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Genetic and Phenotypic Diversity of 2,4-Dichlorophenoxyacetic Acid (2,4-D)-Degrading Bacteria Isolated from 2,4-D-Treated Field Soils

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Forty-seven numerically dominant 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria were isolated at different times from 1989 through 1992 from eight agricultural plots (3.6 by 9.1 m) which were either not treated with 2,4-D or treated with 2,4-D at three different concentrations. Isolates were obtained from the most dilute positive most-probable-number tubes inoculated with soil samples from the different plots on seven sampling dates over the 3-year period. The isolates were compared by using fatty acid methyl ester (FAME) profiles, chromosomal patterns obtained by PCR amplification of repetitive extragenic palindromic (REP) sequences, and hybridization patterns obtained with probes for the *tfd* genes of plasmid pJP4 and a probe (*Spa* probe) that detects a distinctly different 2,4-D-degrading isolate, *Sphingomonas paucimobilis* (formerly *Pseudomonas paucimobilis*). A total of 57% of the isolates were identified to the species level by the FAME analysis, and these isolates were strains of *Sphingomonas*, *Pseudomonas*, or *Alcaligenes* species. Hybridization analysis revealed four groups. Group I strains, which exhibited sequence homology with *tfdA*, *-B*, *-C*, and *-D* genes, were rather diverse, as determined by both the FAME analysis and the REP-PCR analysis. Group II, which exhibited homology only with the *tfdA* gene, was a small group and was probably a subset of group I. All group I and II strains had plasmids. Hybridization analysis revealed that the *tfd* genes were located on plasmids in 75% of these strains and on the chromosome or a large plasmid in the other 25% of the strains. One strain exhibited *tfdA* and *-B* hybridization associated with a plasmid band, while *tfdC* and *-D* hybridized with the chromosomal band area. The group III strains exhibited no detectable homology to *tfd* genes but hybridized to the *Spa* probe. The members of this group were tightly clustered as determined by both the FAME analysis and the REP-PCR analysis, were distinctly different from group I strains as determined by the FAME analysis, and had very few plasmids; this group contained more of the 47 isolates than any other group. The group III strains were identified as *S. paucimobilis*. The group IV strains, which hybridized to neither the *tfd* probe nor the *Spa* probe, were as diverse as the group I strains as determined by the FAME and REP-PCR analyses. Most of group IV strains could not be identified by the FAME analysis. Strains belonging to groups I and III were more frequently recovered from soils that had greater field exposure to 2,4-D, suggesting that they were the best competitors for 2,4-D under field conditions. The selection regimen which we used led to two successful but dissimilar groups; the members of one group were similar at the plasmid level but not at the organism level, and the members of the other group were similar at the organism level. Since the members of the latter group are ecologically successful and have degradative genes unlike *tfd* genes, they deserve more attention.

Large amounts of man-made chlorinated organic chemicals have been used in agriculture as herbicides and pesticides. Among these, 2,4-dichlorophenoxyacetic acid (2,4-D) has received widespread use as a herbicide for more than 40 years. Unlike many of the synthetic compounds released into the environment, 2,4-D is rapidly mineralized by soil bacteria (3, 10, 21, 30, 31). The sizes of populations of microorganisms able to degrade 2,4-D have been estimated by the most-probable-number method (4, 11, 13, 26). Organisms that have been reported to be capable of degrading 2,4-D belong to a number of genera, including *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, *Flavobacterium*, *Pseudomonas*, and *Streptomyces* (2, 6, 8, 14, 20, 25, 32). Furthermore, the 2,4-D degradation pathway in plasmid pJP4, originally isolated in an *Alcaligenes eutrophus* strain, has been extensively studied and described (6, 7, 29).

While there have been many reports of isolation and characterization of biodegrading strains, there have been few studies that have focused on understanding biodegradation at the population level. Such studies should include defining and synthesizing information about the diversity of populations with the biodegradation function, including patterns of relatedness, and understanding how these populations respond to environmental conditions. At least initially, such studies should be done by using a small, homogeneous field environment so that diversity can be defined at least at a local level. We have used 2,4-D degradation as a model for biodegradation function because of the background information available on biodegradability, isolated strains, and genes and because this model can be used with small field plots. Much of the previous work on characterizing 2,4-D-degrading isolates was done in the 1950s and 1960s by using morphological and cultural charac-

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TABLE 1. 2,4-D-degrading bacteria and their sources

Plot(s)	Isolate(s)	Rate of 2,4-D application[a]	Date of soil sample collection[b]
1	2811P	Control	August 1989
4	KI1, 1443	High	
1 and 8	512, 583	Control	May 1990
2 and 7	524, 573	Low	
3 and 6	535, 565	Intermediate	
4 and 5	546, 556	High	
1 and 8	712, 782	Control	July 1990
2 and 7	723, 773	Low	
3 and 6	736, 765	Intermediate	
4 and 5	745, 756	High	
1 and 8	912, 983	Control	September 1990
2 and 7	924, 974	Low	
3 and 6	936, 965	Intermediate	
4 and 5	947, 957	High	
2 and 7	1124, 1173	Low	November 1990
3 and 6	1136, 1165	Intermediate	
4 and 5	1146, 1156	High	
1 and 8	9112, 9182	Control	September 1991
7	9174	Low	
3 and 6	9136, 9166	Intermediate	
4 and 5	91461, 91462, 9157	High	
1	9212	Control	May 1992
2	9224	Low	
3 and 6	9236, 9266	Intermediate	
4 and 5	9247, 9256	High	

[a] Control, no 2,4-D treatment; low, intermediate, and high, 2,4-D was applied at rates of 1, 10, and 100 μg/g of soil, respectively (0.6, 6, and 60 kg/ha, respectively) at the following times: one application in October 1988 and in May, August, October, and December 1989; two applications in May, July, September, and November 1990 and in May, July, September, and November 1991; and three applications in May 1992.

[b] Soil samples for strain isolation were taken 1 week following the previous 2,4-D application.

teristics, and the strains were isolated from heterogeneous environments. Inconsistencies in strain identification and the heterogeneity of the strain sources in previous studies make it difficult to even suggest conclusions concerning patterns of diversity and ecological selection.

In this work we studied the diversity and selection of 2,4-D-degrading bacteria by isolating and characterizing numerically dominant 2,4-D degraders from small agricultural plots over a 3-year period of 2,4-D treatment. We used several genotypic, chemical, and physiological methods to cluster the isolates and evaluate patterns of similarity in response to selection. The results were compared to analyze relationships between the different grouping methods.

MATERIALS AND METHODS

Media and culture conditions. All isolates were maintained on MMO mineral medium (28) containing 2,4-D at a concentration of 500 ppm (500 μg/ml). Peptone-tryptone-yeast extract-glucose medium containing (per liter) 0.25 g of peptone (Difco Laboratories, Detroit, Mich.), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride was used for strain purification and colony production for the repetitive extragenic palindromic PCR (REP-PCR).

Isolation of bacterial strains. Agricultural soil samples were taken from duplicate plots that had not been treated with 2,4-D or had been regularly treated with 2,4-D at different rates (1, 10, and 100 μg/g of soil) from 1988 to 1992 (Table 1); the plots used were the Gene Flow plots at the Long-Term Ecological Research site at the Kellogg Biological Station in Hickory Corners, Mich. Each of the eight subplots was 3.6 by 9.1 m (total sample area, 267 m^2). Samples from the top 15 cm of soil were taken from five locations chosen randomly, sifted through a 2-mm-pore-size sieve, and kept overnight at 4°C prior to use. A 10-g soil sample from each plot was homogenized with 95 ml of a sterilized 0.85% saline solution by shaking the preparation on a rotary shaker at 200 rpm for 20 min. Samples (0.1 ml) of appropriate 10-fold dilutions were inoculated into most-probable-number tubes containing 3 ml of 2,4-D mineral medium (MMO mineral medium containing 500 ppm of 2,4-D). The tubes were incubated at 30°C for 3 weeks, and degradation of 2,4-D was analyzed by high-performance liquid chromatography (16). The culture(s) in the tube(s) containing the highest dilution that exhibited 2,4-D degradation was enriched by two additional transfers into fresh medium. Each enriched culture was streaked onto 2,4-D agar medium (MMO mineral medium containing 500 ppm of 2,4-D, 0.1% Casamino Acids, and 1.5% agar) and incubated at 30°C for 2 to 7 days. Single colonies were then tested for 2,4-D degradation in fresh 2,4-D mineral medium before being checked for purity by streaking onto peptone-tryptone-yeast extract-glucose medium plates. Some cultures failed to produce single colony types able to degrade 2,4-D and were not studied further. Altogether, 47 strains were isolated from the eight plots. These isolates were preserved by freezing them at −70°C in sterile 15% glycerol.

Chemicals. Analytical grade 2,4-D, phenoxyacetic acid, and 2-methyl-4-chlorophenoxyacetic acid were obtained from Sigma Chemical Co., St. Louis, Mo., and 2-chlorophenoxyacetic acid, 4-chlorophenoxyacetic acid, 3-chlorobenzoic acid, and 4-chlorobenzoic acid were obtained from Aldrich Chemical Co., Milwaukee, Wis.

FAME analysis. The isolates were cultured on tryptic soy agar medium (19) at 28°C for 48 to 72 h, and then cells were harvested from the plates by scraping with a sterile glass loop and used for fatty acid methyl ester (FAME) analysis. Saponification, methylation, and extraction were performed by using the procedure described in the MIDI manual (Microbial Identification, Inc.) (27). A cluster analysis was performed by using an in-house cluster program and the MIDI software. Species are identified below if the analysis of three replicates gave a similarity index of ≥0.6.

Colony REP-PCR. The colony REP-PCR was performed as described by de Bruijn (5). Each isolate was grown on a peptone-tryptone-yeast extract-glucose medium plate for 24 to 48 h, and then a small amount of cells was resuspended in 25 μl of PCR mixture (5). After PCR amplification, 8-μl samples of the REP-PCR products were separated by electrophoresis on horizontal 1% agarose gels. All isolates were analyzed at the same time with the same batch of primers.

Genetic diversity analysis. Individual isolates were cultured in 2,4-D broth medium, harvested, and lysed as described by Kado and Liu (18), with some modifications (17). The cells were resuspended in 30 μl of distilled water and lysed by adding 120 μl of lysing solution. The solution was incubated at room temperature for 15 min, heated at 80°C for 1 min, extracted with 1 volume of a phenol-chloroform solution (1:1, vol/vol), and incubated overnight at room temperature. The DNA sample (100 μl) obtained from each cell lysate was subjected to gel electrophoresis to detect and separate plasmid DNA (if present) from linear fragments of chromosomal DNA. Following electrophoresis the DNA was transferred to nitrocellulose hybridization membranes and hybridized with ^{32}P-labelled DNA probes. The *tfdA*, *tfdC*, and *tfdD* gene

probes were subcloned as 587-bp (*Stu*I-*Sma*I), 775-bp (*Sma*I-*Cla*I), and 100-bp (*Hin*dIII-*Xho*I) fragments, respectively, from pJP4. The *tfdB* gene probe has been described elsewhere (13). The *tfd* probes did not hybridize to one of the strains isolated in 1989, strain 1443, so we developed a probe (the *Spa* probe) for this strain by cloning a 6.5-kb *Bam*HI fragment from a large plasmid of strain 1443 into the *Bam*HI site of plasmid pUC19 (22). This fragment was selected because it cross-hybridized under low-stringency conditions to a 2,4-D-degradative plasmid from *Pseudomonas pickettii* 712 that hybridized to the *tfdA* probe but not to the *tfdB*, *tfdC*, and *tfdD* probes. The rationale for this was that the non-*tfdA* 2,4-D genes from p712 might be linked and might have some similarity to the degradative genes of strain 1443. We do not know yet what genes are encoded by the *Spa* fragment.

The *tfd* gene probes were labelled with ^{32}P by using a random primed DNA labelling kit (Boehringer Mannheim, Indianapolis, Ind.), and the *Spa* probe was labelled by using a nick translation kit (Boehringer Mannheim). Prehybridization, hybridization, and posthybridization washes were performed as described by Holben et al. (12), with some modifications. The membranes were prehybridized for at least 24 h at 42°C, and after hybridization, three washes were carried out at room temperature; this was followed by one wash at 65°C for 45 min. This protocol corresponds to moderately high to high stringency (e.g., ≥90% sequence homology). Hybridization signals were detected by autoradiography by using X-Omat AR film (Kodak, Rochester, N.Y.) exposed at −70°C with a Quanta III intensifying screen (Sigma). When necessary, bound probe was stripped from the membranes prior to rehybridization by washing them three times for 15 min with a 0.1% (wt/vol) sodium dodecyl sulfate solution heated to 100°C. Individual isolates were grouped on the basis of their hybridization patterns with these probes.

Degradation phenotype analysis. Representative strains selected from isolates identified as members of the same species by the FAME analysis were cultured in 2,4-D mineral medium. The same medium containing 250 ppm of sodium acetate instead of 2,4-D was used to produce cells not induced to metabolize 2,4-D. Cultures were grown at 30°C and aerated by shaking at 200 rpm in an incubator shaker. Cells in the late log phase were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C, washed twice with an equal volume of 15 mM phosphate buffer (pH 7.0), and resuspended in the same buffer. Aliquots of suspended cells were inoculated into culture tubes, each of which contained MMO mineral medium supplemented with one of the seven structural analogs at a concentration of 250 ppm. The cultures were shaken at 150 rpm at 30°C for 2 weeks, after which the optical density at 550 nm was determined. To determine the degradation of phenoxyacetates, the cultures were centrifuged to remove the cellular material, and the UV absorption was measured.

RESULTS

FAME and taxonomic analysis. The results of the FAME analysis grouped the 47 isolates into 12 groups of strains at Euclidian distances of less than 10 (Fig. 1); it has been suggested that this distance approximates the species level (27). All of the isolates clustered at a Euclidian distance of 48.8. One dominant FAME group (group a) contained 38% of the isolates (18 strains); the overall distribution among the groups was log normal (Fig. 2), a distribution expected for natural microbial populations. The isolates that could be reasonably identified by the FAME results are shown in Table 2.

The FAME analysis results indicated that the group a isolates were either *Pseudomonas paucimobilis* or *Pseudomonas saccharophila* strains. We used the distinctive traits of these two species to establish the identities of the strains. The isolates were determined to be *P. paucimobilis* strains on the basis of the following characteristics: rarely motile in semisolid nutrient medium, yellow pigment produced on nutrient agar, no hydrolysis of starch, and no autotrophic growth with hydrogen. The strains were also gram negative and did not produce fluorescent pigments. Recently, this species has been reclassified as *Sphingomonas paucimobilis* by Yabuuchi et al. (33).

Colony REP-PCR analysis. A colony REP-PCR experiment was performed to distinguish among strains belonging to the same or closely related FAME groups (Fig. 3). The REP-PCR analysis of the isolates revealed that the 47 isolates produced 30 different DNA fingerprint patterns. Identical PCR patterns were observed for strains 1443 and 556, for strains 765, 1124, and 1136, for nine isolates (strains 936, 947, 957, 1146, 1165, 91462, 9174, 9247, and 9256) classified as *S. paucimobilis*, and for strains 1173 and 9157, which were identified as *A. eutrophus*. Among the unidentified isolates, strains 583 and 773 produced identical REP-PCR patterns, strains 9136, 91461, and 9166 produced identical patterns, and strains 9236 and 9266 produced identical patterns. Identical patterns indicate that the strains are very closely related or siblings. Since all of the strains were isolated either at different times or from different plots, their detection frequencies reflect the extents of their dominance in the plots examined.

Only those isolates that were identified by the FAME analysis as *S. paucimobilis* had some bands that were shared (Fig. 3A, lanes 2 through 6 and 8 through 16; Fig. 3B, lanes 18 and 19; and Fig. 3C, lanes 40, 44, 45, and 47). All of the other isolates had no common bands. This observation correlated well with FAME data which grouped the isolates of *S. paucimobilis* together into one FAME group at a Euclidian distance of 6.4. The isolates of *Pseudomonas* and *Alcaligenes* species and the isolates belonging to the unidentified group produced distinct patterns and clustered into several more diverse FAME groups.

Diversity of 2,4-D genes. After gel separation and Southern transfer, chromosomal DNAs and plasmid DNAs (if present) were hybridized to the *tfd* gene and *Spa* probes (Fig. 4). On the basis of their hybridization patterns under high-stringency conditions, the 47 isolates were placed into four groups (Table 3). Hybridization group I included 12 isolates that exhibited sequence homology with all four *tfd* gene probes used; group II contained three isolates that exhibited homology only with the *tfdA* gene probe; group III contained 18 isolates that exhibited homology only with the *Spa* probe; and group IV (all other isolates) contained 14 isolates which did not hybridize with any of the probes used.

All of the isolates in hybridization group I produced clear plasmid bands in agarose gels, and most (75%) of the plasmids detected hybridized with the *tfd* gene probes (Fig. 4A, lane 5, and Fig. 4B through D, lanes 9 and 10). Although plasmids with molecular masses ranging from 2.6 to 350 MDa were reported to be easily detected by the method of Kado and Liu (18), shearing of large plasmids may have occurred during the procedure, which could have accounted for the minor hybridization signals observed with the chromosomal and linear DNA bands in addition to the main signals for plasmid DNA. In some isolates (e.g., 9136, 91461, and 9166) plasmid DNA was clearly detected on the gel, but this DNA did not hybridize to any of the *tfd* gene probes; instead, the chromosomal DNA produced hybridization signals (Fig. 4A, lanes 2, 3, and 6).

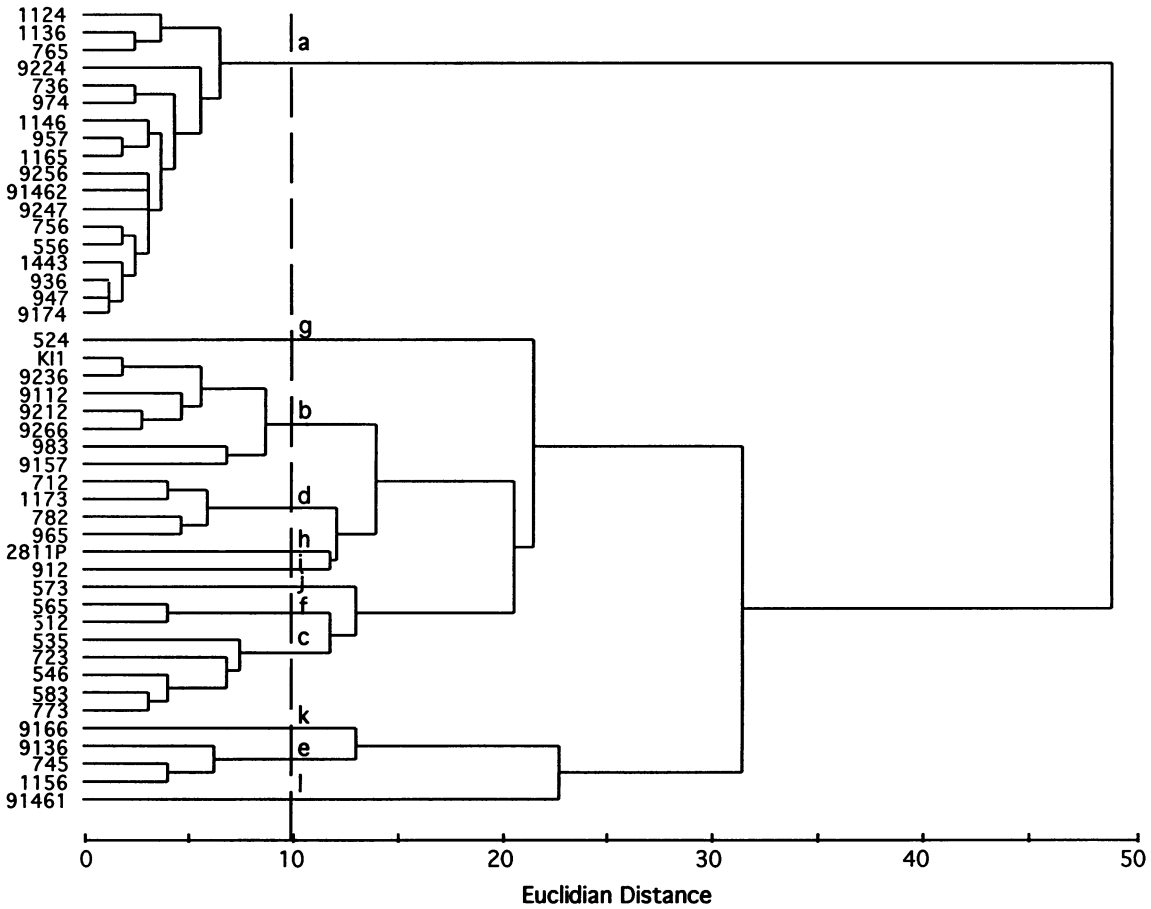

FIG. 1. Dendrogram based on the fatty acid profiles of the isolates. FAME groups a through k were defined at a Euclidian distance of less than 10.

Another interesting strain in group I is strain 9112 (Fig. 4B through D, lanes 14). The plasmid DNA of this strain hybridized to the *tfdA* and *tfdB* gene probes, but the major hybridization signal to the *tfdC* and *tfdD* (data not shown) probes was in the chromosomal DNA band area. It is likely that the genes corresponding to the *tfdA* and *tfdB* probes are located on one plasmid, while the genes corresponding to the *tfdC* and *tfdD* probes are located either on the chromosome or on another large sheared plasmid. The 12 isolates belonging to group I clustered at a Euclidian distance of 31.5 in the FAME analysis, and many of their REP-PCR patterns (Fig. 3A, lane 7, and Fig. 3B, lanes 20, 23, 25, and 28) were quite different from each other, suggesting that these organisms are not closely related.

The isolates in group II accounted for only 6.4% of the total number of isolates, indicating that they were not dominant types in the plots examined. The hybridization patterns of these organisms were more unusual than the hybridization pattern of group I isolate 9112 (see above) in that they did not exhibit sequence homology with the *tfdB*, *tfdC*, and *tfdD* gene probes. These isolates were found to have transferable plasmids encoding 2,4-D-degradative genes (17). Although isolates 712 and 782 were identified as members of the same species, *P. pickettii*, by FAME analysis (Table 2) and were observed to have the same plasmid (data not shown), their REP-PCR patterns were distinct from each other (Fig. 3B, lanes 22 and 24).

The isolates in group III did not hybridize with any of the *tfd*

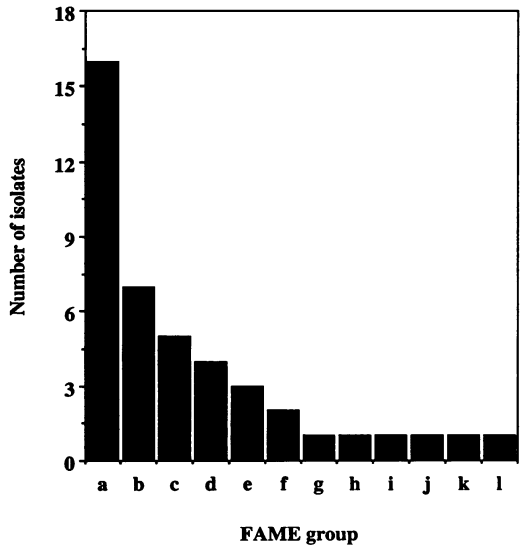

FIG. 2. Frequency distribution of isolates among the FAME groups.

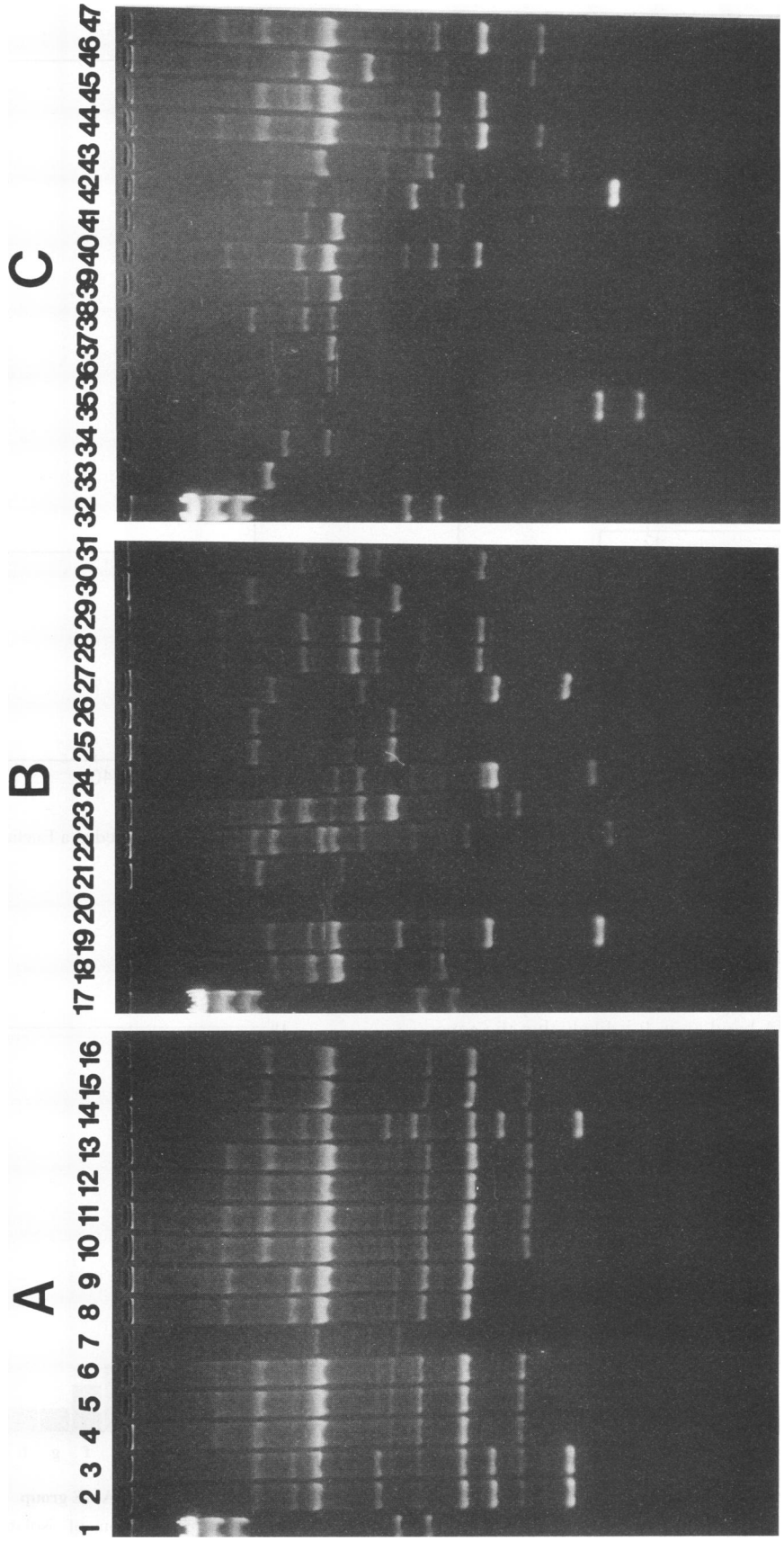
A
B
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47

TABLE 2. Identification of isolates to the species level by FAME analysis

Isolate(s)	FAME group(s)	Identity as determined by FAME analysis
1443, 556, 736, 756, 765, 936, 947, 957, 974, 1124, 1136, 1146, 1165, 91462, 9174, 9224, 9247, 9256	a	*Sphingomonas paucimobilis*
712, 782, 983, 9112	b, d	*Pseudomonas pickettii*
965	d	*Pseudomonas solanacearum*
1173, 9157	b, d	*Alcaligenes eutrophus*
912	i	*Alcaligenes faecalis*
2811P	h	*Alcaligenes paradoxus*
KI1, 512, 524, 535, 546, 565, 573, 583, 723, 745, 773, 924, 1156, 9136, 91461, 9166, 9182, 9212, 9236, 9266	b, c, e, f, g, k, l	Unidentifiable[a]

[a] The isolates could not be identified because of poor matches with profiles in the MIDI library (18 of the 20 strains) or because they did not grow on laboratory medium (isolates 924 and 9182).

genes but hybridized with the *Spa* probe (Fig. 4A and E, lanes 1, 4, and 7), suggesting that the degradative genes of the strains belonging to this group are different from those of group I and II strains. The *Spa* probe was found to be specific for *S. paucimobilis* types and did not exhibit any detectable homology with the plasmid of strain 712 or the *tfd* genes of plasmid pJP4 under high-stringency conditions. Although a plasmid DNA band was not detected in most (83.3%) of the group III isolates by the method of Kado and Liu, these strains may have large plasmids which behave like chromosomal DNA since one isolate (strain 1443) was observed to have a large plasmid (data not shown) when an alternative direct lysis method was used (24). Hence, the hybridization signals detected in the chromosomal areas of gels (Fig. 4A, lanes 1, 4, and 7) may have represented either chromosomal or large-plasmid DNA which had been sheared. The group III isolates clustered at a Euclidian distance of 6.4 in the FAME analysis and had many common bands in their REP-PCR patterns (Fig. 3A, lanes 2 through 6 and 8 through 16; Fig. 3B, lanes 18 and 19; and Fig. 3C, lanes 40, 44, 45, and 47), suggesting that these organisms are closely related. The FAME analysis identified these organisms as *S. paucimobilis*, and the REP-PCR analysis revealed six different patterns produced by them.

The 14 group IV isolates (all other isolates) did not exhibit sequence homology with the DNA probes used (Fig. 4B through E, lanes 11 through 13) and clustered at a Euclidian distance of 31.5 in the FAME analysis (Fig. 1). Only two isolates, 912 and 983, could be identified by the FAME method (*Alcaligenes faecalis* and *P. pickettii*, respectively). These isolates appeared not to be closely related to each other, as indicated by their distinctive REP-PCR patterns (Fig. 3C, lanes 33 through 39, 41 through 43, and 46).

Degradative diversity analysis. Representative strains belonging to the groups identified as members of the same species by the FAME analysis were grown on medium containing 2,4-D or acetate as the sole carbon source and then examined for their ability to degrade other compounds related to 2,4-D (Table 4). The hybridization group I isolates used were more versatile in substrate use than the isolates belonging to the other groups were. Isolate 965, identified as *Pseudomonas solanacearum*, was the most versatile strain among the isolates tested. Whether it was grown on medium containing 2,4-D or acetate, this organism vigorously utilized 2,4-D-related compounds, including phenoxyacetic acid, 4-chlorophenoxyacetic acid, 3-chlorobenzoic acid, and 2-methyl-4-chlorophenoxyacetic acid, as sole carbon sources, as indicated by complete disappearance of the substrates and by substantial cell growth (Table 4). Hybridization group I isolates 1173 and 745 could degrade 2-methyl-4-chlorophenoxyacetic acid and 3-chlorobenzoic acid, respectively. On the other hand, the test isolates belonging to groups II through IV were generally more restricted in their substrate utilization abilities. None of them could degrade any of the 2,4-D-related compounds examined when they were first grown on medium containing acetate (Table 4). The degradation of 4-chlorophenoxyacetic acid by isolates 912, 1443, 1156, 91462, and 1173 only under 2,4-D-adapted conditions indicates that this compound was probably metabolized because of its structural similarity to 2,4-D or 2,4-D pathway intermediates. None of the test isolates could degrade 2-chlorophenoxyacetic acid and 4-chlorobenzoic acid.

Distribution of 2,4-D degraders. The field distribution of the hybridization group members which we isolated was influenced by 2,4-D application rates and repeated 2,4-D treatments (Fig. 5). The group II and IV isolates were obtained more frequently from soils that were not treated with 2,4-D or were treated with a low concentration (1 ppm), whereas the group I and III isolates were more commonly recovered from soils treated with high concentrations of 2,4-D (10 and 100 ppm). This finding was supported by the fact that we observed decreasing ratios of group II and IV isolates to group I and III isolates with increasing rates of 2,4-D application; i.e., the population ratios of isolates belonging to groups II and IV to isolates belonging to groups I and III were 6, 1, 0.33, and 0.15 in soils treated with 0, 1, 10, and 100 ppm of 2,4-D, respectively.

In the early phase of the field experiments, the isolates belonging to groups II and IV were detected as predominant 2,4-D degraders in most of the plots. For example, these isolates accounted for 87% of the isolates obtained from all of the May 1990 soil samples. However, the frequency of occurrence of these isolates decreased to 50 and 37% in July 1990 and September 1990, respectively. Thereafter, after additional 2,4-D treatments, they were rarely detected. In contrast, the group I and III isolates occurred at lower frequencies in the early phases but were encountered more frequently with increasing numbers of 2,4-D treatments, and eventually these isolates accounted for most or all of the isolates recovered in 1991 and 1992.

FIG. 3. REP-PCR patterns of isolates. Lanes 1, 17, and 32, *Hin*dIII-digested lambda DNA standard; lane 2, strain 1443; lane 3, strain 556; lane 4, strain 936; lane 5, strain 947; lane 6, strain 957; lane 7, strain 965; lane 8, strain 1124; lane 9, strain 1136; lane 10, strain 1146; lane 11, strain 1165; lane 12, strain 91462; lane 13, strain 9174; lane 14, strain 9224; lane 15, strain 9247; lane 16, strain 9256; lane 18, strain 736; lane 19, strain 756; lane 20, strain KI1; lane 21, strain 2811P; lane 22, strain 712; lane 23, strain 745; lane 24, strain 782; lane 25, strain 1173; lane 26, strain 1173; lane 27, strain 9112; lane 28, strain 9136; lane 29, strain 91461; lane 30, strain 9157; lane 31, strain 9166; lane 33, strain 512; lane 34, strain 524; lane 35, strain 535; lane 36, strain 547; lane 37, strain 565; lane 38, strain 573; lane 39, strain 583; lane 40, strain 765; lane 41, strain 773; lane 42, strain 912; lane 43, strain 924; lane 44, strain 957; lane 45, strain 974; lane 46, strain 983; lane 47, strain 936.

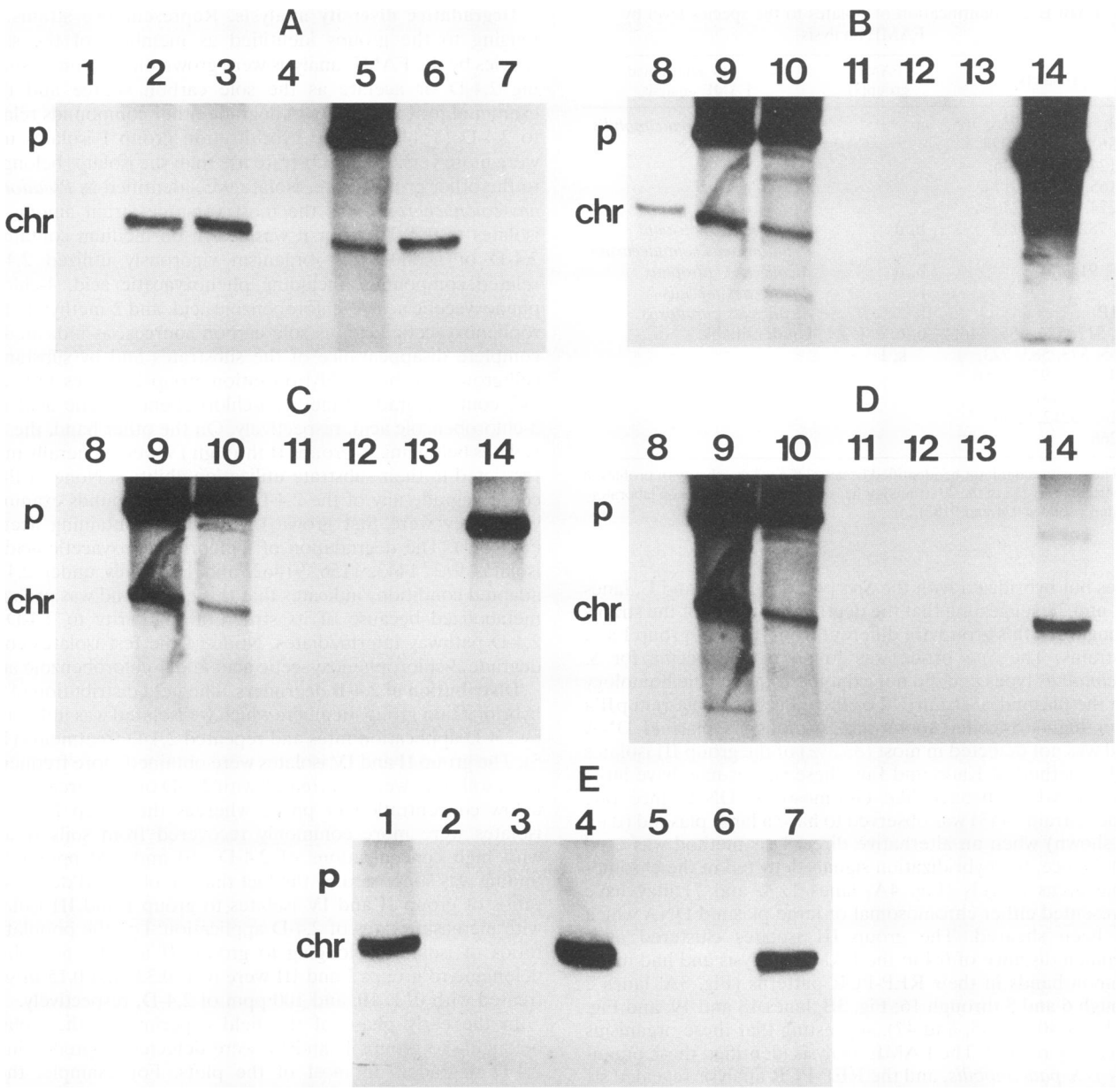

FIG. 4. Autoradiograms of gels after hybridization with the *tfdA* gene (A and B), the *tfdB* gene (C), the *tfdC* gene (D), and the *Spa* probe (E). Lanes 1, strain 1124; lanes 2, strain 9136; lanes 3, strain 91461; lanes 4, strain 91462; lanes 5, strain 9157; lanes 6, strain 9166; lanes 7, strain 9174; lanes 8, strain 2811C; lanes 9, strain 965; lanes 10, strain 1173; lanes 11, strain 912; lanes 12, strain 924; lanes 13, strain 983; lanes 14, strain 9112. The positions of plasmid DNA (p) and chromosomal and linear DNA (chr) are indicated on the left of each gel.

DISCUSSION

We analyzed the diversity of predominant 2,4-D-degrading bacteria isolated at different times from small plot areas that were not treated or treated with 2,4-D at different concentrations by using phylogenic, genotypic, and function classification systems. Species identification by FAME analysis placed 27 of the 47 isolates (57%) in the genera *Sphingomonas*, *Pseudomonas*, and *Alcaligenes*. The remaining 20 strains could not be identified by FAME analysis mainly because of their poor matches with any profile in the MIDI library or because they did not grow on laboratory medium. The characteristics of each hybridization group determined by the different methods are summarized in Table 5.

Hybridization group I, whose members hybridize to *tfdA*, *-B*, *-C*, and *-D* probes, is diverse as determined by the FAME and REP-PCR methods but very narrow in its 2,4-D-degrading genotype as judged by hybridization analysis. The group I strains all contain plasmids, and most of the plasmids exhibit hybridization to at least some *tfd* probes. Other work in our laboratory showed that transmissible plasmids are present in all of the strains of this group that have been tested (23). The group I strains grow well on 2,4-D and also appear to be selected by 2,4-D in the field, which is consistent with the competitiveness conferred by their related plasmid, pJP4 (15). Members of this group also degraded most other aromatic substrates tested. Taken together, these data suggest that the

TABLE 3. Hybridization patterns of isolates with DNA probes

Isolates	Hybridization with the following DNA probes[a]:					Hybridization group
	tfdA	*tfdB*	*tfdC*	*tfdD*	*Spa*	
KI1, 745, 965, 1173, 9112, 9136, 91461, 9157, 9166, 9212, 9236, 9266	+	+	+	+	−	I
2811P, 712, 782	+	−	−	−	−	II
1443, 556, 736, 756, 765, 936, 947, 957, 974, 1124, 1136, 1146, 1165, 91462, 9174, 9224, 9247, 9256	−	−	−	−	+	III
512, 524, 535, 546, 565, 573, 583, 723, 773, 912, 924, 983, 1156, 9182	−	−	−	−	−	IV[b]

[a] The *tfdA*, *tfdB*, *tfdC*, and *tfdD* gene probes were subcloned from plasmid pJP4; the *Spa* probe was subcloned from the large plasmid of strain 1443. +, detectable hybridization signal; −, no detectable hybridization signal under high-stringency conditions.
[b] Hybridization group IV contained all of the isolates that were not placed in hybridization groups I through III.

members of this group contain mobile catabolic plasmids that have spread among many different host organisms. Hybridization group II, whose members hybridize only to the *tfdA* probe, is a small group and is probably a subset of group I since the group II strains are in the same FAME clusters as the group I strains and have plasmids that hybridize to the *tfdA* probe. In both group I and group II, the 2,4-D-degradative genes appear to be located on the chromosome or a large plasmid in 25% of the strains, rather than on smaller plasmids, which has been the case in the strains studied previously.

The most surprising feature of this study was the dominance of a new, tightly clustered class of 2,4-D degraders, group III. The dominance of this group was also confirmed in our microcosm studies in which the probe for this group exhibited the strongest hybridization to soil DNA (16). The group III strains exhibited no detectable hybridization to the *tfd* probes under the conditions which we used. The 18 group III strains clustered in the FAME analysis at a Euclidian distance of 6.4 and differed from all other strains by a Euclidian distance of 48.8. The group III strains produced many common bands in their REP-PCR patterns. The strains that produced identical patterns could be siblings, but if they are siblings, they are siblings produced in nature, not during laboratory enrichment, since each came from a different field sample and was isolated at a different sampling time. The members of this group were also selected by the higher concentrations of 2,4-D used in the field, indicating the ecological success of the group III strains at 2,4-D degradation. Plasmids were rarely detected in members of group III. The *Spa* probe that was used to detect this group was, however, recovered from a very large plasmid. The ecological success of this class of 2,4-D degraders, the DNA sequence that is different from the canonical *tfd* pathway of pJP4, and the lack of small (<350-MDa) plasmids in these strains suggest that this class deserves more attention.

Hybridization group IV, which includes all of the isolates that did not exhibit detectable hybridization to the *tfd* probes or the *Spa* probe, was very diverse, as revealed by the FAME and REP-PCR results. Most of the isolates in this group (86%) could not be identified by the FAME technique. The members of this group also grow slowly on 2,4-D and were not recovered from plots treated with high concentrations of 2,4-D. These results might be expected of an "all-other" group that is diverse and not well adapted to particular selection conditions.

In most previous studies, 2,4-D-degrading bacteria have been isolated by enrichment from undiluted environmental samples in broth cultures containing a high 2,4-D concentration. Under these conditions, strains belonging to hybridization group I are preferably isolated because of their rapid growth (15). This may also explain why most of the 2,4-D-degradative plasmids independently isolated from numerous *Alcaligenes* species exhibited high degrees of similarity to plasmid pJP4 in biophysical and genetic properties (6) and also why most of the 2,4-D-degrading bacteria isolated from various natural water samples exhibited surprising uniformity in their phenotypes, metabolism, and cell structure (1). In our study, the possible bias during the enrichment procedure was reduced by using the highest dilution that produced 2,4-D degradation as the inoculum for enrichment cultures. Some of the active cultures

TABLE 4. Patterns of use of 2,4-D-related compounds by selected isolates

Substrate	Utilization by[a]:																			
	Group I strains						Group II strains				Group III strains				Group IV strains					
	745		965		1173		2811P		712		1443		91462		565		912		1156	
	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate	Grown on 2,4-D	Grown on acetate
2,4-D	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Phenoxyacetic acid	−	−	++	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2-Chlorophenoxyacetic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4-Chlorophenoxyacetic acid	−	−	++	++	++	−	−	−	−	−	++	−	++	−	−	−	++	−	++	−
3-Chlorobenzoic acid	+	+	++	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4-Chlorobenzoic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2-Methyl-4-chlorophenoxyacetic acid	−	−	++	++	++	++	−	−	+	−	−	−	−	−	−	−	−	−	−	−

[a] Selected isolates belonging to hybridization groups I through IV were grown on 2,4-D or on acetate and then tested for the ability to use substrates. ++, >80% reduction in peak height as determined by UV scanning and substantial growth (optical density at 550 nm, >0.13); +, 40 to 60% reduction in peak height and moderate growth (optical density at 550 nm, >0.08); −, <15% reduction in peak height and very scant growth (optical density at 550 nm, <0.01).

TABLE 5. Properties of hybridization groups as determined by different methods

Hybridization group	No. of isolates	FAME profiles[a]	REP-PCR patterns	Plasmid detected	Substrate use patterns[b]
I	12	Diverse (31.5)	Diverse	In all strains	Diverse (1–4)
II	3	Moderately diverse (11.8)	Diverse	In all strains	Similar (0–1)
III	18	Similar (6.4)	Similar	Rarely	Similar (1)
IV	14	Diverse (31.5)	Diverse	Rarely	Similar (0–1)

[a] The values in parentheses are the maximum Euclidean distances among isolates.
[b] The values in parentheses are the numbers of related substrates used by the isolates (six related substrates were tested).

(~10%) failed to produce single colonies that were able to degrade 2,4-D, suggesting that 2,4-D degradation was due to cometabolism, consortia, or strains that were not culturable on laboratory plates. Thus, we did not recover all of the numerically dominant organisms apparently involved in 2,4-D degradation in the field.

Isolates such as 9136, 91461, and 9166, which exhibit sequence homology to the *tfd* genes in the chromosomal band area, appeared to have 2,4-D-degradative genes on the chromosome instead of on the plasmid, although we cannot rule out the possibility that the genes are on very large plasmids. The chromosomal (or large-plasmid) location is in contrast to most of the previous findings (6, 9) that all or most of the 2,4-D genes are typically contained on small-plasmid (<350-MDa) DNA. This observation may be explained by our finding that an entire 2,4-D-degradative plasmid appears to integrate into (strain 2811C [Fig. 4B, lane 8]) and excise from the chromosome (17).

Our hybridization grouping of the isolates not only revealed the substantial genetic heterogeniety among the strains but also was useful in synthesizing microbiological and ecological information on what otherwise would be a highly diverse collection of 2,4-D degraders. The observed gradual shift of the predominant 2,4-D-degrading populations from the group II and IV strains to the group I and III strains in field studies demonstrates how indigenous microbial populations respond to environmental changes. This selection led to one group, group I, whose members were similar at the level of plasmid genotype but not at the level of organism genotype and another group, group III, whose members were similar at the organism genotype level. This example shows that there are two types of successful solutions to strong selection in nature. The former would generally be thought to be more versatile, but under our selection regimen both were similarly successful.

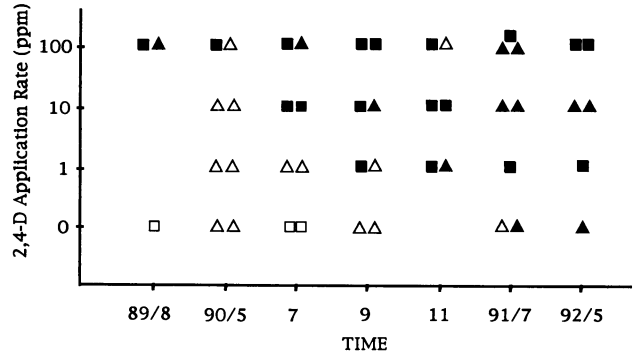

FIG. 5. Distribution of hybridization groups I through IV of 2,4-D degraders isolated from field plots treated with different amounts of 2,4-D at different times from August 1989 to May 1992. Symbols: ▲, group I; □, group II; ■, group III; △, group IV.

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