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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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 Strepto-

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

<table>
<thead>
<tr>
<th>Plot(s)</th>
<th>Isolate(s)</th>
<th>Rate of 2,4-D application &amp; Date of soil sample collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2811P</td>
<td>Control August 1989</td>
</tr>
<tr>
<td>4</td>
<td>K1, 1443</td>
<td>Control High May 1990</td>
</tr>
<tr>
<td>1 and 8</td>
<td>512, 583</td>
<td>Low May 1990</td>
</tr>
<tr>
<td>2 and 7</td>
<td>524, 573</td>
<td>Low May 1990</td>
</tr>
<tr>
<td>3 and 6</td>
<td>555, 565</td>
<td>Intermediate September 1991</td>
</tr>
<tr>
<td>4 and 5</td>
<td>546, 556</td>
<td>High September 1991</td>
</tr>
<tr>
<td>1 and 7</td>
<td>712, 782</td>
<td>Control July 1990</td>
</tr>
<tr>
<td>2 and 7</td>
<td>723, 773</td>
<td>Low September 1990</td>
</tr>
<tr>
<td>3 and 6</td>
<td>736, 765</td>
<td>Intermediate September 1990</td>
</tr>
<tr>
<td>4 and 5</td>
<td>745, 756</td>
<td>High September 1990</td>
</tr>
<tr>
<td>1 and 8</td>
<td>912, 983</td>
<td>Control September 1990</td>
</tr>
<tr>
<td>2 and 7</td>
<td>924, 974</td>
<td>Low September 1990</td>
</tr>
<tr>
<td>3 and 6</td>
<td>936, 965</td>
<td>Intermediate September 1990</td>
</tr>
<tr>
<td>4 and 5</td>
<td>947, 957</td>
<td>High September 1990</td>
</tr>
<tr>
<td>2 and 7</td>
<td>1124, 1173</td>
<td>Low November 1990</td>
</tr>
<tr>
<td>3 and 6</td>
<td>1136, 1165</td>
<td>Intermediate November 1990</td>
</tr>
<tr>
<td>4 and 5</td>
<td>1146, 1156</td>
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</tr>
<tr>
<td>1 and 8</td>
<td>9112, 9182</td>
<td>Control September 1991</td>
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<tr>
<td>7</td>
<td>9174</td>
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<td>3 and 6</td>
<td>9136, 9166</td>
<td>Intermediate September 1991</td>
</tr>
<tr>
<td>4 and 5</td>
<td>91461, 91462, 9157</td>
<td>High September 1991</td>
</tr>
<tr>
<td>1</td>
<td>9212</td>
<td>Control May 1992</td>
</tr>
<tr>
<td>2</td>
<td>9224</td>
<td>Low May 1992</td>
</tr>
<tr>
<td>3 and 6</td>
<td>9236, 9266</td>
<td>Intermediate May 1992</td>
</tr>
<tr>
<td>4 and 5</td>
<td>9247, 9256</td>
<td>High May 1992</td>
</tr>
</tbody>
</table>

* 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.

* 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 m²). 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 Brujin (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 32P-labelled DNA probes. The tfdA, tfIC, and tfID genes
probes were subcloned as 587-bp (Stul-SmaI), 775-bp (SmaI-
ClaI), and 100-bp (HindIII-XhoI) 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 BamHI fragment from a large
plasmid of strain 1443 into the BamHI site of plasmid pUC19
(22). This fragment was selected because it cross-hybridized
under low-stringency conditions to a 2.4-D-degradative plas-
mid 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 32P 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 strin-
gency (e.g., ≥90% sequence homology). Hybridization signals
were detected by autoradiography 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 se-
lected 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.
A 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 deter-
mined. 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 Pseudo-
monas 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. eu-
trophus. 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. paucim-
obilis 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 hybrid-
ization 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).
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 genes.
TABLE 2. Identification of isolates to the species level by FAME analysis

<table>
<thead>
<tr>
<th>Isolate(s)</th>
<th>FAME group(s)</th>
<th>Identity as determined by FAME analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1443, 556, 736, 756, 765 a</td>
<td>Sphingomonas paucimobilis</td>
<td></td>
</tr>
<tr>
<td>936, 947, 957, 974, 1124, 1136, 1146, 1165, 91462, 9174, 9224, 9247, 9256</td>
<td></td>
<td></td>
</tr>
<tr>
<td>712, 782, 983, 9112 b, d</td>
<td>Pseudomonas pickettii</td>
<td>Pseudomonas solanacearum</td>
</tr>
<tr>
<td>965</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1173, 9157 b, d</td>
<td>Alcaligenes eutrophus</td>
<td>Alcaligenes faecalis</td>
</tr>
<tr>
<td>912</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2811P</td>
<td>Alcaligenes paradoxus</td>
<td></td>
</tr>
<tr>
<td>K11, 512, 524, 535, 546 b, c, e, f, g, 565, 573, 583, 723, 745, 773, 924, 1156, 9136, 91461, 9166, 9182, 9212, 9236, 9266</td>
<td>Unidentifiable</td>
<td></td>
</tr>
</tbody>
</table>

* 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).

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. 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 II 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.


**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
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 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 3. Hybridization patterns of isolates with DNA probes

<table>
<thead>
<tr>
<th>Isolates</th>
<th>tfdA</th>
<th>tfdB</th>
<th>tfdC</th>
<th>tfdD</th>
<th>Spa</th>
<th>Hybridization group</th>
</tr>
</thead>
<tbody>
<tr>
<td>K11, 745, 965, 1173, 9112, 9136, 91461, 9157, 9166, 9212, 9226, 9266</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>I</td>
</tr>
<tr>
<td>2811P, 712, 782</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>II</td>
</tr>
<tr>
<td>1443, 556, 736, 756, 765, 936, 947, 957, 974, 1124, 1136, 1146, 1165, 91462, 9174, 9224, 9247, 9247, 9256</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>III</td>
</tr>
<tr>
<td>512, 254, 535, 546, 565, 573, 583, 723, 733, 912, 924, 983, 1156, 9182</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>IV*</td>
</tr>
</tbody>
</table>

* 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.

Hybridization group IV contained all of the isolates that were not placed in hybridization groups I through III.

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Group I strains</th>
<th>Group II strains</th>
<th>Group III strains</th>
<th>Group IV strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>745</td>
<td>965</td>
<td>1173</td>
<td>2811P</td>
</tr>
<tr>
<td>2,4-D</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Phenoxyacetic acid</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
</tr>
<tr>
<td>2-Chlorophenoxyacetic acid</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
</tr>
<tr>
<td>4-Chlorophenoxyacetic acid</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
</tr>
<tr>
<td>3-Chlorobenzoic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4-Chlorobenzoic acid</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
</tr>
<tr>
<td>2-Methyl-4-chlorophenoxy-acetic acid</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
<td>− −</td>
</tr>
</tbody>
</table>

* 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

<table>
<thead>
<tr>
<th>Hybridization group</th>
<th>No. of isolates</th>
<th>FAME profiles</th>
<th>REP-PCR patterns</th>
<th>Plasmid detected</th>
<th>Substrate use patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>12</td>
<td>Diverse (31.5)</td>
<td>Diverse</td>
<td>In all strains</td>
<td>Diverse (1-4)</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>Moderately diverse (11.8)</td>
<td>Diverse</td>
<td>In all strains</td>
<td>Similar (0-1)</td>
</tr>
<tr>
<td>III</td>
<td>18</td>
<td>Similar (6.4)</td>
<td>Similar</td>
<td>Rarely</td>
<td>Similar (1)</td>
</tr>
<tr>
<td>IV</td>
<td>14</td>
<td>Diverse (31.5)</td>
<td>Diverse</td>
<td>Rarely</td>
<td>Similar (0-1)</td>
</tr>
</tbody>
</table>

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

(0.001% - 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 tph 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 heterogeneity 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.

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