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2,4-Dichlorophenoxyacetic Acid-Degrading Bacteria Contain Mosaics of Catabolic Genes

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DNA from 32 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria from diverse locations was probed with the first three genes of the well-known 2,4-D degradation pathway found in *Alcaligenes eutrophus* JMP134(pJP4). The majority of strains did not show high levels of homology to the first three genes of the 2,4-D degradation pathway, *tfdA*, *-B*, and *-C*. Most strains showed combinations of *tfdA*-, *B*-, and *C*-like elements that exhibited various degrees of homology to the gene probes. Strains having the same genomic fingerprints (as determined by repetitive extragenic palindromic PCR) exhibited the same hybridization pattern regardless of the geographic origin of the strain, with the exception of a strain isolated from Puerto Rico. This strain had the same genomic fingerprint as that of numerous other strains in the collection but differed in its hybridization against the *tfdA* gene probe. Members of the β subdivision of the *Proteobacteria* class, specifically *Alcaligenes*, *Burkholderia*, and *Rhodoferax* species, carried DNA fragments with 60% or more sequence similarity to *tfdA* of pJP4, and most carried fragments showing at least 60% homology to *tfdB*. However, many strains did not hybridize with *tfdC*, although they exhibited chlorocatechol dioxygenase activity. Members of the α subdivision of the *Proteobacteria* class, mostly of the genus *Sphingomonas*, did not hybridize to either *tfdA* or *tfdC*, but some hybridized at low stringency to *tfdB*. The data suggest that extensive interspecies transfer of a variety of homologous degradative genes has been involved in the evolution of 2,4-D-degrading bacteria.

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide that is degraded in soils reasonably rapidly. Mineralization is presumably carried out by populations of the numerous bacterial strains that are known to utilize this compound. Since 2,4-D is a xenobiotic compound of recent origin, the way in which genetic elements have been assembled for its catabolism by bacteria is an interesting study in pathway evolution and genetic dissemination.

Alcaligenes eutrophus JMP134, originally isolated in Australia (7, 8), has become the strain used to study the genetics of 2,4-D degraders. It is host to a conjugative plasmid, pJP4, that carries mercury resistance and 2,4-D degradation genes—*tfdA* through *tfdF*. The *tfdA* gene codes for an α-ketoglutarate-dependent dioxygenase that converts 2,4-D to 2,4-dichlorophenol and is located upstream of and transcribed separately from the other *tfd* genes (13, 40). *tfdB* codes for a phenol hydroxylase that converts 2,4-dichlorophenol to dichlorocatechol, and it is downstream of the other *tfd* genes and is also transcribed separately. The *tfdC* gene encodes a 1,2-dichlorocatechol dioxygenase and is found in a single operon with the *tfdDEF* genes. The latter produce enzymes that oxidize the ring cleavage product to β-ketoadipate which is then metabolized by genomically encoded enzymes (45).

All of these genes have been sequenced, and this plasmid-borne pathway is one of the better-understood catabolic pathways. Sequence comparisons confirm that the *tfdCDEF* operon bears strong resemblance to the *clcABD* chlorobenzoate-degradative operon of pAC25 (or its derivative pAC27) found in *Pseudomonas putida* (16, 18) and the *tcbCDEF* chlorobenzene catabolic operon of pP51 found in *Pseudomonas* sp. strain P51 (46). The chlorocatechol dioxygenase (CCD) genes *tfdC*, *clcA*, and *tcbC* share between 54 and 63% DNA similarity, suggesting that these genes evolved from a common ancestor (45).

The *tfd* genes are fairly ubiquitous, having been isolated in disparate places of the world, either on pJP4 or on a different plasmid backbone. Amy et al. (2) describe pJP4 in a different host (EML159) isolated in Oregon. Plasmid-borne *tfd* genes, highly homologous if not identical to those of pJP4 but not necessarily in the same order, have been found in *P. putida* isolated in Estonia (pEST4011 [30]), *Pseudomonas cepacia* isolated in India (pMAB1 [5]), and a *Flavobacterium* species isolated in Florida (pRC10 [6]).

In spite of these reports, all 2,4-D degraders do not necessarily carry plasmid-encoded catabolic genes identical to those of pJP4. Genes less homologous or not homologous to the *tfd* genes are commonly encountered but poorly characterized. Amy et al. (2) describe several strains that show no homology to pJP4. Ka et al. (25) and Matheson (31) have shown that the genes of bacterial populations responding to 2,4-D in a Michigan soil are not highly homologous to the *tfd* genes. Two studies have demonstrated that 2,4-D catabolic determinants are chromosomally encoded in some strains (32, 41).

The purpose of this study was to determine the extent of homology between the well-known 2,4-D degradation genes of the plasmid pJP4 and the organisms in our culture collection. We show that the *tfd* genes cannot be used as universal gene probes for 2,4-D-degradative bacteria and present evidence that recombination has played a major role in the evolution of these catabolic bacteria.

MATERIALS AND METHODS

Isolation of strains. The majority of strains used in this study were isolated from the agricultural soils of the Kellogg Biological Station (KBS), in southwest Michigan, and in the Indian Head Research Station in southern Saskatchewan and are described by Tonso et al. (42). A large group was isolated from activated sludge in Oregon by Amy et al. (2). TFD44 originated from a herbicide wastewater treatment facility in Montana, but nothing is known about how it was isolated (37a). B6-5, B6-9, and B6-10 were isolated directly from lake water microcosms contaminated with 50 μM 2,4-D (14). M1 was isolated from forest

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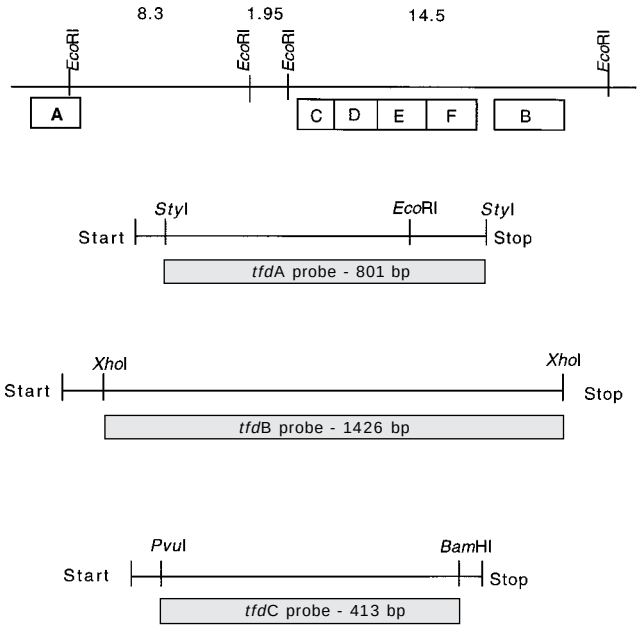

FIG. 1. Diagram showing the layout of *tfdA*, *-B*, *-C*, *-D*, *-E*, and *-F* on pJP4 in relation to the *Eco*RI cuts and the internal cuts used to generate fragments for cloning for the production of *tfdA*, *tfdB*, and *tfdC* probes (23). Distance (in kilobases) is shown over the diagram of *tfd* genes.

soils sampled on the island of Moorea (French Polynesia) and enriched in minimal medium (A+N [49]) supplemented with 50 ppm of yeast extract and 2 mM 2,4-D. Isolate E2w1 originated from soil in the El Loquillo forest of Puerto Rico. Soil was supplemented with 50 ppm of 2,4-D, and degraders were detected by their uptake of ^{14}C-labeled 2,4-D on solid medium (32). Strains K712 and K1443 were isolated from positive most-probable-number dilution tubes used to enumerate 2,4-D degraders in KBS soil (25). BRI6001 was isolated from peat in Quebec (20). Strains were maintained on peptone-tryptone-yeast extract-glucose (PTYG) agar supplemented with 250 ppm of 2,4-D. *Escherichia coli* DH5α and *Comamonas acidovorans* ASB3 were used as non-2,4-D-degrading control strains. The latter was isolated from the wastewater of a pulp and paper mill by enrichment in minimal medium (A+N [49]) supplemented with 1 mM 5-chlorovanillin. All of these strains are available from the laboratory of J. M. Tiedje at the Center for Microbial Ecology or from the laboratory of R. Fulthorpe.

Enzyme assays. Cells were grown for 15 to 18 h in MMO (mineral salts medium [23]) containing 2 g of pyruvate per liter and 0.2 g of 2,4-D per liter as carbon sources. After this period of time, cultures had consumed the pyruvate and were induced to use 2,4-D. The cultures were then harvested by centrifugation and washed twice with 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM dithiothreitol. Since strain M1 was very oligotrophic, a higher biomass yield of the strain was obtained after growth on 1 g of acetate per liter and 0.2 g of 2,4-D per liter. After disruption of the cells by sonication for 2 min at 0°C, cell extracts were separated from whole cells and cell debris by centrifugation at 16,000 rpm for 1 h at 5°C.

Activity of chlorocatechol 1,2-dioxygenase was measured spectrophotometrically at room temperature by monitoring the changes in A_{260} (10). The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 0.25 mM catechol or substituted catechols, and 1.3 mM EDTA. The reaction was started by adding the cell extract. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per min. Molar absorption coefficients were those reported by Dorn and Knackmuss (11).

DNA extraction and hybridization analyses. Cultures (100 ml) of each strain grown on 500 ppm of 2,4-D and 50 ppm of yeast extract in MMO were harvested by centrifugation. Culture fluid was analyzed for the presence of 2,4-D with a Hewlett-Packard 1050 series liquid chromatograph equipped with a C_{18} column and with a UV detector set at 220 nm. The solvent was 70% methanol and 30% dilute phosphoric acid (0.1%). Only those cultures actually mineralizing 2,4-D were used for DNA extraction. Total genomic DNA was extracted from each strain (3). *Eco*RI-digested DNA was separated by electrophoresis through a 0.8% agarose gel and Southern blotted onto Hybond N nylon membranes (Amersham). *Pvu*II digestions of pUC19 clones of the *tfdA*, *tfdB*, and *tfdC* genes from plasmid pJP4 (23) (Fig. 1) were used to make randomly primed digoxygenin-labeled gene probes as described by Fulthorpe and Wyndham (15). In addition, we obtained a 300-bp PCR-amplified fragment of the *clcA* gene from pAC27. Primers corresponding to positions 601 to 632 and 931 to 962 of the *tfdC* sequence of pJP4 (37) were used to amplify *clcA* from *Alcaligenes denitrificans* (19, 34). This *clcA* fragment was also labeled with digoxygenin by the random-priming method.

We carried out hybridizations at three stringencies to estimate sequence similarities between our gene probes and isolates' genes. Because of the difficulty inherent in stripping the nonlabile digoxygenin probe from nylon membranes, we relied on the hybridization conditions, rather than on subsequent stringency washes, to dictate the degree of similarity we wished to detect. We carried out all our hybridizations under conditions that fixed the melting temperature of the duplex we wished to detect at 5°C above our hybridization temperature—62°C. All hybridizations were carried out as recommended by Boehringer Mannheim, and high-, medium-, and low-stringency hybridization solutions contained 50, 20, or 0% formamide and 5, 2, and 1% blocking agent, respectively. Under these conditions, assuming a GC content of 63% (of the *tfdA* gene), we could detect DNA that was >90, >75, or >60% homologous to our gene probes. The color detection method was used to develop the hybridizations. For all our isolates, we hybridized digested and blotted DNA at high, medium, and low stringencies. If the same membrane was used, we carried out the highest stringency first and removed color, but not probe, between each hybridization.

Genomic fingerprinting. Extracted genomic DNA was used to obtain a repetitive extragenic palindromic (REP) PCR fingerprint from each strain by the method of Versalovic et al. (47). Amplification products were separated by electrophoresis on a 1.5% agarose gel for visualization.

Strain identification. As part of a larger study on this isolate collection, the 16S rRNA gene was amplified with rD1 and fD1 as primers (48). Amplification was performed with a Perkin-Elmer 9700 Thermal Cycler. Amplified products were purified with a Gene Clean kit (BIO 101, La Jolla, Calif.). Partial sequences were obtained from each strain from *E. coli* position 519r as previously described by Lane et al. (28). Sequencing was carried out at the Michigan State University Sequencing Facility with the Applied Biosystems model 373A automatic sequencer (Perkin-Elmer Cetus) and fluorescently labeled dye termination. Partial sequences were compared with data in GenBank by using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (1) as well as with the data of the Ribosomal Database Project (29).

RESULTS

General remarks. Previous work on our 2,4-D-degrading isolate collection identified those isolates with identical genomic fingerprints (42). We concentrated this study on those isolates that were unique or representative of a genomic fingerprint group and on isolates that had identical genomic fingerprints but were isolated from different geographic locations. From our collection of 57 2,4-D degraders, we analyzed 32 isolates and 19 genome fingerprint types. The majority of these are shown in Fig. 2. The results of the hybridization studies on unique strains and representative strains are shown in Table 1. Also shown in Table 1 are the results of the sequence-matching searches. For each strain we denote the species or genus showing the closest match to the partial 16S ribosomal sequence we obtained.

Two strains, B6-5 and B6-10, showed no homology to the three *tfd* genes even under low-stringency conditions. Among the remaining 17 fingerprint types, we found 14 different hybridization profiles against the *tfd* probes (Table 1; Fig. 3). With one exception, isolates of the same fingerprint type gave the same hybridization profile regardless of the geographic origin of the strain. On the other hand, some strains with quite different genomic fingerprints gave the same hybridization profile, presumably those carrying the same plasmids. The results are best understood by examining the data in Table 1 and Fig. 3. Figure 4 shows the efficacy of the technique in detecting different levels of homology. The salient features of the data are summarized below.

Strains hybridizing to *tfdA* at high or medium stringency. In the group of strains hybridizing to *tfdA* at high or medium stringency were those strains that were previously determined to carry a type 1 plasmid, i.e., one that hybridized strongly to the entire pJP4 probe (31). All these strains proved to have genes that hybridized at high stringency to *tfdA*. They were all *Alcaligenes*, *Burkholderia*, or *Rhodoferax* species. Within this group, only four strains carried fragments that hybridized to all three pJP4 genes at high stringencies. Only two gave hybrid-

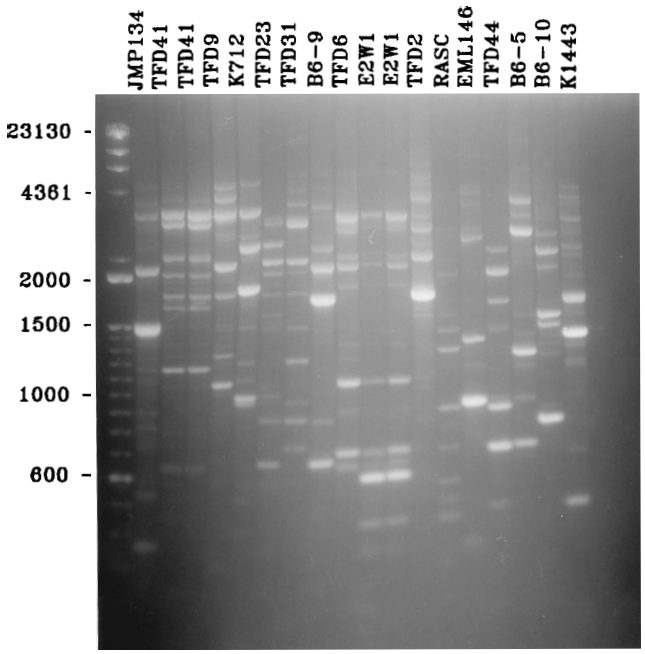

FIG. 2. REP PCR genomic fingerprints for strains examined in this study. See Materials and Methods for details. The leftmost lane contains the size markers, a 100-bp ladder combined with fragments from a *Hin*dIII digestion of lambda DNA. TFD41 and E2w1 were amplified from separate DNA stocks to illustrate the reproducibility of the patterns.

ization patterns identical to that of pJP4. Notably, these strains were from widespread locations (Oregon and Saskatchewan), and each had unique genomic fingerprints. TFD41 (and TFD38 with the same genomic fingerprint) was the only other strain carrying genes hybridizing to *tfdA*, *-B*, and *-C* at high stringencies. Even in this strain, additional homologous fragments, hybridizing at lower stringencies, are found. A 4.5-kb fragment hybridizes at medium stringency to *tfdB*. At low stringency, two fragments (19 and 5.5 kb) hybridize to both *tfdC* and *clcA*. Ongoing studies show that the *tfd* genes of pJP4 are carried in TFD41 by a different plasmid and that some of these genes have undergone duplication (34a).

Also in this group are strains K712 and TFD9, having dissimilar genomic fingerprints, isolated from Michigan and Saskatchewan, respectively. They each have two plasmid bands that hybridize at high stringency to *tfdA*; one hybridizes at medium stringency to *tfdB* and the other hybridizes at low stringency to *tfdC* and *clcA*.

The remaining three strains in this category, all classified as *Rhodoferax fermentans*, all differ significantly from each other. It is also interesting to note that B6-9 is the only strain in our collection to possess a *tfdC*-like element that is more closely related to *clcA* than to *tfdC*.

Strains hybridizing to *tfdA* at low stringency. Strains possessing DNA fragments hybridizing to *tfdA* only at low stringency were common in our collection. Most of these strains were found in the TFD6 siblings (REP group A in Table 1). A 9.5-kb *Eco*RI fragment hybridizes at low stringency to *tfdA* in most of this REP group. A fragment of the same size exhibits high levels of homology to both *tfdB* and *tfdC*. TFD6 siblings were isolated from Michigan, Oregon, Quebec, and Saskatchewan by five different researchers. REP fingerprints of isolates from these different locations were virtually identical. In general, strains with identical genetic fingerprints had identical hybridization patterns, independent of where the strain was isolated, but E2w1 was an exception to this. Strain E2w1 had a typical TFD6-like fingerprint and carried fragments of identical size that hybridized at high stringency to *tfdB* and *tfdC*. However, the *tfdA* gene probe hybridized weakly to an 11.3-kb band rather than the 9.5-kb fragment that was typical for these strains.

A 9.5-kb fragment also hybridizing to *tfdA* at low stringency is present in the second largest, although not geographically widespread, group of degraders—the TFD2 siblings (REP group B in Table 1). All of these strains were isolated at the Michigan KBS. A strain with a unique fingerprint, RASC, carried *tfdA* on a 7-kb fragment that also hybridized at low stringency. RASC was isolated in Oregon but is related to the TFD2 group in two ways. RASC and TFD2 strains all carry homologous plasmids that are not associated with 2,4-D degradation (32, 33). They also carry low-similarity *tfdA* and *tfdB* elements on the same *Eco*RI fragment; in TFD2 this fragment is 9.5 kb and in RASC it is 7.0 kb, but neither of the strain types hybridizes to *tfdC*.

Gene associations. *tfdB* and *tfdC* seem to be more strongly linked to each other than to *tfdA*. For example, fragments that hybridize strongly to *tfdB* also hybridize strongly to *tfdC* in TFD41, TFD6, and E2w1. In contrast, fragments highly homologous to *tfdA* are found independently of strong homologs of *tfdB* and *tfdC*. Three strains that possessed DNA fragments highly similar to *tfdA* were found, but their DNA fragments exhibited only moderate or negligible degrees of similarity to *tfdB* and *tfdC*.

Strains hybridizing to no pJP4 genes or *tfdB* only. All of the other 2,4-D degraders in our collection lack any homology to *tfdA* or *tfdC*. TFD26, TFD44, K1443, and M1 exhibit weak homology to *tfdB*. B6-5 and B6-10 do not hybridize to any of the three gene probes. All of these strains are members of the α subdivision of the *Proteobacteria* class, and except for M1, they are all members of the genus *Sphingomonas*. Of the rRNA sequences found in GenBank, M1 is most closely related to *Rhodopseudomonas palustris*, but it does not share any characteristics with this species.

CCD activity. In Table 2 we show that of the strains we tested, even those that did not hybridize to *tfdC* or *clcA* exhibited CCD activity.

DISCUSSION

In this study we probed 32 2,4-D-degrading isolates representing 19 genomic fingerprint types, including the control strain JMP134, with the *tfdA*, *-B*, and *-C* genes of the well-characterized 2,4-D catabolic plasmid pJP4. Excluding JMP134 itself, only three strains carry highly homologous genes and two of the three carry pJP4 itself. The third strain carries the same genes on a different plasmid backbone. The remaining isolates all exhibit various degrees of homology to the first three *tfd* genes, and the diversity of hybridization patterns is suggestive of a great deal of genetic rearrangement in the evolution of 2,4-D-degrading bacteria.

We are confident that the DNA fragments hybridizing with our *tfd* probes contain functional or ancestral *tfd* genes for several reasons. First, we did not detect any significant bands in non-2,4-D-degrading control strains, *E. coli* or *C. acidovorans* ASB3 (a chloroaromatic-degrading member of the β subdivision of the *Proteobacteria* class). Second, the degree of homology between some of the genes which we predicted from our hybridization experiments corresponds well to recently acquired sequence data. Biochemical studies and cloning and sequencing work have confirmed the presence of *tfdA* activity and a *tfdA*-like sequence in RASC and TFD6 (13, 32, 41). The

TABLE 1. *Eco*RI fragments hybridizing with pJP4- and pAC25-derived DNA probes

Hybridization pattern no.	Size (kbp) of fragments in hybridization pattern[a]				Members of group exhibiting hybridization pattern			
	tfdA	*tfdB*	*tfdC*	*clcA*	Strain	Type[b]	Origin[c]	Identification[d]
1	10.6***, 8.3***	14.5***	14.5***	14.5*, 8*	JMP134	u	AU	*Alcaligenes eutrophus*
					EML159	u	OR	*Burkholderia* sp.
					TFD39	u	SK	*Burkholderia* sp.
2	6.3***, 3.5***	3.5**	6.3*	6.3*	TFD9	u	SK	*Alcaligenes xylosoxidans*
					K712	u	MI	*Pseudomonas andropogonis*
3	4.5***, 2.9***	19***, 16***, 9*, 4.5**	19*, 16***, 5.5*	5.5*	TFD41, TFD38	C	MI	*Alcaligenes eutrophus*
4	7.2***, 3.5***	3.5*	6.3*	NH	TFD23	u	MI	*Rhodoferax fermentans*
5	15.5**, 2.3*	NH	NH	NH	TFD31	u	SK	*Rhodoferax fermentans*
6	19.4**, 14.4**, 2.3**	NH	19.4*	19.4***	B6-9	u	ON	*Rhodoferax fermentans*
7	9.5*	9.5***	9.5***	9.5*	TFD6, TFD14	A	MI	*Burkholderia mallei*
					TFD15, TFD17, TFD18	A	OR	*Burkholderia mallei*
					TFD20, TFD21, TFD27	A	SK	*Burkholderia mallei*
					BRI6001	A	PQ	*Burkholderia mallei*
8	11.3*	9.5***	9.5***	NH	E2w1	A	PR	*Burkholderia mallei*
9	9.5*	9.5*	NH	NH	TFD2, TFD4, TFD7, TFD8, TFD36	B	MI	*Burkholderia* sp.
10	7.0*	7.0*, 3.4*	NH	NH	RASC	u	OR	*Burkholderia* sp.
11	NH	12*	NH	NH	M1	u	FP	*Rhodopseudomonas palustris*
12	NH	23*	NH	NH	EML146	u	OR	*Sphingomonas* sp.
13	NH	>23*, 8*	NH	NH	TFD44	u	MN	*Sphingomonas* sp.
14	NH	8*	NH	NH	K1443	u	MI	*Sphingomonas* sp.
15	NH	NH	NH	NH	B6-5	u	ON	*Sphingomonas* sp.
16	NH	NH	NH	NH	B6-10	u	ON	*Sphingomonas* sp.

[a] All fragments in each hybridization pattern were found in all members of the group exhibiting that hybridization pattern. The stringency at which hybridization was detected is indicated by the asterisks as follows: ***, high stringency (>90% sequence similarity); **, medium stringency (75 to 90% sequence similarity); *, low stringency (60 to 75% sequence similarity). NH, no homology.

[b] REP PCR fingerprint type, where u stands for unique and A, B, and C are REP fingerprints with multiple representatives.

[c] Origin of strain. See Materials and Methods for details and sources. Abbreviations: AU, Australia; OR, Oregon; SK, Indian Head Research Station, Saskatchewan, Canada; MI, KBS, Michigan; ON, Ontario, Canada; PQ, Montreal, Quebec, Canada; PR, Puerto Rico; MN, Montana; FP, Moorea, French Polynesia.

[d] Closest species as determined by alignment of partial 16S rRNA genes with known genes in GenBank (33).

tfdA sequences from these strains proved to be identical to each other and 72% similar to *tfdA* in pJP4. The hybridization work presented in this study predicted that the two genes would share between 60 and 75% sequence similarity. Finally, primers specific to areas of sequence conservation between the two *tfdA* sequences were synthesized and used to amplify and sequence DNA from all the other fingerprint types in this collection. All those strains strains showing some hybridization with *tfdA* yielded 300 bp of *tfdA*-like sequence, and all those that did not hybridize gave no amplification product (48a).

The appearance of highly similar *tfd* genes in different bacterial strains and species is most parsimoniously explained by horizontal genetic exchange events. Horizontal transfer followed by sequence divergence is thought to explain the high degrees of similarity between toluene and napthalene degradation genes, xylene and benzene degradation genes, toluene

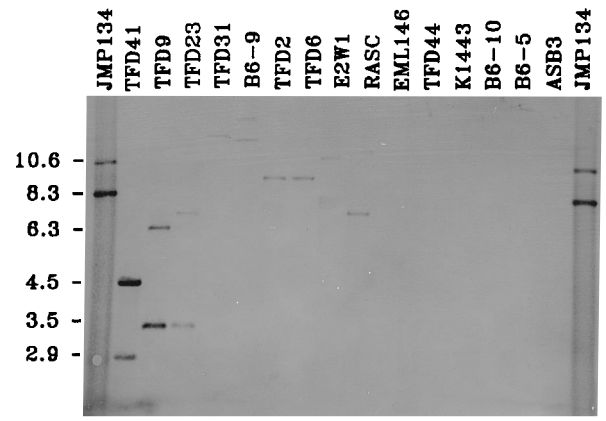

FIG. 3. Results of low-stringency hybridization of *tfdA* against all the unique or representative strains in our collection. Band sizes (in kilobases) were determined from migration distances calibrated to migration of *Hin*dIII-digested lambda DNA on the original gel.

and biphenyl degradation genes, as well as between the chlorocatechol degradation genes of pJP4 itself and pAC27. In many of these cases, gene orders are not conserved, or open reading frames are found inserted or deleted (for a review, see reference 45).

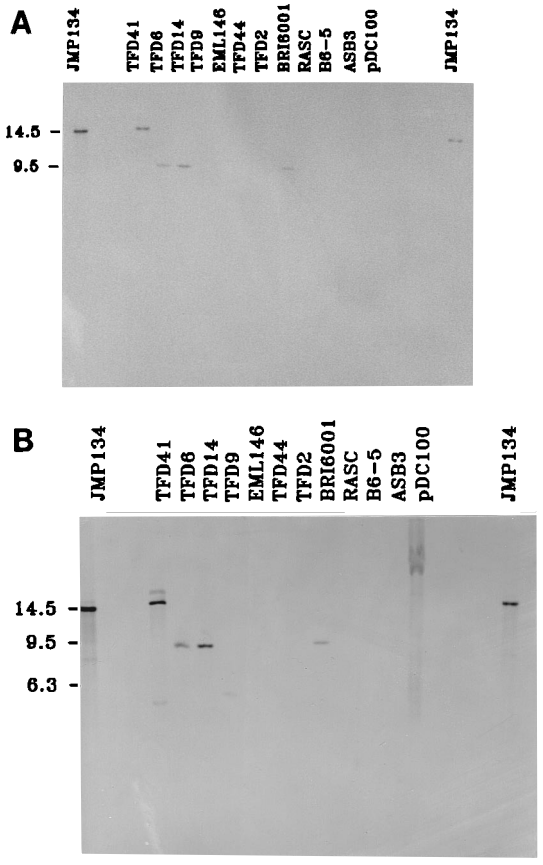

FIG. 4. Results of high-stringency (A) and low-stringency (B) hybridization of *tfdC* against some of the strains in our collection. Note that under low-stringency conditions, extra bands appear in TFD41, TFD9, and pDC100, the plasmid carrying the *clcABCDEF* operon. *clcA* is 57% homologous to *tfdC*. Band sizes (in kilobases) were determined from migration distances calibrated to migration of *Hin*dIII-digested lambda DNA on the original gel.

TABLE 2. Relative activities of chlorocatechol 1,2-dioxygenases with different chlorinated catechols in cell extracts of 2,4-D-degrading strains which do not hybridize with *tfdC* and *clcA* gene probes

Strain	Activity (nmol/mg of protein/min) of enzyme with 3,5-dichlorocatechol	Relative activity[a] of enzyme with:		
		3-Chlorocatechol	4-Chlorocatechol	Catechol
EML146	120	51	57	84
TFD44	97.4	45	87	31
B6-10	55.4	65	81	67
K712	51.2	50	63	48
TFD2	45.8	45	74	39
RASC	38.0	78	72	51
K1443	27.2	41	54	42
M1	20.2	29	62	55

[a] Activity expressed as a percentage of the enzyme activity with 3,5-dichlorocatechol (which was set at 100%).

The existence of pJP4 in various species is well documented, and it is known to be a conjugative plasmid (7). The presence of *tfd* genes on other conjugative plasmid backbones (2, 5, 6, 30, 43) and their chromosomal locations on TFD6 and RASC (32, 41) and probably other strains (4) demonstrate a high degree of mobility of this gene family. Interspecies transfer of plasmid-borne genes probably accounts for the existence of homologous *tfd* genes in the different species, but we do not yet understand the mechanism that accounts for the mobility of the *tfd* catabolic units within any one genome. Transposons are known to account for the mobility of toluene and chlorobenzoate degradation genes (15, 35, 44), but this has not been established for the 2,4-D genes. Ghosal and You have demonstrated the presence of invert duplications of some of the catabolic genes of pJP4 under selection with 3-chlorobenzoate and the presence of tandem duplications of the same genes in the related plasmid pJP2 (17). Repeated sequences have been implicated in these duplication events, but no clear-cut mechanism has been established.

The hybridization patterns seen in this strain collection cannot be explained by the simple movement of a large piece of DNA that contains the catabolic *tfd* genes. We find combinations of high- and low-similarity genes together in various strains. The genes also appear to be in different orders. In pJP4, *tfdA* is found at a point greater than 10 kb away from the *tfdCDEF* and *tfdB* genes which are found on a single 14.5-kb fragment (Fig. 1). In the TFD6 group, highly similar *tfdB*- and *tfdC*-like genes are found on a 9.5-kb fragment but not along with a low-similarity *tfdA*-like gene on the same-size fragment, i.e., less than 10 kb away (if we make the reasonable assumption that the elements lie on the same, rather than just the same-size, *Eco*RI fragment). The absence of a high-similarity *tfdA*-like gene suggests that the *tfdB* and *tfdC* genes have transferred into these strains independently of the canonical pJP4 *tfdA* gene. Conversely, a *tfdA* element highly similar to that of pJP4 appears in two quite different strains, TFD9 and TFD23, but is found with low-similarity *tfdB* and *tfdC* elements. The sequence conservation of the *tfdA* element is indicated not only by the high-stringency conditions under which it was detected but also by the conservation of the *Eco*RI site within it. In both of these hosts, one end of the *tfdA* element lies on a 3.5-kb fragment, as does the *tfdB* element. In these strains, *tfdA* is no longer 10 kb from *tfdB* and there is no intervening *Eco*RI digestion site and *tfdC* is no longer found between the two

elements but on a separate *Eco*RI fragment. The diversity of associations of low- and high-similarity elements suggests that there are mechanisms for independent movement of these genes, and it is possible that some strains have recruited the individual genes from different sources, rather than as a preassembled whole. This hypothesis is consistent with the observation that *tfdA* appears to have been recruited independently on the basis of its distance from the other *tfd* genes as they are carried on pJP4 (40).

The *tfdA*-like genes of TFD6 and RASC have been shown to be chromosomal and identical to each other (32, 41). This result makes this *tfdA*-like gene the dominant type in our collection. From the similarity of the hybridizing fragment sizes and from preliminary amplification studies, we suspect that the *tfdA* gene in the TFD2 group is again identical to that of TFD6 and RASC but is associated in each of these three strains with different *tfdB* and *tfdC* elements. In RASC, the *tfdA*-like gene is part of a larger chromosomal unit that has been shown to transfer to *P. cepacia* via an unknown mechanism (33), which may be part of the reason for the apparent high mobility of this element.

Strains that hybridize to *tfdB* are more common than strains that hybridize to *tfdA* or *tfdC* in this collection. The *tfdB* gene encodes phenol hydroxylase. Other phenol hydroxylases have been isolated from *Trichosporan cutaneum* (26), *Bacillus stearothermophilus* (9), as well as from numerous pseudomonad species (27, 36, 38, 39). However, to date, only the enzyme from *Pseudomonas* sp. strain EST1001 shows significant amino acid homology with the gene product of *tfdB*, and it also shares a similar narrow substrate range (36). Different phenol hydroxylases can have very different substrate ranges, and the genes may share NAD binding areas but exhibit very little structural sequence similarity (27, 50). It is clear that there have been multiple origins of phenol hydroxylase activity, so it is not surprising that some strains do not show any homology to *tfdB*. Conversely, genes related to *tfdB* clearly have been important to the majority of strains in this collection.

We assume that fragments hybridizing to *tfdC* are genes encoding CCDs. These same fragments also hybridize to *clcA*, often showing similarities midway between the two gene probes. Conversely, the absence of fragments hybridizing to the *tfdC* and *clcA* probes does not mean that CCD genes are absent—only that they cannot be detected under low-stringency hybridization conditions. All of the strains which we investigated that did not hybridize with *tfdC* or *clcA* did exhibit CCD activity nonetheless, proving they are less than 60% homologous to the known genes or that some CCD genes have derived from a different ancestral gene. It is not uncommon for isofunctional genes to be less than 60% homologous, depending on how much of the protein structure needs to be conserved in order to maintain the enzymatic function. It is not clear at this time whether the CCDs in B6-5, B6-10, K1443, and others are analogous or homologous to those found in pJP4 and pAC27.

Known gene probes are insufficient to detect these CCD genes in environmental samples or strains, because any further reduction in stringency conditions leads to problematic nonspecific probe binding. We found that a very slight reduction of the hybridization temperature from 62 to 60°C could lead to nonspecific binding to the lambda marker. The absence of a good probe for CCD genes has implications beyond our abilities to detect 2,4-D degraders. The detection of CCD is of considerable importance because of the central role that these enzymes play in the degradation of many other simple chlorinated aromatic compounds (21). A search for these less similar CCDs will help in finding more universal detection tools for these important enzymes.

Apparently, there is a reservoir for heterologous but isofunctional 2,4-D-degrading genes in the α subdivision of the *Proteobacteria* class. In our collection, strains EML 146, TFD44, B6-5, B6-10, K1443, and M1 are all members of this group, and all but M1 are members of the genus *Sphingomonas*. The *Sphingomonas* strains are clearly of ecological importance, since they were easily isolated from lake water, sewage sludge, and soil systems alike. Furthermore, they became the most dominant 2,4-D degraders in KBS soils in response to long-term 2,4-D additions (25). We found that these strains may hybridize weakly to *tfdB* but not at all to *tfdA* or *tfdC*. While they do not show homology to *tfdC* or to *clcA*, they do exhibit CCD activity. In this laboratory, researchers have been unable to find *tfdA*-like enzymatic activity in these strains by established methods (13, 41a).

There may be a distinct phylogenetic limitation on the functional host range of the canonical *tfd* genes. The remainder of our strains in the collection hybridized to some extent with *tfdA* and to various degrees with the other two probes. They were all members of the β subdivision of the *Proteobacteria* class—either *Burkholderia* species, *Alcaligenes* species, or *Rhodoferax fermentans*. This is consistent with what is known about the host range of pJP4. The plasmid can transfer to and be maintained in members of the α, β, and γ subdivisions of the *Proteobacteria* class (including *E. coli*, *Rhodopseudomonas sphaeroides*, *Agrobacterium* species, *Rhizobium* species, *P. putida*, *Pseudomonas fluorescens*, *A. eutrophus*, and *Alcaligenes paradoxus* [7]). However, not all recipients of the plasmid can express the *tfd* genes. Efficient expression may be limited to the members of the β subdivision of the *Proteobacteria* class (*Alcaligenes* sp., *Burkholderia* sp., and "*Pseudomonas oxalaticus*" [= *Alcaligenes* sp.] [12, 24]). Expression has been reported in one *P. putida* strain (γ subgroup [7]) but not in another (22). Active *tfd* genes have been found in a "*Flavobacterium*" species, but this is a polyphyletic genus (6).

It is clear that several homologs of at least three of the pJP4 2,4-D degradation genes exist and that they can be found in various combinations in different degradative strains. We have probably only uncovered a fraction of the number of different gene arrangements possible, given that the units seem to be able to mix and match, at least within the β subdivision of the *Proteobacteria* class, much like the pieces of a mosaic. This suggests that each gene in the 2,4-D degradative pathway may have evolved as part of disparate pathways and were only modified and recombined at a later date in response to selection from 2,4-D. The gene arrangement on plasmid pJP4, as globally distributed as it is, is only one of the possible mosaic constructions that allow the degradative pathway to proceed. At this point we have no evidence to suggest if any of the arrangements we see are ancestral to others nor any idea what functional differences the sequence changes make to the efficiencies of the actual enzymes.

In summary, we have shown that, contrary to data published to date (5–7, 30), organisms degrading 2,4-D do so by virtue of a high diversity of genes, some that are very similar in sequence to the well-known *tfd* genes of the catabolic plasmid pJP4 and others that are not. The many variants of the *tfd* genes and many nonhomologous genes have been recombined in a variety of different ways in many different strains. This group of bacteria should prove to be an interesting study in gene recruitment and pathway assembly when they are better characterized.

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