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Characterization of Diverse 2,4-Dichlorophenoxyacetic Acid-Degradative Plasmids Isolated from Soil by Complementation

EVA M. TOP,[1,2] WILLIAM E. HOLBEN,[3] AND LARRY J. FORNEY[1]*

National Science Foundation Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824[1]; Laboratory of Microbial Ecology, Department of Biochemical and Microbial Technology, Faculty of Agricultural and Applied Biological Sciences, University of Ghent, Ghent, Belgium[2]; and The Agouron Institute, La Jolla, California 92037[3]

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The diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degradative plasmids in the microbial community of an agricultural soil was examined by complementation. This technique involved mixing a suitable *Alcaligenes eutrophus* (Rif^r) recipient strain with the indigenous microbial populations extracted from soil. After incubation of this mixture, Rif^r recipient strains which grow with 2,4-D as the only C source were selected. Two *A. eutrophus* strains were used as recipients: JMP228 (2,4-D$^-$), which was previously derived from *A. eutrophus* JMP134 by curing of the 2,4-D-degradative plasmid pJP4, and JMP228 carrying pBH501aE (a plasmid derived from pJP4 by deletion of a large part of the *tfdA* gene which encodes the first step in the mineralization of 2,4-D). By using agricultural soil that had been treated with 2,4-D for several years, transconjugants were obtained with both recipients. However, when untreated control soil was used, no transconjugants were isolated. The various transconjugants had plasmids with seven different *Eco*RI restriction patterns. The corresponding plasmids are designated pEMT1 to pEMT7. Unlike pJP4, pEMT1 appeared not to be an IncP1 plasmid, but all the others (pEMT2 to pEMT7) belong to the IncP1 group. Hybridization with individual probes for the *tfdA* to *tfdF* genes of pJP4 demonstrated that all plasmids showed high degrees of homology to the *tfdA* gene. Only pEMT1 showed a high degree of homology to *tfdB*, *tfdC*, *tfdD*, *tfdE*, and *tfdF*, while the others showed only moderate degrees of homology to *tfdB* and low degrees of homology to *tfdC*. These results indicate that this soil microbial community submitted to selective pressure (application of 2,4-D) for several years maintained a diversity of self-transferable plasmids carrying diverse genes encoding 2,4-D degradation.

Catabolic pathways required for the degradation of man-made chemicals are often encoded by transferable plasmids. The spread of plasmid-borne pathways involved in the degradation of organic compounds suggests that plasmids may be an important means for the dissemination of these genes and for the evolution of new functions under selective pressure (45).

During the past decade, large amounts of chlorinated compounds have been released into the environment as pesticides, fire retardants, refrigerants, and solvents, etc. One example of a chlorinated aromatic compound used in large amounts in agriculture is the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Genes encoding 2,4-D degradation are often located on conjugative plasmids (5, 6, 10, 28, 35). One such plasmid, pJP4 from *Alcaligenes eutrophus* JMP134, has been extensively studied and has become a model for the study of 2,4-D degradation. It has been shown that pJP4 encodes enzymes for the conversion of 2,4-D to chloromaleylacetate (Fig. 1). Enzymes responsible for further metabolism of chloromaleylacetate to tricarboxylic acid cycle intermediates are apparently encoded by chromosomal genes of JMP134 (12, 33). The catabolic (*tfdABCDEF*) and regulatory (*tfdR* and *tfdS*) genes have been localized, sequenced, and characterized (11, 12, 17–19, 22, 30, 31, 37, 41, 48). Studies in which other 2,4-D catabolic plasmids were compared with pJP4 have shed some light on the mechanisms by which catabolic pathways are acquired and evolve in microorganisms (5, 6, 35). Much remains unknown, however, about the diversity, evolutionary relationship, and horizontal dissemination of different 2,4-D-degradative plasmids within and among microbial populations in the environment.

All 2,4-D plasmids studied so far have been isolated from pure cultures that were obtained from environmental samples. However, this requires the isolation of bacteria on synthetic media and is therefore limited to those strains that are culturable under laboratory conditions. More recently, a method has been developed to capture transferable plasmids from microorganisms of a natural habitat, including putatively nonculturable ones, by conjugation. This general method was first described by Bale et al. (2) as exogenous plasmid isolation and involves mixing a suitable recipient strain with a natural microbial community and incubating the mixture to allow plasmid transfer to occur. Transconjugants are selected as recipients that have acquired a new genetic marker (14). Until now, this approach has been used successfully only for the isolation of mercury resistance plasmids (2, 3, 42). We describe variations of this approach used to isolate plasmids encoding genes for 2,4-D degradation from agricultural soils. Derivatives of *A. eutrophus* JMP134 unable to degrade 2,4-D were used as recipients, or genetic sinks, for these catabolic genes. The diversity of the isolated plasmids and the catabolic genes that they carry was investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. eutrophus* JMP134 harbors plasmid pJP4, carrying the necessary genes for 2,4-D (Fig. 1) and 3-chlorobenzoate degradation (10). The recipient strains used as genetic sinks in the plasmid capture experiments were *A. eutrophus* JMP228 and JMP228(pBH501aE). JMP228, a rifampin-resistant derivative of JMP134 that has been cured of pJP4, was obtained from J. M. Pemberton. JMP228 was used to capture plasmids which carry all of the genes coding for the conversion of 2,4-D to chloromaleylacetate by conjugation and complementation with chromosomal genes, yielding growth on 2,4-D as the

* Corresponding author. Mailing address: NSF Center for Microbial Ecology, Michigan State University, 540 Plant and Soil Sciences Building, East Lansing, MI 48824-1325. Phone: (517) 353-9021. Fax: (517) 353-2917. Electronic mail address: 21394ljf@msu.edu.

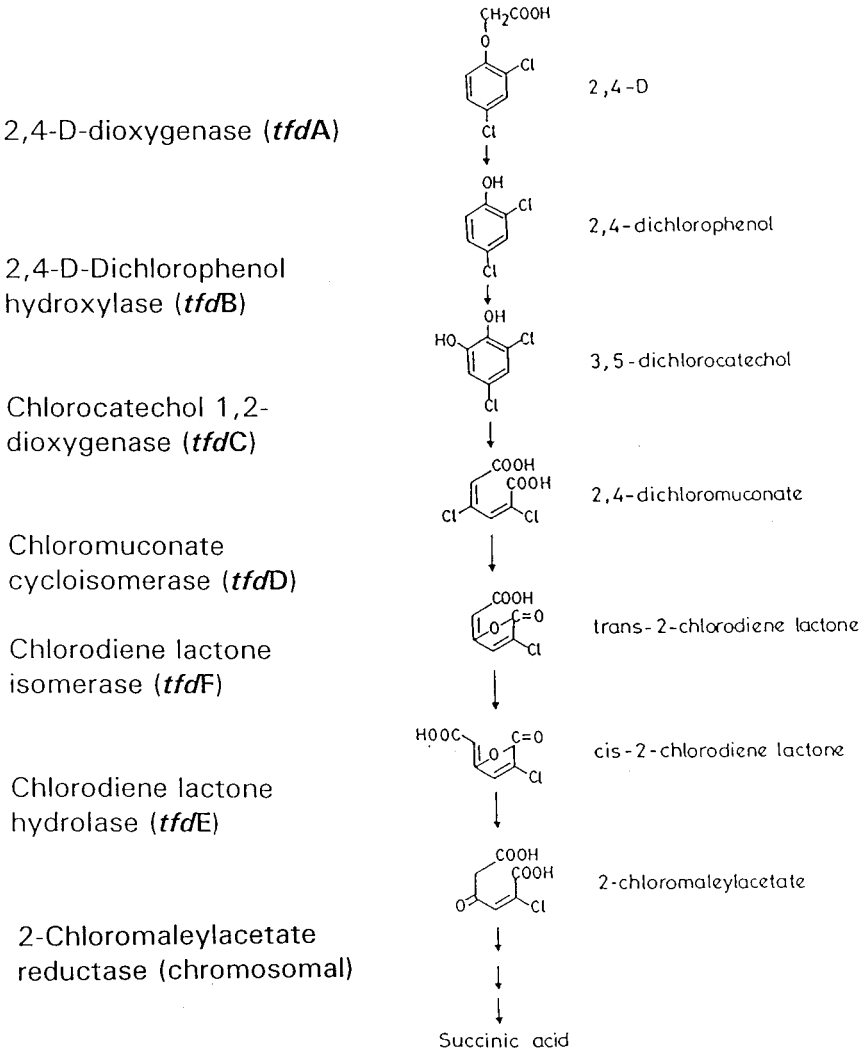

FIG. 1. Pathway for degradation of 2,4-D in strain JMP134. The figure is adapted from publications by Bhat et al. (5) and Fukumori and Hausinger (15) with permission from the publisher.

only carbon and energy source. Plasmid pBH501aE is a derivative of pJP4, obtained through a site-specific deletion of the 566-bp *Nru*I fragment of the *tfdA* gene into which an *nptII* (kanamycin resistance) cassette has been inserted. This strain was used in experiments designed to capture plasmids requiring only that they carry a *tfdA*-like gene since the rest of the 2,4-D catabolic pathway is located on pBH501aE and the chromosome of JMP228.

A. eutrophus JMP228n is a nalidixic acid-resistant mutant of JMP134 cured of pJP4 (44). *Escherichia coli* CM120(RP4) is a *trpA229-tonB* mutant of W3110 (1) and harbors the IncP1 plasmid RP4 (Tra^+ Tc^r Km^r Ap^r) (8, 52).

Media and culture conditions. JMP228 was plated and maintained on Luria-Bertani (LB) medium (43) containing 100 μg of rifampin per ml. The medium used for JMP228(pBH501aE) also contained kanamycin (100 μg/ml) to maintain selection for the pBH501aE plasmid. 2,4-D-degrading transconjugants were selected and maintained on MMO mineral medium (47) supplemented with 2,4-D at a concentration of 250 or 1,000 μg/ml and containing 100 μg of rifampin per ml. 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) and filter sterilizing the solution with a 0.22-μm-pore-size filter. Cycloheximide (200 μg/ml) was added to the plate media used in the soil experiments to inhibit fungal growth. Overnight cultures of recipient strains for the plasmid capture experiments were grown in LB medium; when appropriate, kanamycin (100 μg/ml) was added to select for plasmid pBH501aE. All broth cultures and plates were incubated at 30°C; broth cultures were shaken on a rotary platform at ca. 200 rpm.

Soil samples. Soil samples used in this study were obtained from plots that were regularly treated with 2,4-D at a rate of 100 mg of 2,4-D/kg of soil from 1988 on and from adjacent, untreated control plots at the National Science Foundation Long-Term Ecological Research site at the Kellogg Biological Station (Hickory Corners, Mich.) (25). Soil samples (2,4-D treated) collected in the spring of 1992 were used in the first plasmid capture experiments. Samples of 2,4-D-treated soil and nontreated control soil were collected in the fall of 1993 for use in the second plasmid capture experiments. Soil samples were stored at 4°C until use and were preincubated at room temperature (23 ± 2°C) for 2 to 4 weeks prior to the start of the mating experiments.

Plasmid capture from soil via conjugation. Soil samples (5 or 10 g) were diluted 10-fold in a sterile saline solution (0.85% NaCl) and shaken on a rotary shaker for 1 h. The soil particles were allowed to settle for 30 min, after which the bacterial cell suspension was decanted into sterile centrifuge tubes and bacterial cells were collected by centrifugation in a Sorvall SS34 rotor at 10,000 rpm for 10 min. The supernatant was discarded, and the cell pellet was resuspended in 0.1 volume of 1/10 strength LB broth (LB 1/10). One milliliter of this soil bacterial suspension was mixed with 1 ml of an overnight culture of the recipient strain [JMP228 or JMP228(pBH501aE)] which was previously diluted 1:1,000 in LB 1/10. This mating mixture was poured onto an LB plate, left open to dry in a laminar flow hood, and incubated overnight at 30°C to allow conjugation to occur. In order to assess possible background growth of bacteria that are not real transconjugants, 1 ml of the soil bacterial suspension and both recipient cultures were also plated separately. After 18 to 22 h, cells were harvested from the plates by resuspending them in 5 ml of sterile saline with a sterile spatula. The cell suspension was serially diluted in saline, and 0.1-ml samples were plated on MMO plates supplemented with 2,4-D (250 and 1,000 μg/ml) and rifampin (100 μg/ml). Transconjugant colonies were picked after at least 4 days of incubation and purified by using the same medium.

TABLE 1. Number of total heterotrophs and 2,4-D degraders in different soil samples and number of 2,4-D$^+$ transconjugants obtained after matings with these soils

Soil treatment and mating system[a]	No. of:		
	Total heterotrophs (CFU/g of soil)[b]	2,4-D degraders (MPN/g of soil)[c]	2,4-D$^+$ transconjugants (CFU/ml)
(2,4-D-treated soil 1 + JMP134) × JMP228	ND	1.1×10^{7}[d]	1.8×10^{8}
2,4-D-treated soil 1			
Alone	3.0×10^{6}	ND	<10
× JMP228			3.0×10^{4}
× JMP228 (pBH501aE)			3.3×10^{3}
2,4-D-treated soil 2			
Alone	5.1×10^{5}	7.0×10^{4}	<10
× JMP228			1.9×10^{4}
× JMP228 (pBH501aE)			8.0×10^{3}
Nontreated soil			
Alone	3.5×10^{6}	9.8×10^{2}	<10
× JMP228			<10
× JMP228 (pBH501aE)			<10

[a] Soils 1 and 2 were two 2,4-D-treated soil samples, collected at different times, and used in two independent mating experiments; ×, mating of soil with indicated strain. Concentrations of genetic sinks JMP228 and JMP228(pBH501aE) were ca. 10^6 CFU/ml before the mating and ca. 10^9 CFU/ml after the mating.
[b] ND, not determined.
[c] MPN, most probable number.
[d] The number of 2,4-D degraders was determined by plating MMO containing 1,000 μg of 2,4-D per ml; the number of CFU was several log units higher than that for uninoculated soil, and the colony morphology was that of JMP134.

Quantitation of total aerobic, heterotrophic, and 2,4-D-degrading bacterial populations. The total heterotrophic count of the soil samples was determined by plating 0.1-ml samples of appropriate 10-fold dilutions of soil bacterial suspensions onto R2A medium (Difco) containing cycloheximide (100 μg/ml). To determine the most probable number of 2,4-D-degrading bacteria in soil, 0.5-ml samples of serial dilutions of soil bacterial suspensions were inoculated into 4.5 ml of 1/10 strength R2A broth containing 250 μg of 2,4-D per ml. The tubes were incubated at room temperature for 2 weeks on a rotary shaker (ca. 150 rpm). One milliliter of culture was filtered through a Millipore Millex-GS syringe filter and analyzed for 2,4-D by high-performance liquid chromatography as described previously (23).

Plasmid DNA isolation and restriction pattern analysis. Individual colonies were cultured in LB broth, and plasmid DNA isolation was performed with 1.5 ml of culture essentially as described previously (29, 53). A modification of that procedure consisted of precipitation of DNA from the upper (aqueous) phase at −20°C after phenol-chloroform extraction. Precipitation was performed either overnight with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol or for a few hours with 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol. The DNA pellet was then resuspended in 20 μl of sterile deionized water. These plasmid DNA samples were then digested with one or more restriction enzymes by using the manufacturer's specifications, and restriction fragments were analyzed on a 0.8% agarose gel essentially as described previously (43).

Hybridization analyses. DNA probes directed against internal regions of the six structural genes for 2,4-D metabolism (*tfdABCDEF*) that are encoded on the pJP4 plasmid were described previously (23). The *tfdR* gene probe is an internal fragment of *tfdR* of pJP4 (59). The *repP* probe is composed of the 750-bp *Hae*II fragment of vector pCT7 (also referred to as pULB2420 [7]), which contains the *inc*/*rep* functions of the IncP1 plasmid RK2 (50), and was purified by using the Geneclean kit (Bio 101, La Jolla, Calif.) as described by the manufacturer. All probes were labeled with digoxigenin d-UTP by using a nonradioactive DNA-labeling kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Southern blots were performed by using nylon membranes (Hybond N; Amersham, Arlington Heights, Ill.) according to the supplier's instructions. Hybridizations were performed as recommended by Boehringer Mannheim, and strong, medium, and weak hybridization solutions contained 50, 20, or 0% formamide and 5, 2, and 2% of the blocking agent, respectively. This allowed us to detect DNA that was >90, >75, or >60% homologous to the gene probes (16). Color development was performed with the nonradioactive DNA detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

Plate matings between pure cultures. Strains to be mated were grown overnight in LB broth, and plate matings were carried out overnight on LB agar plates as described previously (34).

REP-PCR. Repetitive extragenic palindromic (REP) elements are highly repeated and conserved elements that are present in the genomes of most or all bacterial genera (57). The use of REP-specific oligonucleotide primers in the PCR with chromosomal DNA of different strains yields very characteristic banding patterns after size fractionation of PCR products on agarose gels (9, 57). This approach was used to verify that the transconjugants obtained were indeed derived from the genetic sinks JMP228 and JMP228(pBH501aE) and thus result in the same REP-PCR banding pattern. The REP-PCR method was performed as described by de Bruijn (9) except that 1 μl of glycerol stocks of the different transconjugants was mixed with 24 μl of the PCR mixture. After PCR amplification, 10-μl samples of the REP-PCR products were separated by gel electrophoresis on a horizontal 1% agarose gel.

RESULTS

Capture of 2,4-D-degradative plasmids from 2,4-D-treated agricultural soils. The efficiency of capturing catabolic plasmids from soil by using JMP228 was estimated by evaluating the recovery of pJP4 from soil seeded with ca. 10^7 CFU of *A. eutrophus* JMP134(pJP4) per g 2 days prior to the mating experiment. High numbers of 2,4-D$^+$ JMP228 transconjugants (ca. 10% of the total number of potential recipients) were obtained. This shows that our method efficiently captures 2,4-D-degradative plasmids when the soil contains a high concentration of a strain, isogenic to the genetic sink, that carries a self-transferable 2,4-D-degradative plasmid.

To assess the diversity of self-transmissible plasmids that carry genes for 2,4-D degradation in indigenous bacterial populations in 2,4-D-treated soil, two independent plasmid capture experiments were performed with soil samples collected at different times. A control soil sample not previously treated with 2,4-D was also included to assess the relationship between the history of 2,4-D exposure and the presence of transferable 2,4-D-degradative plasmids. In both experiments, comparable numbers of 2,4-D-degrading transconjugants were obtained from matings between the soil bacterial populations from samples of 2,4-D-treated soil and either JMP228 or JMP228(pBH501aE) (Table 1). No differences in the number of transconjugants were observed when results from selective media containing either 250 or 1,000 μg of 2,4-D per ml were compared. The initial number of recipients (i.e., genetic sink bacteria) in each mating was always ca. 10^6 CFU/ml. In all experiments, the numbers of total heterotrophic bacteria and recipient bacteria increased dramatically during the overnight mating on LB medium (from ca. 10^6 to ca. 10^9 CFU/ml), suggesting that the number of transconjugants obtained resulted from both plasmid capture and outgrowth of newly formed transconjugants. When soil with no genetic sink was

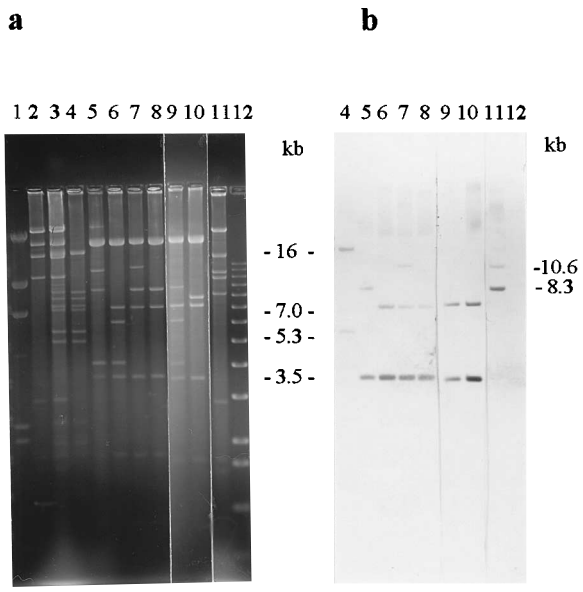

FIG. 2. (a) Agarose gel electrophoresis of plasmids pEMT1 to pEMT7 after digestion with *Eco*RI; (b) Southern blot hybridized with *tfdA* probe at high stringency. Lanes: 1, λ *Hin*dIII; 2, pBH501aE; 3, pBH501aE + pEMT1; 4, pEMT1; 5, pEMT2; 6, pEMT3; 7, pEMT4; 8, pEMT5; 9, pEMT6; 10, pEMT7; 11, pJP4; 12, 1-kb ladder.

plated on the same selective medium, only a few colonies (ca. 30 CFU/ml) were obtained. These could be easily distinguished from the JMP228 transconjugants by their different colony morphology. No transconjugant colonies were detected in matings between the untreated control soil bacterial populations and either JMP228 or JMP228(pBH501aE) (Table 1). The most probable number of 2,4-D degraders in this soil was 100-fold lower than that in the 2,4-D-treated soil. This difference in potential donor populations may determine the ability to capture 2,4-D-degradative plasmids from the soil microbial community.

Characterization of transconjugants and their plasmids. All 11 JMP228 transconjugants from the first plasmid capture experiment that were examined were found to harbor a plasmid. Four different patterns of *Eco*RI fragments were observed, and representatives of each type were designated pEMT1 to pEMT4 (Fig. 2). From plasmid capture experiments with the second 2,4-D-treated soil sample, 33 2,4-D^+ JMP228 transconjugants were examined. Three novel plasmid types were identified by restriction fragment analysis and designated pEMT5, pEMT6, and pEMT7 (Fig. 2). Two plasmid types already identified in the first experiment (pEMT1 and pEMT3) were also recovered. When 17 of the 19 JMP228 transconjugants isolated from a single dilution plate from the second plasmid capture experiment were screened for plasmids, the following plasmid distribution was observed: pEMT1 was found in five transconjugants, pEMT3 in seven, pEMT5 in three, pEMT6 in one, and pEMT7 in one. The *Eco*RI restriction pattern of pEMT1 is clearly very different from each of the other plasmid patterns, all of which have several bands in common. The sizes of pEMT1 and pEMT3, which was chosen as the representative plasmid of the group of related plasmids (pEMT2 to pEMT7), were estimated to be ca. 97 and ca. 63 kb, respectively, on the basis of the sum total of *Eco*RI, *Eco*RV, and *Bgl*II restriction fragment patterns (data not shown). Colonies harboring pEMT1 always grew faster and more extensively than those harboring the other plasmids (pEMT2 to pEMT7), both in liquid and on solid medium with 2,4-D.

All 8 of the JMP228(pBH501aE) transconjugants from the first experiment that were examined and each of the 36 JMP228(pBH501aE) transconjugants obtained from the second plasmid capture experiment contained both pBH501aE and pEMT1, as both *Eco*RI patterns were observed (Fig. 2). The fact that both plasmids were coresident, even after several transfers on MMO medium supplemented with 2,4-D (250 or 1,000 μg/ml) and rifampin (100 μg/ml) without kanamycin, suggests that they are compatible and that pEMT1 does not belong to the IncP1 incompatibility group.

The seven different JMP228 transconjugants, harboring pEMT1 through pEMT7, and the JMP228(pBH501aE) transconjugant with pEMT1 were further characterized. The pattern of REP-PCR fragments from chromosomal DNA has been shown to be very characteristic for different bacterial strains (9, 57). Comparison of the REP-PCR patterns of the different transconjugants to that of JMP228 confirmed that they were authentic JMP228 transconjugants (data not shown). Subsequent plate mating experiments between pure cultures of each transconjugant and JMP228n confirmed that the 2,4-D^+ phenotype could be transferred by conjugation. Restriction analysis of plasmid DNA from the transconjugants of these plate matings confirmed the presence of the corresponding plasmids in the JMP228n transconjugants. This indicates that the enzymes required to mineralize 2,4-D are encoded on the captured plasmids.

The plasmid incompatibility group of the seven different plasmid types was assessed by using the *repP* gene probe, allowing detection of IncP1 plasmids, and genetic analysis. pEMT1 did not show homology to the *repP* probe, even at low stringency. The observation that pEMT1 was stably maintained in JMP228 harboring the IncP1 plasmid pBH501aE further supports the notion that pEMT1 is not an IncP plasmid. Plasmids pEMT2 to pEMT7, however, hybridized to the *repP* probe at high stringency, indicating that these plasmids belong to the IncP1 group. The *Eco*RI fragment that hybridized to the *repP* probe was smaller than that of pJP4 (Table 2). Transfer of RP4 (IncP1) from *E. coli* CM120(RP4) to JMP228(pEMT3) resulted in the loss of pEMT3 from JMP228, indicating that

TABLE 2. Hybridization of different plasmids to *tfdA*, *tfdB*, *tfdC*, and *repP* after digestion with *Eco*RI

Probe	Hybridization pattern[a]							
	pEMT1	pEMT2	pEMT3	pEMT4	pEMT5	pEMT6	pEMT7	pJP4
tfdA	16 + 5.3***	8.3 + 3.5***	7.0 + 3.5***	7.0 + 3.5***	7.0 + 3.5***	7.0 + 3.5***	7.0 + 3.5***	10.6 + 8.3***
tfdB	16***	3.5**	3.5**	3.5**	3.5**	3.5**	3.5**	14.5***
tfdC	16***	11*	6.0*	11.2 + 8.5*	8.5*	8.5 + 6.0*	7.5*	14.5***
repP	NH	21.0***	21.0***	21.0***	21.0***	21.0***	21.0***	36***

[a] *Eco*RI fragment size(s) (in kilobases). The homology to the corresponding probe is indicated by asterisks: ***, >90%; **, 75 to 90%; *, 60 to 74%; NH, <60% (no homology).

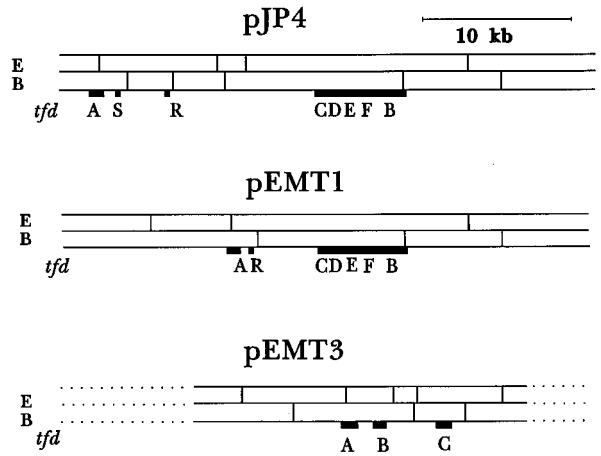

FIG. 3. Comparison of locations of *tfdA*, *tfdB*, and *tfdC* genes on restriction digest map of catabolic regions of pJP4, pEMT1, and pEMT3. The locations of *tfdD*, *tfdE*, *tfdF*, and *tfdR* genes on pJP4 and pEMT1 are also compared. E, *Eco*RI; B, *Bam*HI. The map of pJP4 is adapted from publications by Kaphammer and Olsen (30) and Matrubutham and Harker (37) with permission from the publisher.

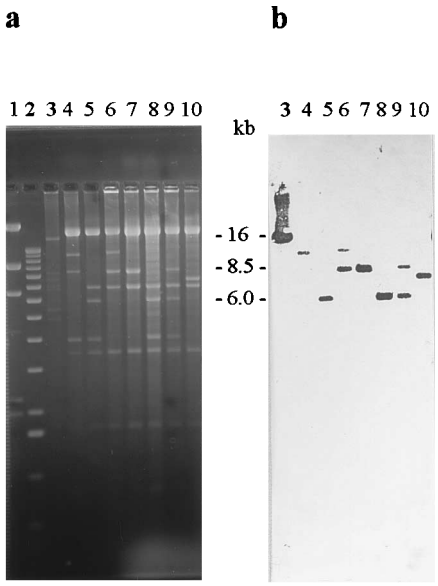

FIG. 4. (a) Agarose gel electrophoresis of plasmids pEMT1 to pEMT7 after digestion with *Eco*RI; (b) Southern blot hybridized with *tfdC* probe at low stringency. Lanes: 1, λ *Hin*dIII; 2, 1-kb ladder; 3, pEMT1; 4, pEMT2; 5 and 8, pEMT3; 6, pEMT4; 7, pEMT5; 9, pEMT6; 10, pEMT7.

the plasmids are incompatible and that pEMT3 belongs to the IncP1 group.

The plasmid-encoded 2,4-D degradation pathways carried on pEMT1 to pEMT7 were analyzed by Southern hybridization of digested plasmid DNA with probes based on the *tfd* genes of the catabolic pathway on pJP4. Probes based on the *tfdA*, *tfdB*, and *tfdC* genes, coding for the first three enzymes of the pathway, were hybridized to each of the plasmids. pEMT1 and pEMT3 were also probed with the *tfdD*, *tfdE*, *tfdF*, and *tfdR* gene probes to further compare similarities and differences between each other and with pJP4. pEMT1 showed high degrees of homology (90 to 100% sequence similarity [16]) to *tfdA*, *tfdB*, *tfdC*, *tfdD*, *tfdE*, *tfdF*, and *tfdR* (Table 2; some data not shown). As in the case of pJP4, two *Eco*RI bands from pEMT1 hybridized to *tfdA*, a result which indicates that the *tfdA* sequence on this plasmid has an *Eco*RI site as does the *tfdA* gene of pJP4 (Fig. 2). However, the sizes of both restriction fragments hybridizing to the *tfdA* gene probe differed from those of pJP4. The *tfdB* to *tfdF* and *tfdR* genes are located on a 16-kb *Eco*RI fragment, which also contains a part of the *tfdA* gene (Fig. 3). This is different from the gene organization on pJP4, in which neither of the two fragments that carry *tfdA* also encodes *tfdB* or *tfdC* (Table 2; Fig. 3). Digestion of pEMT1 with *Bam*HI yielded only three fragments: *Bam*HI-A, a very large fragment with an estimated size of ca. 80 kb; *Bam*HI-B, 10.2 kb; and *Bam*HI-C, 6.4 kb. The *tfdA* probe hybridized to the large fragment, whereas the *tfdB* to *tfdF* probes hybridized to the 10.2-kb fragment (Fig. 3). At medium stringency, *tfdB* also hybridized to the 6.4-kb fragment (data not shown), possibly indicating that *tfdB* spans a *Bam*HI site (Fig. 3). This is analogous to the *tfdB* gene of pJP4, which is mainly located on an 11.9-kb *Bam*HI site and spans the *Bam*HI site into the next 6.4-kb *Bam*HI site (Fig. 3). Although the gene sequences and the gene organization within the catabolic operon of pEMT1 appear quite similar to those of pJP4, the restriction fragment patterns and the location of *tfdA* are clearly different. Also, the backbones of the plasmids are very different, as pJP4 is an IncP1 plasmid and pEMT1 is not.

Plasmids pEMT2 to pEMT7 were also hybridized with the *tfdA*, *tfdB*, and *tfdC* probes, and pEMT3 was also probed with *tfdD*, *tfdE*, *tfdF*, and *tfdR* probes. Again, all plasmids had two *Eco*RI bands that hybridized to *tfdA* at high stringency, indicating that the *tfdA*-like genes on these plasmids also have an *Eco*RI site (Fig. 2). Yet, the sizes of these two *Eco*RI fragments were different from those of pEMT1 and pJP4. For each plasmid (pEMT2 to pEMT7), the *tfdA* probe hybridized to 3.5- and 7-kb bands, with the exception of pEMT2, in which the larger band was 8.3 kb (Fig. 2). For each of these plasmids, a moderate degree of homology (75 to 90%) to *tfdB* of pJP4 was shown by a 3.5-kb *Eco*RI fragment that also contains homology to *tfdA*. Hybridization of *Bam*HI-digested DNA from pEMT2 to pEMT7 showed that the *tfdA*- and *tfdB*-like genes are clustered on an 8.1-kb *Bam*HI fragment (Fig. 3). The close linkage of *tfdA* and *tfdB* on these plasmids differs from that found on pEMT1 and pJP4, where they are separated by ca. 9 and 17 kb, respectively (Fig. 3). The *tfdC* gene probe of pJP4 hybridized to another *Bam*HI fragment (3.6 kb) of plasmids pEMT2 to pEMT7 and only at low stringency (60 to 75% homology). This shows that the *tfdC* homologs were not located between *tfdA* and *tfdB*, as is the case with pJP4 and pEMT1 (Fig. 3), and thus that the gene organization of plasmids pEMT2 to pEMT7 differs from that of pEMT1 and pJP4. Hybridization of *Eco*RI-digested plasmid DNA showed that *Eco*RI fragments carrying a *tfdC* homology differed in pEMT2 through pEMT7 (Fig. 4). A more precise location of *tfdC* relative to *tfdA* and *tfdB* on plasmid pEMT3 was determined and compared with pJP4 and pEMT1 (Fig. 3). Hybridization of pEMT3 with the probes derived from *tfdDEF* and *tfdR* of pJP4 showed that pEMT3 has only a low degree of homology to *tfdD* and *tfdF*, no detectable homology to *tfdE*, but a high degree of homology to *tfdR* (data not shown).

DISCUSSION

We have successfully used a modified exogenous plasmid isolation concept to capture plasmids that carry genes enabling degradation of 2,4-D from soil bacterial communities. This approach has previously been used in the isolation of mercury resistance plasmids (2, 3, 42), but to our knowledge, this is the

first report of successful capture of catabolic plasmids by using this approach. Complementation between the genes carried by the newly acquired plasmids and host genes allowed the genetic sinks to use 2,4-D as the sole carbon and energy source. The use of such genetic sinks to capture plasmids from natural microbial communities does not require isolation or even growth of the original hosts, avoiding problems related to culturability of the majority of soil bacteria. This method may not allow for the recovery of 2,4-D pathways which are located on plasmids with a narrow host range, on nonconjugative plasmids, or on the chromosome (36, 49). However, the possibility that these pathways could be recovered through mobilization with helper plasmids and recombination or transposition, although at very low frequencies, cannot be ruled out. The success of this method to capture a variety of 2,4-D-degradative plasmids encourages further attempts to isolate other plasmid-borne catabolic pathways from bacterial populations in other environmental habitats.

The number of 2,4-D-degrading transconjugants obtained in these plasmid capture experiments appears to be related to the most probable number of 2,4-D^+ strains in soil, which in turn is related to the history of exposure of the soil to 2,4-D (Table 1). Several studies have shown that microbial communities in environments subjected to selective pressure caused by pollutants have higher numbers of plasmid-bearing strains than do control sites (4, 20, 40). However, the function of these plasmids was not always clear. This work indicates that the number of strains harboring 2,4-D-degradative plasmids was likely lower in the nontreated control soil than in the 2,4-D-treated soil and agrees well with the results of prior studies in this laboratory (23, 25).

Seven different plasmids encoding genes for 2,4-D degradation were isolated from the 2,4-D-treated soil used in this study. The ability of the transconjugants to transfer their plasmid and 2,4-D^+ phenotype via subsequent rounds of conjugation indicates that these plasmids carry the 2,4-D-degradative genes and are conjugative and therefore had probably transferred themselves into JMP228 without the need of self-transmissible, mobilizing helper plasmids.

Plasmid pEMT1 obtained in this study is a non-IncP1 plasmid that has catabolic genes that show high degrees of homology to the *tfdABCDEFR/S* pathways of the IncP1 plasmid pJP4. Differences between pEMT1 and pJP4 other than the replication origin are the size (ca. 96 kb compared with 86 kb), the restriction digest fragment patterns, and the distance between *tfdA* and *tfdC* (ca. 13 kb on pJP4 [41] and ca. 5 kb on pEMT1 [Fig. 3]). Other 2,4-D-degradative plasmids have also been compared with the canonical pJP4 plasmid. Chaudry and Huang (6) found that the size (45 kb) and restriction pattern of pRC10, a plasmid encoding 2,4-D degradation in a *Flavobacterium* sp., were different from those of pJP4, although the plasmid showed homology to the regions of pJP4 on which the degradative genes (*tfdABCDEF*) are located. Similarly, Maë et al. (35) characterized plasmid pEST4011, which shows homology to the catabolic genes of pJP4 but has a considerably different size, order of catabolic genes, and restriction pattern. Bhat et al. (5) observed that, although the physical map of their 2,4-D-degradative plasmid pMAB1 differed from those of pJP4 and pRC10, the fine map of the catabolic region showed a high degree of similarity to that of the *tfdCDEF* operon of pJP4 and the *tfdC* gene sequence was identical to the *tfdC* sequence of pJP4. Taken together, these reports and ours indicate that very similar (maybe identical) catabolic genes or complete pathways are found on large plasmids which have different backbones. This suggests that these plasmids might serve as vectors that shuttle genes required for the metabolism of 2,4-D between different microbial populations. This means of catabolic gene transfer is analogous to the TOL and NAH catabolic plasmids that confer the ability to metabolize toluene and naphthalene, respectively (13, 21, 58, 60).

Previous studies have also shown that catabolic genes can reside on transposable genetic elements and be transferred among bacterial populations as modules. For example, Tn*4651* and Tn*4653* are catabolic transposons that encode the toluene-xylene-degradative genes of the TOL plasmid pWW-O (55) and Tn*4655*, which encodes the naphthalene catabolic genes on the NAH plasmid (54). Similarly, Tn*5280* of plasmid pP51 encodes genes required for the metabolism of chlorobenzene (56), and Tn*5271* of plasmid pBRC60 encodes genes required for the degradation of 3-chlorobenzoate (39). Although the clustering of genes on pJP4, pAC27, and pP51 suggests that these genes have moved among bacterial strains as a module (56), there is no direct evidence that the *tfd* genes are located on a transposable element.

The 2,4-D-degradative genes of pEMT2 to pEMT7 and their organization differ considerably from those of pJP4 and pEMT1, on the basis of DNA homology studies. The plasmids, however, belong to the same IncP1 incompatibility group as pJP4. The incompatibility group was determined by replicon typing and confirmed by mating experiments with the IncP1 plasmid RP4. Only the *tfdA* gene probe of pJP4 hybridized at high stringency to these plasmids, while *tfdB* and *tfdC* hybridized only at medium and low stringency, respectively. The location of *tfdA* close to the *tfdB*-like gene is in contrast with the large distance between these genes on pJP4 (41) and also on pEMT1. Also, the position of the *tfdC*-like gene outside the region between *tfdA* and *tfdB* on pEMT3 is different from the gene organization of pJP4 and pEMT1 (Fig. 3). Diversity among genes encoding 2,4-D degradation in soil microbial populations has also been demonstrated elsewhere (16, 23–28, 36, 49). Whether these apparently nonhomologous genes evolved independently or have a common origin remains an unanswered question. More comparative studies of the different catabolic pathways could shed more light on the mechanisms of evolution of these genes.

Our finding that very different plasmids, present in the same soil, carry different *tfdBCDEF* genes (pEMT1 compared with pEMT2 to pEMT7) but very similar (maybe identical) *tfdA* genes suggests that plasmids may also serve as shuttle vectors for parts of catabolic pathways used for the evolution of new metabolic capabilities. Recruitment of *tfdA* has been suggested from studies with pJP4, because of the wide separation with the *tfdCDEFB* cluster (48). Evolution of degradative pathways by assembly of distinct parts of the pathway has been suggested by Franklin et al. (13) after the observation that the catabolic genes of the toluene degradation plasmid, TOL, were separated into upper and lower pathway gene clusters. A similar clustering was also observed on the NAH plasmid, which encodes naphthalene degradation (60).

Experiments with the same soils, performed 2 to 3 years prior to our experiments, indicated the presence of natural host strains that harbored plasmids identical or related to pEMT2 to pEMT7 (25–28). One isolate obtained in those earlier experiments, *Pseudomonas pickettii* 712, was shown to carry plasmid pKA4 (27, 28), which when hybridized with the *tfdA* gene probe revealed the same 3.5-kb band as that found on our plasmids pEMT2 to pEMT7. *P. pickettii* 712 was further characterized by Fulthorpe et al. (16) and was shown to have a hybridization pattern similar to that of pEMT3 when hybridized with *tfdA*, *tfdB*, and *tfdC*. Indeed, when we transferred pKA4 from *P. pickettii* 712 to JMP228 by conjugation, the *Eco*RI restriction pattern was identical to the restriction pat-

tern of pEMT3 (unpublished data). These data suggest that the same plasmid was still present in that soil more than 3 years later and could be transferred to *A. eutrophus* JMP228 in our soil mating experiment. Southern hybridizations of total soil bacterial DNA from the same field plots with the probe derived from *tfdA* revealed bands of 3.5, 5, and 7 kb (24). The 3.5- and 7-kb bands correspond with the hybridization patterns of plasmids pEMT3 to pEMT7. The 5-kb band could correspond with the 5.3-kb band of our plasmid pEMT1, which probed positive with *tfdA*. This indicates that the *tfdA*-carrying DNA obtained with our plasmid capture method is representative of the total amount of *tfdA* homologous DNA present in the soil.

Both IncP1 and non-IncP1 plasmids encoding 2,4-D degradation were present in the soil samples. The incompatibility group designation of several 2,4-D-degradative plasmids described in the literature is still unknown (5, 35). Of the six 2,4-D plasmids isolated by Don and Pemberton (10), four belonged to the IncP1 group and two were not IncP1 or IncW. Although IncP1 plasmids are the most intensively studied broad-host-range plasmids and may have the widest transfer and replication range among the conjugative plasmids (38, 46, 51), little is known about their prevalence in natural microbial communities. A recent study has shown that some but not all of the conjugative, mobilizing plasmids isolated from soil belong to the IncP1 group (52). Further, Kobayashi and Bailey (32) found that none of the 79 plasmid-containing strains isolated from the phyllosphere of sugar beets reacted with the *repP* probe, while 25 hybridized to *repFIIA* or *repFIB*.

When JMP228(pBH501aE) was used as genetic sink, all transconjugants obtained harbored pBH501aE and one additional plasmid, pEMT1, which was also found in many JMP228 transconjugants. The observation that pEMT1 is not an IncP plasmid, while pEMT2 through pEMT7 are, explains why it was the only plasmid recovered when JMP228 harboring IncP1 plasmid pBH501aE was used as the genetic sink. The use of a genetic sink which harbors a plasmid has the disadvantage that it cannot acquire and maintain plasmids of the same incompatibility group. This could, however, be used to advantage to avoid capturing plasmids belonging to a specific incompatibility group. Since pEMT1 conferred growth on 2,4-D as the sole carbon and energy source in JMP228, it contains the whole complement of genes analogous to the *tfd* pathway on pJP4.

This study illustrates the use of a genetic sink as a useful method to isolate diverse plasmids encoding 2,4-D degradation from soil microbial communities. This approach can be extended to isolate and characterize plasmids which encode other catabolic functions. Such studies should enhance our knowledge about the diversity, evolution, and horizontal dissemination of catabolic genes and broad-host-range plasmids in the environment.

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