Comparison of 2,4-Dichlorophenoxyacetic Acid Degradation and Plasmid Transfer in Soil Resulting from Bioaugmentation with Two Different pJP4 Donors

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A pilot field study was conducted to assess the impact of bioaugmentation with two plasmid pJP4-bearing microorganisms: the natural host, Ralstonia eutropha JMP134, and a laboratory-generated strain amenable to donor counterselection, Escherichia coli D11. The R. eutropha strain contained chromosomal genes necessary for mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D), while the E. coli strain did not. The soil system was contaminated with 2,4-D alone or was cocontaminated with 2,4-D and Cd. Plasmid transfer to indigenous populations, plasmid persistence in soil, and degradation of 2,4-D were monitored over a 63-day period in the bioreactors. To assess the impact of contaminant reexposure, aliquots of bioreactor soil were reamended with additional 2,4-D. Both introduced donors remained culturable and transferred plasmid pJP4 to indigenous recipients, although to different extents. Isolated transconjugants were members of the Burkholderia and Ralstonia genera, suggesting multiple, if not successive, plasmid transfers. Upon a second exposure to 2,4-D, enhanced degradation was observed for all treatments, suggesting microbial adaptation to 2,4-D. Upon reexposure, degradation was most rapid for the E. coli D11-inoculated treatments. Cd did not significantly impact 2,4-D degradation or transconjugant formation. This study demonstrated that the choice of donor microorganism might be a key factor to consider for bioaugmentation efforts. In addition, the establishment of an array of stable indigenous plasmid hosts at sites with potential for reexposure or long-term contamination may be particularly useful.

Increased public awareness of environmental pollution has brought to the forefront the need for new technologies to help mitigate deterioration of environmental quality. Contaminated sites are often bioaugmented with bacteria with specific properties or capabilities, such as metal resistance or contaminant degradation. However, in many instances, introduced microorganisms do not survive well due to stresses inherent in the soil environment, including competition from indigenous microorganisms. Another approach to bioaugmentation is to inoculate the contaminated site with microorganisms carrying self-transmissible plasmids containing genes in resistance and/or degradation. These plasmids may be transferred to indigenous microorganisms that possess the characteristics necessary for growth and survival in the soil environment and, thus, establish a stable array of hosts for the plasmids.

Transfer of large catabolic plasmids from an introduced donor to indigenous microbial recipients has been evaluated previously (3–5, 7, 12, 19). Furthermore, enhanced remediation has been attributed to such transfers (4, 5, 12, 19). Use of this bioremediation approach for sites that contain both organic and metal contaminants may be of particular interest, since the presence of metals has been shown to significantly reduce, if not inhibit, organic degradation (15, 17, 20).

In the United States alone, approximately 37% of sites contaminated with an organic substance also contain inorganic contaminants such as heavy metals (W. Kovalich, Abstr. 4th World Congr. Chem. Eng., p. 281–295, 1991). Bioaugmentation studies of cocontaminated sites have focused on the introduction of a microorganism that is both metal resistant and capable of organic degradation. Strains constructed via plate matings have been shown to be capable of degrading 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of nickel and zinc (17). Although this study was conducted in broth, it demonstrated that it is possible for organic biodegradation to occur in the presence of metal stress. Several studies have also shown that it is possible to isolate metal-resistant microbial populations from metal-contaminated soils as well as from soil with no metal contamination. It stands to reason that transfer of a catabolic plasmid to metal-resistant indigenous recipient populations may be another approach to facilitate remediation of cocontaminated sites.

Studies of gene transfer in soil have typically been conducted in microcosms or columns containing between 100 grams and a few kilograms of soil. Although these studies are useful in assessing the potential for gene transfer, their relatively small scale may not produce results representative of soil in the field. In addition, the controlled laboratory conditions do not realistically reflect environmental conditions. Thus, the scale of this study was increased to the intermediate field scale and involved incubations within greenhouses.

Plasmid pJP4 was used in this model system to assess catabolic-plasmid dissemination in contaminated soil. Two pJP4 hosts were used as inocula for field soil bioreactors: the natural host, Ralstonia eutropha JMP134, and a laboratory-generated strain amenable to donor counterselection, Escherichia coli D11. Plasmid pJP4 is 80 kb in size and contains genes whose products degrade 2,4-D to 2-chloromaleylacetic acid. Further degradation from 2-chloromaleylactic acid to succinic acid is achieved by chromosomally encoded proteins (13, 16, 18). Genes whose products result in resistance to mercuric ions and...
phenyl mercury acetate are also found within this plasmid (6). The *R. eutropha* host degraded 2,4-D, while the *E. coli* host lacked chromosomal genes necessary for complete mineralization. Furthermore, it is not known whether the genes involved in the conversion of 2,4-D to 2-chloroanisole are expressed in the *E. coli* host. Both donors have been shown to be able to transfer plasmid pJP4 to indigenous soil populations. Inoculation with *R. eutropha* was considered to be both cell bioaugmentation and gene augmentation since this organism can both utilize the degradative genes on the plasmid and transfer the plasmid. In contrast, inoculation with *E. coli* D11 was considered to be only gene augmentation since the host itself could not degrade 2,4-D but could transfer the plasmid. Numerous laboratory microcosm studies have been conducted with these donors (3, 5, 11, 12; D. T. Newby and I. L. Pepper, unpublished data). The foci of these studies included plasmid transfer to indigenous populations, plasmid and donor transport, and 2,4-D degradation.

There were two main objectives for this bioaugmentation study. The first objective was to evaluate plasmid pJP4 transfer from two introduced pJP4 donors to indigenous microbes in soil contaminated with 2,4-D alone and in soils cocontaminated with 2,4-D and Cd. Associated subobjectives included comparisons of transconjugant generation and identity and of plasmid persistence occurring with the different soil treatments. A key difference between these pJP4 donors was their ability (*R. eutropha*) or inability (*E. coli*) to mineralize 2,4-D. Thus, the second main objective was to assess the effects of cell bioaugmentation in conjunction with gene augmentation (*R. eutropha* donor) or of gene augmentation alone (*E. coli* donor) of the soil microbial gene pool on degradation of 2,4-D.

### MATERIALS AND METHODS

#### Soil

A surface soil not previously exposed to 2,4-D and characterized as a sandy loam with a pH of 6.5 was collected from Madera Canyon Recreational Area of the Coronado National Forest near Tucson, Ariz. Soil was sieved (5-mm pore size) and stored at the field site for 6 days prior to contamination and inoculation of individual bioreactors. The moisture content during the storage period was the same as that at the time of collection, 3% gravimetric (8% water-holding capacity).

#### Field bioreactors

Each 20-liter polypropylene bioreactor (46 cm by 76 cm) contained 12.5 kg (dry weight) of Madera Canyon soil. A description of bioreactor treatments, all conducted in triplicate, is shown in Table 1. Inoculants and amendments were applied to soil aliquots while soil was being mixed in a cement mixer. To avoid cross-contamination, treatments were done according to the following protocol: treatment 1, treatment 4, mixer rinsed with tap water, treatment 2, treatment 5, mixer rinsed with 10% bleach and rinsed with tap water; treatment 3, and treatment 6. Appropriate volumes of 1% stock solutions of CaCl2 and 2,4-D were added to bioreactors to achieve the appropriate contaminant level(s) for each treatment. The 2,4-D stock solution was added to controls to compensate for nutrient and Hg supplements added with the inoculum to soil aliquots. Tap water was used to achieve comparable initial moisture contents among all treatment groups and to maintain the moisture level at approximately 25% gravimetric moisture content (64% water-holding capacity) throughout the course of the study.

<table>
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<th>Treatment group</th>
<th>2,4-D (500 µg of dry soil)</th>
<th>Cadmium (100 µg of dry soil)</th>
<th><em>E. coli</em> D11 (106 CFU of dry soil)</th>
<th><em>R. eutropha</em> JMP313 (106 CFU of dry soil)</th>
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The bioreactors were incubated in two greenhouses located at the University of Arizona Campbell Avenue Agricultural Station, Tucson, Ariz. Inoculated bioreactors were incubated in the greenhouse, while noninoculated controls were incubated in another. Bioreactors inoculated with different donors were spatially separated within the greenhouse. Greenhouses were used to separate controls from treatments and to try to equalize the thermal conditions for all bioreactors. Temperatures within the greenhouses ranged from 3 to 47°C, however, those in the greenhouse near the 3°C temperature extremes. Based on ambient temperatures, the greenhouse temperatures ranged from 10 to 20°C the majority of the time.

Two soil cores (45.7 cm by 2.5 cm) were collected weekly from each bioreactor. To eliminate cross-contamination, the soil core was disinfected with 10% bleach and rinsed with tap water between samplings. Soil from the cores was homogenized and analyzed to determine 2,4-D levels and the number of cultivable donor and transconjugant cells. Microorganisms and 2,4-D were extracted from the soil by the addition of 6 g of moist soil (5 g of dry soil) to a 47.5-mL extraction solution blank (6 µM Zwittergent detergent and 0.2% sodium dodecyl sulfate [2]) followed by shaking for 5 min at speed 100 on a Multi-wrist shaker (Lab-Line Instruments, Inc., Melrose Park, Ill.).

**Microcosms.** On day 49, moist soil (100 g dry weight) from each bioreactor was plated on 2-broth agar (Difco Laboratories, Detroit, Mich.) medium amended with 25 ppm of Hg (added as HgCl2) and on 2,4-D indicator plates, allowing for the selection of *E. coli* D11 and transconjugant isolates, respectively. The 2,4-D indicator plates contained 112 mg of MgSO4·7H2O, 500 mg of Na2HPO4, 340 mg of KH2PO4, 14 mg of CaCl2·2H2O, 0.22 mg of FeCl3·6H2O, 500 mg of NH4Cl, 500 mg of 2,4-D, 80 mg of cobin, 13 mg of methylene blue, and 20 g of purified agar per liter of distilled water. Cells that mineralized the 2,4-D in this medium formed dark purple colonies on these plates due to the concomitant pH change. *E. coli* D11 formed dark-purple colonies with a metallic green sheen on the Hg-Endo agar.

Isolates capable of 2,4-D degradation (*R. eutropha* donor and presumptive transconjugants) were selected from the 2,4-D indicator plates with the highest soil dilution streaked for isolation on PH plates and 0.5 g of yeast extract, 11.1 g of CaCl2, 25 mg of Hg [added as HgCl2], and 15 g of agar liter of distilled water [2], and then used as an inoculum for PH broth (same composition as PH agar, except without agar and with only 5 mg of Hg, added as HgCl2). The presence of plasmid pJP4 within these isolates was determined by PCR analysis in the presence of mercury. Overnight PH broth cultures of each isolate were centrifuged at 5,220 × g for 5 min, and the pellets were resuspended in saline. Aliquots (500 µl) of each suspension were added to 3-mL volumes of 2,4-D indicator broth. This broth contained (per liter of distilled water) 12 mg of MgSO4·7H2O, 5 mg of ZnSO4·7H2O, 2.5 mg of Na2MoO4·2H2O, 340 mg of KH2PO4, 305 mg of Na2HPO4, 14 mg of CaCl2·2H2O, 22 mg of FeCl3·6H2O, 500 mg of NH4Cl, 500 mg of 2,4-D, and 0.004% bromthymol blue, at pH 7.0. In addition, 100 µl of each cell suspension was lysed via boiling at 98°C for 10 min prior to use as a template for two PCR-based analyses. Another 500-µl portion was stored at 4°C for plasmid analysis. Enterobacterial repetitive intergenic consensus (ERIC) PCR was performed on each sample as described by Versalovic et al. (21) to generate a molecular fingerprint of each isolate. Primers ERICIR and ERIC2 were used. The presence of the pJP4 plasmid-borne ftdf gene was confirmed in isolates with unique ERIC fingerprints by PCR amplification of a 205-bp portion of this gene (11). A modified miniscreen for large plasmids was used to assess the presence of an 80-kb plasmid within these isolates (14). Transconjugants were identified via amplification of the 16S rRNA gene (rDNA) within each isolate (J. McQuaid, personal communication) followed by DNA sequencing of the resulting amplification products. PCR products were first purified by using a QIAquick PCR purification kit (Qiagen). The forward primer was subsequently used for dye termination PCR sequencing, which was performed at the University of Arizona’s Laboratory of Molecular Systematics and Evolution sequencing facility. Sequence analysis was performed with advanced BLAST 2.0 program (1) found on the National Center for Biotechnology Information’s World Wide Web site (http://www.ncbi.nlm.nih.gov). Product and plasmid DNA was visualized by using an AlphaImager 2000 gel imager (Alpha Innotech Corp., San Leandro, Calif.) following gel electrophoresis and ethidium bromide staining.

**ERICIR and ERIC2** (Difo, Detroit, Mich.) primers were used for the enumeration of heterotrophic microorganisms extracted from soil as described above. Heterotrophic populations within bioreactors were monitored on
days 0, 21, 42, and 63, and those within microcosms were monitored on day 14 (day 68 overall). Plates were incubated at 28°C for 6 days.

Quantitative of 2,4-D biodegradation. The concentration of 2,4-D was monitored through the use of a Waters Associates LC Module 1 high-performance liquid chromatography (HPLC) system, with the wavelength set at 235 nm, and a Waters C18 column (3.9 mm by 150 mm). Elution was isocratic, and the mobile phase was acetonitrile-acidified water (50:50, vol/vol) with a flow rate of 1 ml min⁻¹. Waters Millennium²¹ version 3.05 software was utilized for peak integrations. Phosphoric acid was used to acidify the water to pH 2.6. For analysis of 2,4-D within soil, a 1.0-ml aliquot of the vortexed soil-extraction solution was placed in a 1.2-ml microcentrifuge tube and centrifuged at 16,000 × g for 10 min. Any necessary dilutions were made with extracting solution. All samples were filtered through 0.45-μm-pore-size polypropylene filters prior to HPLC analysis. Several Madera Canyon soil samples that were unoinoculated and unamended with Cd and/or 2,4-D (blanks) were analyzed to evaluate natural soil components that absorbed at this wavelength.

RESULTS

Bacterial isolation and differentiation. E. coli D11 donor cells formed dark-purple colonies with a metallic green sheen within 2 days on Endo medium amended with 25 ppm of Hg.ERIC PCR and tfdB PCR confirmed that isolates were indeed E. coli D11 cells. Presumptive transconjugants capable of mineralizing 2,4-D formed dark-purple colonies, often with a metallic sheen, within 5 days of incubation on 2,4-D indicator plates. The R. eutropha JMP134 donor formed dark-purple colonies (no sheen) on this medium, and they were morphologically distinct from presumptive transconjugants. E. coli D11 cells did not grow on the 2,4-D indicator plates. Presumptive transconjugants were randomly selected from the highest-dilution 2,4-D indicator plates for confirmation of plasmid presence and isolate identification. The use of the highest-dilution plates ensured that dominant transconjugant populations were selected and reduced the likelihood of plate matings. All 2,4-D-degrading isolates that produced unique ERIC fingerprints, contained a plasmid similar in size to pJP4, hadings. All 2,4-D-degrading isolates that produced unique ERIC 

(i) Control. No presumptive transconjugant or pJP4 donor cells were detected for any control treatments. Complete bio-

degradation of 2,4-D within control bioreactors occurred within 49 days when the treatment lacked Cd and within 63 days for soil also containing Cd. In microcosms, degradation was complete within 14 days for all treatment groups.

(ii) E. coli D11-inoculated treatment groups (pJP4 gene augmentation). Plasmid pJP4 donor cells and presumptive transconjugants were culturable throughout the entire study from bioreactor soil inoculated with E. coli D11, both in the absence and in the presence of Cd (Fig. 1B and E, respecti-

ly). With both treatments, the number of E. coli D11 donor cells decreased gradually from the initial inoculum, 10⁶ CFU g of dry soil⁻¹, by approximately 2 orders of magnitude during the course of the study. Presumptive transconjugant colonies isolated on the 2,4-D indicator plates were morphologically diverse. These colonies were all considered presumptive transconjugants since the E. coli donor was unable to grow on that medium. Presumptive transconjugant populations initially consisted of 10⁵ CFU g of dry soil⁻¹ and increased in number by approximately 1 order of magnitude over the next 2 weeks. By day 21, approximately 7 × 10⁶ CFU of presumptive transconjugants g of dry soil⁻¹ were observed. Only a slight decrease in this number was observed over the next 6 weeks. Degradation of 2,4-D within bioreactors inoculated with E. coli D11 occurred at rates similar to that of controls. Degradation of 2,4-D was complete 49 (no Cd) to 56 (Cd) days after inocu-

Reamendment of soil with 500 μg of 2,4-D g of dry soil⁻¹ impacted microbial populations as shown in Fig. 2B and E. Within microcosms, the number of E. coli D11 cells decreased to only 10⁵ CFU g of dry soil⁻¹ 2 weeks after soil was spiked with 2,4-D. In contrast, presumptive transconjugant populations increased in number, reaching a maximum observed level of 4 × 10⁷ CFU g of dry soil⁻¹ 3 days after the 2,4-D spike. The morphological diversity of presumptive transconjugant colonies remained high. In the microcosms, E. coli D11-inoculated soil demonstrated the highest rate of 2,4-D degradation of all the treatment groups, with approximately 90% of the 2,4-D degraded within 3 days and complete degradation achieved within 7 days (Fig. 3).

(iii) Ralstonia-inoculated treatment groups (pJP4 cell bio-

augmentation and gene augmentation). The R. eutropha JMP134 inoculum was added to soil at approximately 5 × 10⁵ CFU g of dry soil⁻¹. Fig. 1C and F show that the number of 2,4-D degraders increased from this value to 10⁷ CFU g of dry soil⁻¹. The 2,4-D-degrading populations remained at this level throughout the remainder of the study. Nearly all colonies isolated on the 2,4-D indicator plates had the same morphology as the R. eutropha pJP4 donor. No E. coli D11 donor cells were detected in soil subjected to these treatments. Cell bio-

augmentation with R. eutropha JMP134 (Fig. 1C and F) re-

sulted in significantly increased rates of 2,4-D degradation compared to those in the corresponding control and E. coli D11-augmented bioreactors (Fig. 3). Approximately 98% of the 2,4-D was degraded within 21 days, with no 2,4-D detected by day 28.
The number of 2,4-D degraders did not change significantly when microcosms were spiked with 500 μg of 2,4-D g of dry soil\(^{-1}\) (Fig. 2C and F). However, 1 week after the spike, 2,4-D-degrading populations on the 2,4-D indicator plates were morphologically diverse. This suggested the presence of presumptive transconjugants. Degradation of 2,4-D was rapid within these microcosms. Approximately 50% of the added 2,4-D was degraded within 3 days, and it was completely degraded within 7 days (Fig. 3).

**Characterization of 2,4-D-degrading populations.** Approximately 14 colonies selected randomly from 2,4-D indicator plates for each treatment group and from each sampling time...
were further characterized. Growth of these isolates on PH plates confirmed their Hg resistance. Molecular fingerprints of each isolate were generated via ERIC PCR. Fingerprint analysis of these isolates confirmed that none was *E. coli* D11. The distribution of ERIC fingerprints over time was used to semi-quantitatively assess transconjugant diversity within bioreactors (Fig. 4) and within microcosms (Fig. 5).

In all, 34 different ERIC fingerprints of presumptive transconjugants from *E. coli* D11-treated soil were observed. Isolates from bioreactor soil generated 30 of these fingerprints. Dominant recipient populations in the 2,4-D (Fig. 4A) and the 2,4-D- and Cd (Fig. 4B)-amended soils were similar. Isolates with ERIC fingerprints A and A2 predominated in both treatments. Other prevalent isolates generated ERIC fingerprints D, H, J, K, W, Z, and 2. Isolates with ERIC fingerprint K were detected more frequently in the presence of Cd than in its absence. Fewer transconjugant fingerprints were observed within 2,4-D-spiked microcosms (Fig. 5A and B) than were seen within the corresponding bioreactors. Although only 15 ERIC fingerprints were observed, 4 of these fingerprints (12, 14, 17, and 18) were not detected in bioreactors. Dominant populations generated ERIC fingerprints A, A2, and J.

Analysis of *R. eutropha* JMP134-inoculated bioreactors (Fig. 4C and D) revealed that >95% of the 2,4-D degraders were not transconjugants but rather were surviving donor organisms represented by ERIC fingerprint R. However, limited numbers of transconjugants were detected in both treatment groups. These transconjugants generated ERIC fingerprints A, G, S, W, 2, and 4. Upon addition of 2,4-D to soil removed from these bioreactors and placed in microcosms, the proportion of transconjugants increased significantly (Fig. 5C and D). Depending on the sampling day and treatment, transconjugants comprised between 27 to 73% of the population. Ten ERIC fingerprints were generated by these transconjugants, with ERIC fingerprints A and A2 detected most frequently. Only ERIC fingerprint G was novel compared to those found in *E. coli* D11-inoculated treatments.

All transconjugants analyzed were identified, via sequencing of 16S rDNA, as belonging to either the *Burkholderia* or *Ralstonia* genus. *Burkholderia graminis* was the most prevalent microorganism. The identities of transconjugants isolated for this diversity analysis are shown in Table 2.

**FIG. 2.** Assessment of levels of presumptive transconjugants, *E. coli* D11 donor cells, and heterotrophic cells and 2,4-D concentrations in Madera Canyon soil microcosms. (A) Treatment 1 (2,4-D); (B) treatment 2 (2,4-D and *E. coli*); (C) treatment 3 (2,4-D and *R. eutropha*); (D) treatment 4 (2,4-D and Cd); (E) treatment 5 (2,4-D, Cd, and *E. coli*); (F) treatment 6 (2,4-D, Cd, and *R. eutropha*). The data points are the means of three replicate microcosms; error bars indicate standard deviations. Microcosm days 0, 3, 7, and 14 correspond to bioreactor days 51, 54, 58, and 65.

**DISCUSSION**

This study was conducted at the intermediate field scale level in order to approximate field conditions more closely than is possible in smaller-scale laboratory experiments. The extreme fluctuation of the greenhouse temperature resulted from large diurnal temperature changes, with high temperatures being amplified by the greenhouse enclosure. Although observed fluctuations in temperature were extreme, they were more representative of field conditions than incubation at a constant temperature as is the common practice for laboratory experiments.

Assessment of the microbial populations over time (Fig. 1) provided insight into treatment-induced shifts in soil microbial ecology. The increase in the heterotrophic populations in all bioreactors on days subsequent to day 0 can be attributed to an increase in moisture, from 8 to 64% water-holding capacity, and the addition of a carbon source, 2,4-D. *E. coli* D11 donor cells declined in number in soil inoculated with this organism. In contrast, the *R. eutropha* JMP134 inoculum increased by 1 order of magnitude and remained at the elevated level throughout the study. There exist several plausible explanations for the observed difference in donor survival. First, *E. coli* D11 is not a naturally soilborne microorganism and thus may be more susceptible to environmental stresses, such as the extreme temperature fluctuations observed in the greenhouses and competition from indigenous populations. Second, it is
likely that the difference in survival is due in part to the ability of the *R. eutropha* donor to degrade 2,4-D and thus compete more effectively than *E. coli* D11 with the indigenous populations to establish a niche for itself. Finally, only pJP4-harboring *E. coli* was enumerated on the Endo medium amended with HgCl₂. Thus, it is possible that some of the decline in *E. coli* numbers was due to curing of pJP4 from *E. coli* D11. The survival of both donor organisms was significant since it demonstrated that even in the absence of gene transfer, plasmid pJP4 persisted within both donor microorganisms for extended periods of time in the soil.

This study revealed that plasmid transfer occurred from both of the introduced donors to indigenous recipients (Fig. 4 and 5). However, the level of transconjugants present at specific times differed significantly depending on the pJP4 donor. Donor counterselection facilitated the detection of plasmid transfer from *E. coli* D11 even at low levels, such as were evident at days 0, 7, and 14. In contrast, *R. eutropha* JMP134 remained culturable (10⁷ CFU g of dry soil⁻¹) in bioreactors. Low-frequency transfer events are difficult to observe when the donor survives at or above the order of magnitude of transconjugants because of the high soil dilution that must be plated in order to obtain distinct colonies. Thus, *R. eutropha* survival at this level limited the detection of transconjugants. The percentage of transconjugants detected in *R. eutropha*-inoculated bioreactors when the total population of presumptive transconjugants in all inoculated treatments was 10⁷ CFU g of dry soil⁻¹ indicated that transconjugant populations were not as prevalent as they were in bioreactors inoculated with *E. coli* D11. This may be the result of differences in the conjugation efficiencies of the two donors. Plasmid pJP4 transfer has been found to be more efficient with *E. coli* JMP397 than with the natural host, *R. eutropha* JMP134 (J. M. Pemberton, personal communication). Similarly, *E. coli* D11 may be more efficient at transferring pJP4 than is *R. eutropha*. This hypothesis was not tested in vitro due to the questionable applicability of these

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**FIG. 3.** Degradation of 2,4-D in Madera Canyon soil, presented as the percentage remaining of the initial 2,4-D added to each bioreactor or microcosm. The data points and error bars show the means and standard deviations based on data from three replicate microcosms.
results to gene transfer events in a nonsterile soil environment. In addition, the difference in the donors' abilities to derive benefit from the catabolic genes encoded on the plasmid may play a role. Since *E. coli* D11 cannot degrade the herbicide, it may have been energetically favorable for it to cure itself of the plasmid. Although conjugation is believed to be the mechanism of transfer, it is possible that transformation of released plasmids also occurs. However, transformation of an intact 80-kb plasmid is unlikely, and yet all transconjugants analyzed contained an 80-kb plasmid, suggesting that conjugation is the mechanism of transfer. In contrast, plasmid pJP4 conferred a competitive advantage to the *R. eutrophus* donor, making the maintenance of this plasmid within this microorganism favorable.

The increase in transconjugant populations in all treatments following the second addition of 500 mg of 2,4-D g of dry soil$^{-1}$ was likely the result of growth of transconjugants already present in the bioreactor soil (Fig. 2). However, the appearance of previously undetected transconjugant populations suggested that additional transfer from the pJP4 donor to indigenous populations and/or successive gene transfer events between indigenous populations occurred.

Similarly, the diversity of ERIC fingerprints indicated that presumptive transconjugants did not arise simply from a single transfer event followed by growth but that numerous gene transfer events occurred at some point(s) in time (Fig. 4 and 5). The semiquantitative analysis of transconjugant diversity showed that transconjugant populations varied slightly depending on the treatment. However, many of the dominant recipient populations were the same regardless of the pJP4 donor or the contaminants present. Recipients with ERIC fingerprints A and A2 were prevalent in all treatment groups, and those with J and Z fingerprints were prevalent in most.

Transconjugants were identified as members of one of two common soil genera, either *Burkholderia* or *Ralstonia* (Table 2). *Burkholderia* populations were predominant, with five different species being identified. In previous studies of gene transfer in Madera Canyon soil (12; Newby and Pepper, unpublished data), the majority of transconjugants were also found to belong to the genus *Burkholderia*. These recipients may be somehow predisposed to take up plasmid DNA, or they may simply be present in larger numbers in the soil and thus be more likely to come in contact with pJP4 donors. Taken together, these results suggested that the identity of potential soil recipient populations played a larger role in determining transconjugants than did the donor or the contaminant level.

It is significant that at distinct times in all inoculated soils, transconjugant numbers reached levels of approximately 10% of the culturable heterotrophic populations. In addition, absolute transconjugant numbers were probably higher than those observed. Only cultivable transconjugants were detected by plating on 2,4-D indicator medium. Viable but nonculturable transconjugants, in addition to any transconjugants lacking the chromosomally encoded genes necessary for complete 2,4-D mineralization or unable to express the plasmid-encoded genes, would have escaped detection. Although there has been little direct evidence regarding the occurrence of maleylacetate reductase genes among indigenous populations, the ubiquity and diversity of soil microbial populations capable of mineralizing 2,4-D suggest that these genes are probably relatively common (8–10).

This study demonstrated the potential for increased rates of 2,4-D degradation following bioaugmentation of bioreactors (Fig. 1) and of microcosms (Fig. 2) with pJP4-harboring microorganisms. The impact of the different treatments on degradation is most easily seen in Fig. 3, in which degradation in...
all bioreactors and microcosms is compared. Although no presumptive transconjugants were detected on the 2,4-D indicator plates for control bioreactors or microcosms, degradation of 2,4-D in these treatment groups suggested the presence of indigenous 2,4-D degraders or consortia of microorganisms capable of carrying out the degradation. In addition, growth of light-purple pinpoint colonies on 2,4-D indicator plates for controls was indicative of the presence of such populations.

The increased 2,4-D degradation rate in bioreactors inoculated with *R. eutropha* JMP134 can be explained by the fact that this bacterium has a chromosomal complement to the genes on plasmid pJP4 that enable it to mineralize 2,4-D. This catabolic ability undoubtedly contributed to the establishment of a competitive niche for the inoculant. In contrast, the *E. coli* pJP4 host does not contain this complement and, thus, cannot completely mineralize the herbicide. The inability of *E. coli* D11 to degrade 2,4-D was reflected by similar levels of 2,4-D degradation in soils inoculated with this microorganism and in controls.

Microbial populations within all treatment groups were found to degrade 2,4-D more rapidly in the microcosms spiked with 2,4-D (Fig. 2) than within corresponding bioreactors (Fig. 1). Increased degradation rates upon reexposure to a contaminant have been well documented. There are several plausible explanations for this. For instance, during initial herbicide exposure, a favorable niche for microorganisms capable of degrading 2,4-D might have been established, and thus their numbers increased. It is also possible that genes involved in degradation were turned on or mutated. In yet another scenario, plasmid pJP4 may have been transferred to indigenous populations in inoculated soils. The rapid degradation of 2,4-D in all microcosms masked any lag in degradation that may have resulted from the presence of Cd. In contrast to bioreactor results, degradation of 2,4-D was faster in soils inoculated with *E. coli* D11 than in *R. eutropha* JMP134-inoculated soils. This reversal in relative rates of 2,4-D degradation may be attributed to the presence of different populations of pJP4 hosts and, thus, 2,4-D degraders. In *E. coli* D11-inoculated bioreactors, substantial populations of transconjugants (10^6 CFU g of dry soil^-1^) persisted at the time soil was removed and respiked with 2,4-D for setup of microcosms. These indigenous pJP4 hosts were well adapted to the soil environment. At the same time, *R. eutropha* JMP134 remained culturable (10^7 CFU g of dry soil^-1^) in bioreactors inoculated with that microorganism, limiting detection of transconjugants. However, visual examination of low-dilution 2,4-D indicator plates suggested that there were about 10^3 CFU of presumptive transconjugants g of dry soil^-1^ in these treatment groups. Increased rates of 2,4-D degradation upon a second herbicide exposure probably re-

![FIG. 5. Semiquantitative analysis of presumptive transconjugant diversity in microcosms on sampling days 7 and 14. (A) Treatment 2 (2,4-D and *E. coli*); (B) treatment 5 (2,4-D, Cd, and *E. coli*); (C) treatment 3 (2,4-D and *R. eutropha*); (D) treatment 6 (2,4-D, Cd, and *R. eutropha*). ERIC fingerprints, in terms of percentages of presumptive transconjugants analyzed per sample day, are plotted against sampling day.](http://aem.asm.org/)

### TABLE 2. Identities of transconjugants as determined from DNA sequencing of a 16S rDNA fragment amplified via PCR

<table>
<thead>
<tr>
<th>Isolate identity</th>
<th>ERIC fingerprint(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burkholderia caribiensis</td>
<td>B, H, O, V, 5, 17</td>
</tr>
<tr>
<td>Burkholderia glathei</td>
<td>I, W</td>
</tr>
<tr>
<td>Burkholderia graminis</td>
<td>A, A2, C, D, J, K, N, P, Q, S, X, Z, Za, 1, 2, 3, 4, 6, 8, 11, 12, 14, 15</td>
</tr>
<tr>
<td>Burkholderia kuranei</td>
<td>16</td>
</tr>
<tr>
<td>Burkholderia phenazinium</td>
<td>M, Y, 9, 10, 13</td>
</tr>
<tr>
<td>Ralstonia eutropha</td>
<td>R, 18</td>
</tr>
<tr>
<td>Ralstonia gilardi</td>
<td>G</td>
</tr>
</tbody>
</table>

* Homology levels range from 97 to 99% and reflect the similarity between the query and database sequences.
sulted, at least in part, from the catabolic activity of these transconjugant populations. These results suggest that indigenous plasmid hosts (transconjugants) can be more effective remediators than an inoculated host, even when initially present in smaller numbers.

The minimal impact of Cd on 2,4-D degradation and on gene transfer observed in this study was not surprising. Josephson and Pepper found that complete degradation of 2,4-D in Madera Canyon soil inoculated with 10⁵ CFU of *Alcaligenes eutrophus* JMP134 g of dry soil⁻¹ was delayed only 1 week at Cd amendment levels of 60, 120, and 240 µg g of dry soil⁻¹ (unpublished data). Furthermore, sorption isotherm studies with 100 µg of Cd added g of dry Madera Canyon soil⁻¹ revealed that less than 1 ppm of the added Cd was bioavailable (K. L. Josephson, personal communication). However, 100 µg of Cd g of dry soil⁻¹ is much higher than background levels and thus can be considered significant contamination.

The results from this field study are significant from a microbial ecology standpoint for a number of reasons. This study considered significant contamination.

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REFERENCES