-D applied to soil was quickly degraded by indigenous microbial populations. As determined by slot blot analyses of DNA from a Michigan soil, the increase in hybridization signal in response to 2,4-D treatments was greater with the *Spa* probe than with the *tfdA* probe. In contrast, the DNA from a Saskatchewan soil exhibited an increase in hybridization signal with the *tfdA* probe. This indicated that a population with 2,4-D-degradative gene sequences different from the *tfdA* gene sequence was dominant in the Michigan site, but not in the Saskatchewan site. A Southern blot analysis of DNA from Michigan soil showed that the dominant 2,4-D-degrading population was *S. paucimobilis* 1443. A less dominant 2,4-D-degrading population was detected with the *tfdA* probe; further analysis revealed that this population was a *Pseudomonas pickettii* 712. These gene probe analyses revealed that an important population carrying out 2,4-D degradation was not detected when the canonical *tfdA* gene probe was used. After a series of new strains were isolated, we identified a probe to detect and identify the dominant members of this new group.

In the past, in soil microbiology studies workers typically either measured a process or studied model isolates, but rarely was a connection between these two approaches established. We have explored the use of nucleic acid-based methods involving total bacterial community DNA extracted from soil to identify populations that grow in response to a new carbon addition. We used 2,4-dichlorophenoxyacetic acid (2,4-D) as a model carbon source because probes for structural genes (*tfdA*, *tfdB*, *tfdC*, *tfdD*, and *tfdE*) which detect some 2,4-D-degrading populations have been identified (9) and this chemical serves as a growth substrate for a moderate number of indigenous soil organisms (4, 5). In a previous study performed with a Canadian soil, we showed that an increase in the population of 2,4-D degraders in response to 2,4-D addition corresponded to an increase in hybridization of the soil bacterial community DNA to the *tfdA* and *tfdB* gene probes (9). The correspondence between hybridization signal and population density and the simple band pattern observed in Southern blot analyses suggested that a single population with sequences homologous to the *tfdA* and *tfdB* genes had accounted for 2,4-D degradation. This may not be typical, however, as more diversity in natural populations would be expected. In this study we examined a Michigan soil with a dominant 2,4-D-degrading population that does not exhibit sequence homology with the *tfd* probes and used the gene probe method to help identify a

novel dominant 2,4-D degrader, as well as to study the response of a secondary 2,4-D-degrading population.

## MATERIALS AND METHODS

**Bacterial strains and media.** Strains of *Pseudomonas pickettii* and *Sphingomonas paucimobilis* capable of utilizing 2,4-D as a sole source of carbon were isolated from the Gene Flow plots at the Long-Term Ecological Research site at the Kellogg Biological Station (KBS) in Hickory Corners, Mich. (11). *Escherichia coli* JM83 has been described elsewhere (2). *S. paucimobilis* and *P. pickettii* strains were cultivated in MMO mineral medium (14) containing 2,4-D at a concentration of 500 ppm (500 μg/ml) at 30°C. *E. coli* JM83 was cultivated in Luria broth (12) at 37°C. Plasmid DNA was isolated by using the method of Hirsch et al. for large plasmids (7) or the method of Maniatis et al. for small plasmids (12).

**Soil treatment.** Stock solutions (20 mg/ml) of analytical grade 2,4-D (Sigma Chemical Co., St. Louis, Mo.) were prepared by dissolving 2,4-D in 0.1 M $NaH_2PO_4$ buffer (pH 7.0) and were stored at 4°C until they were used. Soil (Typic Hapludalfs, fine loamy, mixed, mesic) (13) with no history of 2,4-D treatment was obtained from the untreated control plot within the Gene Flow study area at the KBS Long-Term Ecological Research site. The soil was stored at field moisture levels at 4°C until it was used. Soil samples (500 g) which had been sifted through a 2-mm-pore-size sieve were transferred to sterile polyethylene wide-mouth bottles. The soil water content was adjusted to 25% (wt/wt) by adding sterile, distilled water. 2,4-D in phosphate buffer was added to a concentration of 250 ppm and thoroughly mixed in two bottles; two control bottles received only phosphate buffer. The disappearance of 2,4-D from soil was monitored as described below, and when 2,4-D was degraded, the soil was respiked with 2,4-D for each of five

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cycles of degradation. At the end of each degradation cycle, 50-g subsamples were taken from both the 2,4-D treated and control soils. Total soil bacterial DNA was extracted by the bacterial fractionation method, which involves the separation of bacterial cells from soil particles by differential centrifugation followed by lysis of the recovered cells (8). DNA from Saskatchewan soils that had been treated with 2,4-D and extracted by the same procedure (9) was used for comparison.

**Measurement of 2,4-D in soil.** The 2,4-D in soil was analyzed by vortexing soil with an equal volume of sterile distilled water and then centrifuging the preparation. The supernatant was filtered through a Millipore Millex-GS syringe filter and analyzed for 2,4-D with a Hewlett-Packard series 1050 high-performance liquid chromatography equipped with a Lichrosorb RP-18 column (Anspec Co., Ann Arbor, Mich.) and a UV detector set at 230 nm; methanol–0.1% phosphoric acid (60:40) was used as the eluant.

**Hybridization analysis.** To analyze 2,4-D-degrading microbial populations, the *tfdA* gene was chosen from among the five structural genes (*tfdA*, *tfdB*, *tfdC*, *tfdD*, and *tfdE*) of plasmid pJP4 (1, 3) because it appears to be more specific to the 2,4-D degradation pathway of plasmid pJP4 than the other genes (6, 9, 11). The *tfdA* and *Spa* (*S. paucimobilis*) probes are described in an accompanying paper (11). All restriction enzymes and T4 DNA ligase were purchased commercially and were used according to the manufacturer's specifications. Cloned plasmids in *E. coli* JM83 were amplified and isolated by the method of Maniatis et al. (12). The cloned probe fragments were isolated as restriction fragments from the pUC19 vector sequences on a 0.7% agarose gel, purified with a Geneclean kit (Bio 101, Inc., La Jolla, Calif.), and labelled with $^{32}$P by using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) or a nick translation kit (Boehringer Mannheim) according to the manufacturer's specifications. Labelled probes were separated from unincorporated nucleotides prior to use with a spun column (12). The probes were used at a concentration of approximately $10^6$ cpm/ml of hybridization fluid.

For slot blot experiments, total soil bacterial DNA (0.25 μg) dissolved in TE (10 mM Tris-Cl, 1 mM EDTA) was denatured for 10 min at 100°C, chilled on ice, and then transferred onto nitrocellulose hybridization membranes by using a Bio-Dot SF manifold (Bio-Rad Laboratories, Richmond, Calif.). The membranes were then placed DNA side up on a piece of (Whatmann 3MM) filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min and then neutralized by transfer to a piece of filter paper soaked with a solution containing 1.5 M NaCl, 0.5 M Tris-Cl, and 1 mM EDTA for 1 min. The DNA was then fixed to the membranes with a UV cross-linker (Stratagene, La Jolla, Calif.).

For Southern blot experiments, soil community DNA (1.5 μg) was digested with appropriate restriction endonucleases according to the manufacturer's specifications, size fractionated by electrophoresis through horizontal 0.7% agarose gels, and transferred to nitrocellulose hybridization membranes by the capillary technique (12). The DNA was fixed to the membrane with a UV cross-linker.

Prehybridization, hybridization, and posthybridization washes were performed as described in an accompanying paper (11). Hybridization signals were visualized with a Beta-scope radioactive blot analyzer (Betagen Corp., Waltham, Mass.) or by autoradiography with X-Omat AR film (Kodak, Rochester, N.Y.) exposed at −70°C, using a Quanta III intensifying screen (Sigma). The exposure times were 1 to 7 days depending on the intensity of the radioactive signal.

## RESULTS

**Degradation of 2,4-D in soil.** The first treatment of KBS soil with 2,4-D resulted in slow 2,4-D degradation, and it took 3 to 4 weeks for the added 2,4-D to be degraded completely (data not shown). The rates of degradation for subsequent additions of 2,4-D were much higher; it took 1 week or less for complete degradation for each of the four additional 2,4-D amendments.

**Gene probe hybridization.** When soil bacterial community DNA was probed with the *tfdA* gene probe in slot blot analyses, no significant hybridization was observed with KBS soil DNA except for a weak signal obtained after the fifth treatment of KBS soil (Fig. 1A, lane a), while a strong hybridization signal was observed with DNA from the Saskatchewan soil (Fig. 1A, lanes c and d). Apparently, 2,4-D-degrading microbial populations with DNA sequences very different from the *tfdA* sequence had become dominant in the KBS soil. Thus, we sought to identify the successful new 2,4-D degraders in the KBS soil. Since we had isolated a group of predominant 2,4-D degraders that did not hybridize to *tfd* genes (11), we used the *Spa* probe for this group to analyze the KBS soil DNA. We observed significant hybridization of this probe to DNA from KBS soils treated one to five times with 2,4-D (Fig. 1B, lanes a and b), while no increase in hybridization signal was observed after repeated 2,4-D treatments with the Saskatchewan soil DNA (Fig. 1B, lanes c and d).

To confirm that the hybridization signals observed in slot blot analyses were due to 2,4-D-degrading microbial populations and to identify specific 2,4-D-degrading isolates responsible for the hybridization signals, soil DNA from KBS soil was hybridized to the *Spa* probe after restriction endonuclease digestion (Fig. 2). After a single treatment and after four additional treatments of soil with 2,4-D, two hybridization bands (5.0 and 11.3 kb) were detected with this probe only in 2,4-D-treated samples. The positions of the hybridization bands remained constant throughout the experiment, indicating that these bands represent a single, stably maintained population.

Since each lane in Fig. 2 contained the same amount of total bacterial community DNA (1.5 μg), the intensity of the hybridization at each time point was proportional to the amount of target DNA in each sample. A quantitative hybridization analysis revealed that the size of this population increased about twofold between the first and fifth 2,4-D treatments.

Soil community DNA obtained after the fifth treatment with 2,4-D and DNA isolated from *S. paucimobilis* 1443 produced identical hybridization band patterns when three different restriction endonucleases were used (Fig. 3). This suggests that *S. paucimobilis* 1443 was one of the dominant 2,4-D degraders throughout the experiment.

Since there was also hybridization with the *tfdA* probe (albeit weak) (Fig. 1), we tried to identify the responsible *tfdA*-hybridizing strain(s). A single band of hybridization to *tfdA* (8.8 kb) was detected on Southern blots of bacterial community DNA after the second through the fifth additions of 2,4-D (Fig. 4). The DNA from soils not treated with 2,4-D and the DNA from soils after the first 2,4-D treatment exhibited no significant hybridization to the *tfdA* gene probe (Fig. 4). Of 47 2,4-D-degrading strains isolated from the test plot, 15 hybridized to the *tfdA* probe (14). Since the KBS soil community DNA did not hybridize to the *tfdB*, -*C*, or -*D* probe (data not shown), the *tfdA* probe hybridization signal observed on Southern blots might represent one or more of the strains belonging to group II (11), which comprises the KBS isolates that exhibit sequence homology only to the *tfdA* probe. Plasmid DNA from
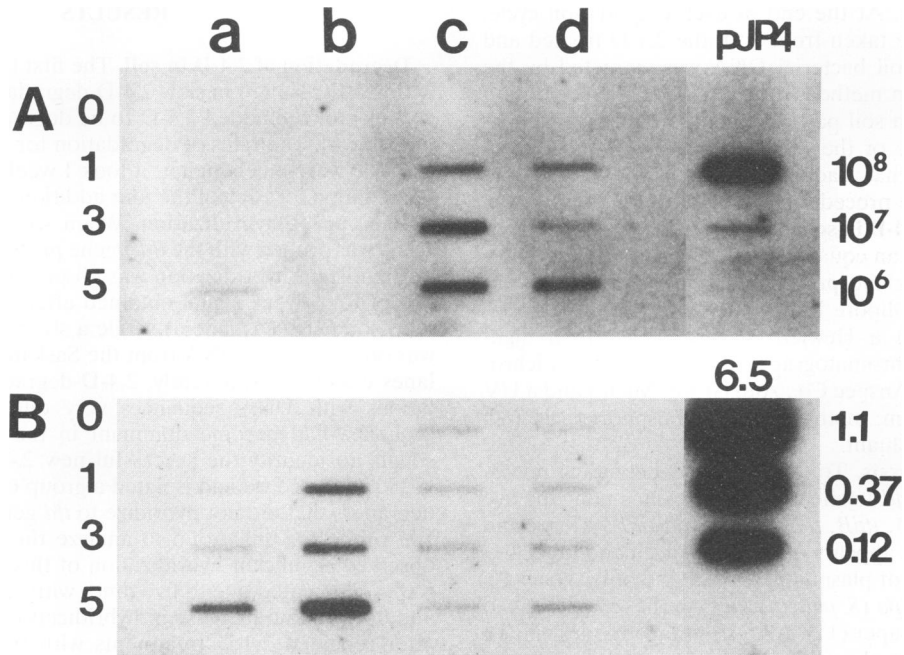
FIG. 1. Hybridization of DNAs from KBS (lanes a and b) and Saskatchewan (lanes c and d) soils to the *tfdA* probe (A) and the *Spa* probe (B). Lanes a through d contained 0.25 µg portions of soil DNA extracted after zero, one, three, and five additions of 2,4-D, respectively. The following positive controls were included: KBS control soil DNA containing $10^8$, $10^7$, and $10^6$ cells of *Pseudomonas cepacia* DBO1(pJP4) per g of soil (lane pJP4); 1.1, 0.37, and 0.12 ng of the *Spa* probe (lane 6.5).

*P. pickettii* 712, one of the group II isolates, was compared with total soil bacterial DNA obtained after the fifth 2,4-D treatment in Southern blot analyses by using the *tfdA* gene probe (Fig. 5). Matching band patterns were obtained for these two DNA samples when the samples were digested with three different restriction endonucleases, suggesting that *P. pickettii* was the dominant *tfdA*-homologous 2,4-D-degrading organism in the soils after the second through fifth treatments.
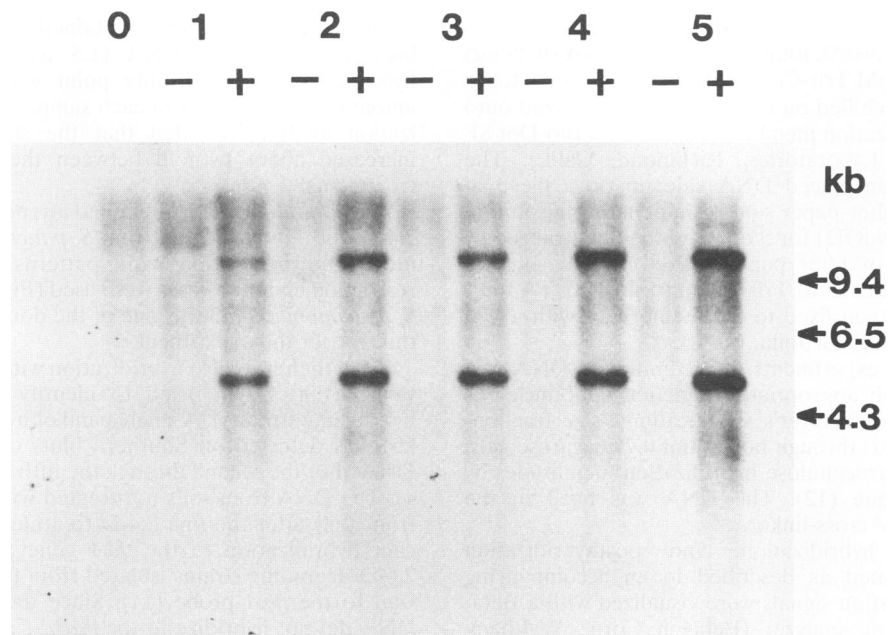


FIG. 2. Detection of indigenous 2,4-D-degrading microbial populations in soil by Southern blot analysis with the *Spa* probe. Soil DNA samples (1.5 µg) were digested with *Hin*dIII, size fractionated by agarose gel electrophoresis, and then transferred to nitrocellulose membranes. 0, 1, 2, 3, 4, and 5, DNA samples from soils after zero, one, two, three, four, and five treatments with 2,4-D, respectively. +, 2,4-D treated; −, no 2,4-D treatment (controls).
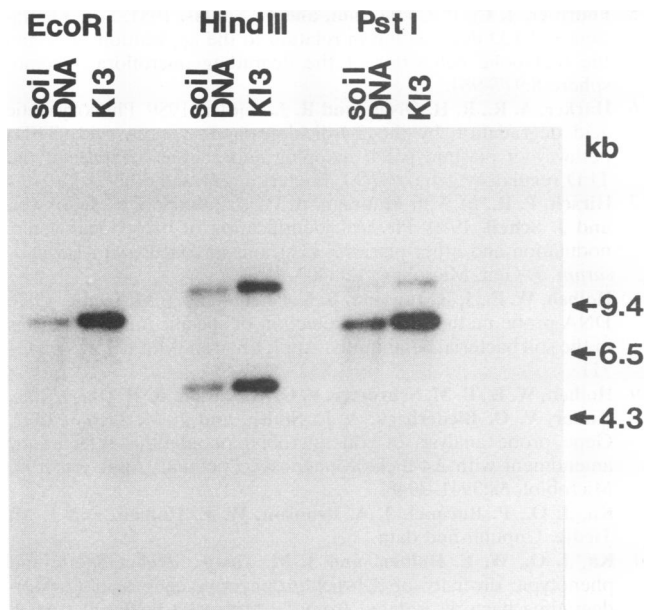
FIG. 3. Hybridization bands obtained from soil bacterial community DNA and *S. paucimobilis* 1443 DNA (KI3) digested with three different restriction endonucleases, size fractionated by agarose gel electrophoresis, and hybridized with the *Spa* probe. The DNA used was the DNA isolated after five 2,4-D treatments.
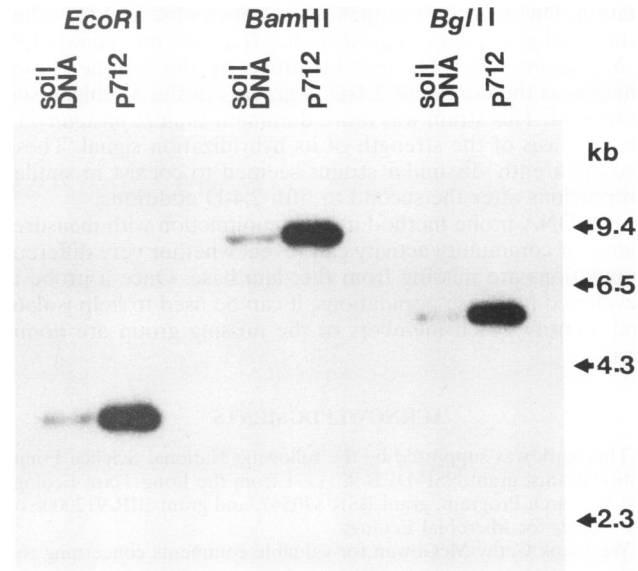
FIG. 5. Hybridization bands obtained from soil bacterial community DNA and *P. pickettii* 712 plasmid DNA (p712) digested with three different restriction endonucleases, size fractionated by electrophoresis, and hybridized with the *tfdA* gene probe. The soil DNA used was the DNA isolated after five 2,4-D treatments.

## DISCUSSION

The DNA from the 2,4-D-treated KBS soils exhibited no or only weak hybridization to the *tfdA* gene, while the DNA from the Saskatchewan soil exhibited the expected strong hybridization to *tfdA*. The population densities of the 2,4-D degraders estimated by the most-probable-number method were similar (ca. $5.0 \times 10^7$ organisms per g of soil) in the two soils (9, 10), indicating that there was no difference in the densities of the populations probed. Hence, organisms with different DNA sequences for 2,4-D degradation must exist in the Michigan soil. We tried to resolve the discrepancy between the high most-probable-number counts and the weak hybridization signal with *tfdA* by using the *Spa* probe for the group III isolates from the test site, since these organisms exhibited no

hybridization to *tfdA* genes (11). The *Spa* probe that defines this group exhibited increasing hybridization to the soil DNA in response to 2,4-D treatment and therefore could be used to detect and identify the missing 2,4-D degraders. We do not know yet what genes are encoded by the *Spa* fragment, but the hybridization response following 2,4-D addition shows that this probe does track an important population with this function.

Our results also illustrate that a single probe often may not be sufficient to monitor populations with a specific function unless the target genes have highly conserved sequences. The KBS soil contains at least three different groups of 2,4-D degraders (11); thus, even the two probes which we used (the *tfdA* gene probe and the *Spa* probe) may not completely monitor all of the indigenous 2,4-D-degrading microbial pop-
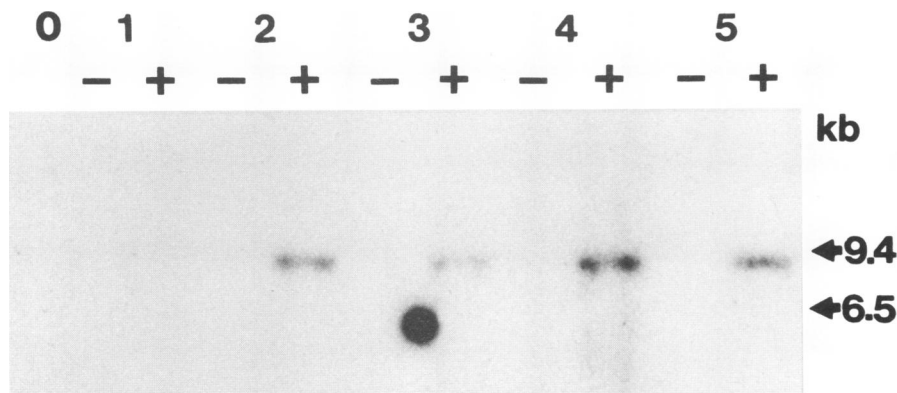


FIG. 4. Detection of indigenous 2,4-D-degrading populations in soil by Southern blot analysis with the *tfdA* probe. 0, 1, 2, 3, 4, and 5 total soil bacterial community DNA isolated after zero, one, two, three, four, and five treatments with 2,4-D, respectively. +, 2,4-D treated; −, no 2,4-D treatment (controls). DNA samples (1.5 μg) were digested with *Bam*HI, size fractionated by agarose gel electrophoresis, and then transferred to nitrocellulose membranes.

ulation. Divergence of sequences has been observed for other functional genes in ecological studies (e.g., the *mer* gene) (15).

*S. paucimobilis* 1443 was identified by the Southern blot analysis as the dominant 2,4-D degraders in the Michigan soil examined. This strain was more dominant than *P. pickettii* 712 on the basis of the strength of its hybridization signal. These two apparently dissimilar strains seemed to coexist in similar proportions after the second to fifth 2,4-D additions.

The DNA probe method used in conjunction with measurements of community activity can reveal whether very different populations are missing from the data base. Once a probe is developed for these populations, it can be used to help isolate and identify which members of the missing group are dominant.

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