Isolation and Characterization of a New Plasmid from a *Flavobacterium* sp. Which Carries the Genes for Degradation of 2,4-Dichlorophenoxyacetate†

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A *Flavobacterium* sp. (strain 50001), capable of degrading 2,4-dichlorophenoxyacetate (2,4-D), 2-methyl-4-chlorophenoxyacetate, and 2-chlorobenzoate and imparting resistance to mercury, harbored a degradative plasmid, pRC10. Cured strains of the *Flavobacterium* sp. lost the plasmid as well as the ability to degrade these chlorinated compounds. Comparison of this plasmid with the well-characterized 2,4-D-degradative plasmid pJP4 from *Alcaligenes eutrophus* showed regions of homology between the two plasmids. Restriction fragments of plasmid pRC10 which shared homology with the regions conferring 2,4-D-degradative genes (*tfd*) of plasmid pJP4 were cloned into a broad-host-range plasmid and studied in *Pseudomonas putida*. From the results obtained, the cloned DNA fragment expressed the genes for 2,4-D monooxygenase (*tfdA*) and 2,4-dichlorophenol hydroxylase (*tfdB*). In spite of the similarity in function, the size (45 kilobases) and restriction pattern of plasmid pRC10 were considerably different from those of pJP4 (80 kilobases). This may be due to the difference in the microbial background during evolution of the two plasmids.

Many of the microorganisms capable of metabolizing synthetic organic compounds harbor large degradative plasmids (15, 22, 36). The degradative plasmids which are involved in the catabolism of toluene and naphthalene are the best-characterized plasmids among this group (11, 16, 18, 26, 27, 35). Recently, plasmids involved in the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) have also been under considerable investigation (1, 8-10, 29). These plasmids range from 80 to 120 kilobase pairs (kb) in size and have been predominantly isolated from members of the genus *Alcaligenes*, obtained from soil or aquatic environments (1, 8). One of these plasmids, pJP4 from *Alcaligenes eutrophus*, has been investigated more extensively (9, 10, 29).

Several other microorganisms belonging to a variety of different bacterial genera, such as *Acinetobacter*, *Arthrobacter*, *Corynebacterium*, *Flavobacterium*, and *Pseudomonas*, have also been shown to degrade 2,4-D and related phenoxyacetates (2, 4, 8, 12, 14, 28, 30-32, 34; A. W. Ogram, M.S. thesis, University of Florida, Gainesville, 1984). Unlike *Alcaligenes*, little information is available on the mechanism of 2,4-D degradation by these microorganisms. Furthermore, the occurrence and nature of the 2,4-D-degradative plasmids in these microorganisms are not known. It should be of interest to isolate the plasmids from microorganisms other than *Alcaligenes* spp. and to compare them with the well-characterized plasmid pJP4. This may help us to understand how plasmids with the same function have evolved in different microbial backgrounds.

In this paper, we present initial results of a study designed to investigate the mechanism of degradation of 2,4-D in a previously isolated *Flavobacterium* sp. (Ogram, M.S. thesis). This bacterium resembles *A. eutrophus* in degradative properties (8) and was found to harbor a degradative plasmid, pRC10. This plasmid showed considerable differences in size and restriction patterns from plasmid pJP4 (from *A.*

eutrophus). We report the physical map of pRC10 and compare its regions of homology with those of pJP4, as well as the cloning of its degradative genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacteria and plasmids used in this study are listed in Table 1.

Media and growth conditions. The *Flavobacterium* sp. and *Alcaligenes eutrophus* strains were grown at 28°C aerobically in minimal medium (MM) (5) supplemented with 2,4-D or other compounds as the sole source of carbon. *Escherichia coli* strains were grown in LB medium (5). The medium used for growing *Pseudomonas putida* has been described elsewhere (34). A nalidixic acid-resistant mutant of *P. putida* was obtained by selecting resistant colonies on heavily inoculated LB agar plates containing nalidixic acid (50 µg/ml) (Sigma Chemical Co.). The autoclaved medium was supplemented with antibiotics and metal salts by using sterile stock solutions as needed.

Detection and isolation of plasmid DNA. An estimated 10⁸ exponentially growing cells were suspended in E buffer (21) containing 25% sucrose, lysozyme (2 mg/ml) and RNase (100 µg/ml). The cell suspension was then poured into the front wells of an 0.7% agarose gel in E buffer. A second series of wells immediately behind the front wells were filled with a lysis solution containing 1% sodium dodecyl sulfate (SDS) and 0.5% agarose in E buffer. Electrophoresis was first carried out for 30 min at 20 V for electrophoretic transfer of SDS into the front wells for cell lysis and then for 3 h at 100 V for fractionation of the DNA. *E. coli* plasmids were isolated by the method of Birnboim and Doly (3). Plasmid DNA from the *Flavobacterium* sp. and *A. eutrophus* was isolated by a previously described method (6).

The *Flavobacterium* sp. was cured of the plasmid by treating the exponentially growing cells (in LB) with streptomycin (100 µg/ml) overnight. The cells were then diluted, plated on LB agar plates, and checked for growth on MM containing 2,4-D and for the presence of the plasmid.

Analysis of DNA fragments and restriction mapping. Re-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Plasmid	Relevant traits	Source or reference
<i>Flavobacterium</i> sp.			
50001	pRC10	2,4-D ⁺ Hg ^r	Ogram, thesis
50183	Cured	2,4-D ⁻ Hg ^s	This study
<i>Alcaligenes eutrophus</i>			
50007 (JMP134)	pJP4	2,4-D ⁺ Hg ^r	J. M. Pemberton (8)
50009 (JMP228)	Cured	2,4-D ⁻ Hg ^s Str ^r	J. M. Pemberton
<i>Escherichia coli</i>			
50008 (HB101)		<i>leu-6 pro thi-2 recA lacY hsdR rpsL</i>	21
S17-1		Mobilizing strain	R. Simon (23)
<i>Pseudomonas putida</i>			
PRS2015		Prototroph	A. M. Chakrabarty (33)
Plasmids			
pLO1193		Broad host range, <i>mob</i> (RSF1010), <i>mob</i> (RP4), Tc ^r Cm ^r	L. O. Ingram (7)
pKL400		Ap ^r <i>galK</i>	K. McKenney
pRC220		<i>Hind</i> III-B of pJP4 cloned into pBR322	This study
pRC10EA		<i>Eco</i> RI-A of pRC10 cloned into pBR322	This study
pRC10AF		Ap ^r <i>tfdA</i>	This study
pRC10PF		Tc ^r <i>tfdB</i>	This study
pRC301		Tc ^r <i>tfdA tfdB</i>	This study
pRC305		Cm ^r <i>tfdA</i>	This study
pRC310		Cm ^r <i>tfdB</i>	This study

striction endonucleases were purchased from U.S. Biochemical Corp., Cleveland, Ohio. Enzyme digestions were performed as specified by the manufacturer. Samples were subjected to agarose gel electrophoresis for fractionation of DNA. Sizes of restriction fragments were determined with reference to bacteriophage lambda DNA digested with *Hind*III or with both *Hind*III and *Eco*RI. Mapping of plasmid pRC10 was carried out by comparing the results of digestion with pairwise combinations of enzymes as well as partial digestions.

Ligation, transformation, and agarose gel electrophoreses were performed as described previously (21). For transfer of plasmids into microorganisms by conjugation, a procedure similar to that described by Simon et al. (23) was used. Overnight-grown cells were diluted 1:50 in 10 ml of the medium in a 250-ml flask and then incubated for 5 h at 30°C. Donor and recipient cells of approximately the same density were mixed in Eppendorf tubes (ratio, 1:1) and centrifuged for 30 s. The pellet was carefully suspended in 50 µl of medium, transferred onto nitrocellulose filters (pore size, 0.2 µm; diameter, 25 mm) which had been placed on prewarmed fresh plates, and incubated at 30°C for matings. After overnight incubation the cells were suspended, diluted in the medium, and plated on solid medium containing appropriate antibiotics for donor counter selection.

Southern hybridization. Hybridization was carried out by a modified Southern method (26). To prepare probes, DNA fragments were labeled by nick translation (21) with [³²P]dATP as the labeled precursor.

Oxygen uptake assays. Cell cultures (250 ml) were grown to late log phase in MM containing 2,4-D for the *Flavobacterium* sp. and *A. eutrophus* JMP134. Strains harboring recombinant plasmids were grown overnight in LB medium containing appropriate antibiotics, harvested by centrifugation, washed three times in cold MM, and suspended in MM to an optical density at 420 nm (A_{420}) of 30. Enzyme analysis was performed at 30°C (29). Protein was determined by the method of Lowry et al. (20). The assay was carried out in a volume of 1.5 ml. The reaction mixture contained MM saturated with oxygen and cell suspension (1:9) to a final A_{420} value of 3. After the background level of oxygen uptake was estimated, the reaction was started by adding a final

concentration of 1 mM 2,4-D or 0.2 mM 2,4-dichlorophenol, and the linear decrease in oxygen concentration was measured over 15 min. Controls were run simultaneously with the strains without plasmids.

RESULTS

Properties of the *Flavobacterium* sp. The *Flavobacterium* sp. has been reported to degrade 2,4-D (Ogram, thesis). It was further observed that this bacterium was resistant to HgCl₂ (147 µM) and can also utilize additional chlorinated compounds, including 3-chlorobenzoate (3-CBA) and 2-methyl-4-chlorophenoxyacetic acid (MCPA), as a sole source of carbon. Such growth characteristics are similar to the properties of *A. eutrophus* (8). The *Flavobacterium* sp. was resistant to several antibiotics, including lincomycin (25 µg/ml), tetracycline (30 µg/ml), carbenicillin, kanamycin, mikamycin, and neomycin (50 µg/ml), and ampicillin (100 µg/ml), as well as to heavy metals such as cobalt, copper, and zinc (added as 40 µg of the chloride or sulfate salt per ml). Since *A. eutrophus* harbors a plasmid, pJP4 (9, 10, 29), coding for 2,4-D-degradative genes (Fig. 1), it was conceivable that the *Flavobacterium* sp. contained a similar plasmid. The presence of a single plasmid, pRC10 (Table 2), was confirmed by plasmid detection and isolation from this microorganism. The growth of the *Flavobacterium* sp. in the presence of streptomycin as a curing agent resulted in a putative cured strain (at a frequency of 1 to 2%) which had simultaneously lost both pRC10 and its ability to grow on the chlorinated compounds. However, the antibiotic resistance properties of the *Flavobacterium* sp. were not affected by curing of the plasmid. The cured strain (50183) did not revert to the 2,4-D⁺ phenotype at detectable levels as tested (<1 in 10¹⁰), nor did it retain the ability to utilize 3-CBA or MCPA or resistance to Hg. Also, enzyme activities for 2,4-D monooxygenase and 2,4-dichlorophenol hydroxylase were detected in wild-type but not in cured derivatives of the *Flavobacterium* sp. (Table 3).

Physical map of plasmid pRC10. By using a combination of restriction analysis, gene cloning, and Southern hybridization (as described below), a physical map of pRC10 was developed (Fig. 2). This map is relatively simpler than the

FIG. 1. Proposed 2,4-D-degradative pathway and the enzymes and genes encoding them (adapted from references 9, 10, 12, 14, 28, and 31).

TABLE 2. Comparison of restriction fragments of pRC10 and pJP4^a

Fragment	Size (kb)					
	<i>Bam</i> HI		<i>Eco</i> RI		<i>Hind</i> III	
	pRC10	pJP4	pRC10	pJP4	pRC10	pJP4
A	35.0	39.4	31.0	28.8	23.0	34.7
B	8.0	14.4	9.6	14.5	16.0	22.2
C	2.0	11.9	4.5	10.6	3.5	8.0
D		6.5		9.9	3.0	6.4
E		3.6		8.3		4.1
F		3.3		3.2		2.0
G				1.9		1.6
H				1.7		1.4
I				1.2		
Total	45.0	79.1	45.1	80.1	45.5	80.4

^a Sizes of DNA fragments of pRC10 were estimated by using bacteriophage lambda DNA cleared with restriction enzymes as standards as described in the text. Sizes of DNA fragments of pJP4 were taken from Don and Pemberton (9).

map of pJP4. As with its relative size, pRC10 has approximately half as many restriction sites as does pJP4.

DNA hybridization. The homology between pRC10 and pJP4, a well-characterized 2,4-D-degradative plasmid (10, 29), was investigated by Southern hybridization. First, pRC10 was used as a probe, and it hybridized to all of the restriction fragments of pJP4 except fragments *Bam*HI-D, *Eco*RI-A and -G, *Hind*III-A and -E to -H, and *Pst*I-C, -F, -G, and -H. This indicated that pRC10 had homology only with the regions of pJP4 encoding 2,4-D-degradative enzymes (data not shown). Subsequently, the location of the degradative genes on plasmid pRC10 was determined. This was accomplished by preparing two probes comprising isolated DNA fragments *Eco*RI-B and *Eco*RI-*Xba*I (from *Hind*III-B) of pJP4, which contain *tfdB*-*tfdF* genes and a part of the *tfdA* gene, respectively, and hybridizing them with the Southern blots of pRC10. It was found that the former probe hybridized with several of the restriction fragments of pRC10. However, the fragment *Eco*RI-A of pRC10 alone showed the most homology (Fig. 3A and B). This probe also hybridized weakly with fragments *Eco*RI-C and -D of pJP4, because the DNA used for preparation of the probe was slightly contaminated with these fragments. The fragments *Eco*RI-C and -D of pJP4 did not hybridize with pRC10 (data not shown). The

TABLE 3. Enzyme activities of microorganisms

Strain	Plasmid	Relative activity (%) ^a	
		2,4-D monooxygenase	2,4-Dichlorophenol hydroxylase
<i>Flavobacterium</i> sp.			
50001	pRC10	100	100
50183	None	<1	<1
<i>A. eutrophus</i>			
JMP134	pJP4	90	92
JMP228	None	<1	<1
<i>P. putida</i> (N1d ⁺)			
	pRC301	90	41
	pRC305	80	ND ^b
	pRC310	ND	65

^a Expressed as percentages of the activities obtained with strain 50001 harboring plasmid pRC10.

^b ND, Not determined.

FIG. 2. Physical maps of plasmids pJP4 and pRC10, harboring 2,4-D-degradative genes in common. Regions of homology in pRC10 to *EcoRI* fragment B (*tfdB-tfdF*) and the *EcoRI-XbaI* segment from *HindIII*-B (part of *tfdA*) of pJP4 are shown by arrows.

second probe, (*EcoRI-XbaI*), which comprised only a part of *tfdA*, also hybridized to restriction fragments *AccI*-F, *EcoRI*-A, *HindIII*-A, *PstI*-C and -I, and *PvuI*-F of pRC10 (Fig. 3A and C).

Cloning and location of *tfd* genes. The fragment *EcoRI*-A of pRC10 which showed homology with pJP4 was cloned into pKL400 (Fig. 4). The resulting plasmid, designated pRC10EA, contained *tfdA* and *tfdB* and possibly other degradative genes as well (see below). *E. coli* carrying pRC10EA exhibited low levels of 2,4-D monooxygenase (*tfdA*) activity as judged by the *in vitro* release of ^{14}C from ^{14}C -labeled 2,4-D (data not presented). Since the fragment

EcoRI-A of pRC10 is quite large (31 kb) and also hybridized to fragment *EcoRI*-B of pJP4, it was reasonable to presume that it also harbored other degradative genes. The fragment *EcoRI*-A was then cloned into plasmid pLOI193 (Fig. 4) in *E. coli* S17-1, and the resulting plasmid (pRC301) was transferred into *P. putida* (Nld^r) by conjugation to study expression of the degradative genes. The results (Table 3) show that *P. putida* harboring pRC301 exhibited 2,4-D monooxygenase and 2,4-dichlorophenol hydroxylase activities (*tfdB*).

To subclone the *tfdA* and *tfdB* genes, the following strategy was used. First, the 2.3-kb fragment (*AccI*) isolated from fragment *EcoRI*-A of pRC10 which hybridized to the frag-

FIG. 3. Southern blot analysis for localization of degradative genes on pRC10. (A) DNA gel profile of plasmids pJP4 and pRC10. (B) Southern blot probed with *EcoRI* fragment B harboring the *tfdB-tfdF* genes of pJP4. To isolate probe DNA, *EcoRI* restriction digests of pJP4 were fractionated by agarose gel electrophoresis, and *EcoRI* fragment B was electroeluted. (C) Southern blot probed with the *EcoRI-XbaI* fragment containing part of the *tfdA* gene of pJP4. Plasmid pRC220 was used to isolate probe DNA by restriction digestion and electroelution. Plasmids: undigested (lanes 1, pJP4, and 2, pRC10) and digested with *AccI* (lanes A, pJP4, and B, pRC10); *EcoRI* (lanes C, pJP4, and D, pRC10); *HindIII* (lanes E, pJP4, and F, pRC10); *PstI* (lanes G, pJP4, and H, pRC10); and *PvuI* (lanes I, pJP4, and J, pRC10). Molecular weight standards, bacteriophage lambda DNA cleaved with *HindIII*, are shown in lane K. Arrow in panel b, lane 2, indicates the low-intensity band of pRC10.

FIG. 4. Construction of plasmids. The inserts are represented by heavy lines. The restriction sites shown are: A, *AccI*; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Pt, *Pst*I; Pv, *Pvu*I; and X, *Xba*I.

ment *Eco*RI-*Xba*I of pJP4 (Fig. 3C) was cloned into pBR322. The resulting plasmid, designated pRC10AF, was then digested with *Pst*I and cloned into the *Pst*I site of pLOI193 to generate plasmid pRC305 (Fig. 4). *P. putida* harboring pRC305 exhibited 2,4-D monooxygenase activity (Table 3). Second, the 1.4-kb fragment (*Pvu*I) again isolated from the digests of *Eco*RI-A of pRC10 was cloned into the *Pvu*I site of pBR322. The resulting plasmid, pRC10PF, was then digested with *Bam*HI and cloned into the *Bam*HI site of pLOI193 to generate plasmid pRC310 (Fig. 4). *P. putida* harboring pRC310 showed 2,4-dichlorophenol hydroxylase activity (Table 3).

DISCUSSION

We have isolated a new degradative plasmid, pRC10, from a *Flavobacterium* sp. capable of metabolizing 2,4-D and other chlorinated compounds such as 3-CBA and MCPA. The size and restriction pattern (Table 2 and Fig. 3A) of this plasmid were found to be considerably different from those of previously reported plasmids with similar functions isolated from species of *Alcaligenes* (1, 8). However, plasmid pRC10 showed homology (Fig. 2) with regions of pJP4 (fragments *Eco*RI-B and *Hind*III-B) coding for the degradative genes (*tfd*ABCDEF) (9, 10, 29). When these fragments or their parts were used as probes containing the *tfd* genes, they all hybridized with fragment *Eco*RI-A of pRC10 (Fig. 3). Cloning of fragment A, first into pKL400 (plasmid pRC10EA) and then into pLOI193 (plasmid pRC301), revealed that it carried at least the first two genes (*tfdA* and *tfdB*) of the pathway of 2,4-D catabolism in this *Flavobac-*

terium sp. However, the restriction map of this fragment did not match the fragments (*Eco*RI-B and *Hind*III-B) of pJP4 which harbor the known *tfd* genes (Fig. 4). From this fragment, we were able to subclone two genes, *tfdA* and *tfdB*, by using a broad-host-range plasmid, pLOI193. The cloned genes were expressed in *P. putida* (Table 3). To determine whether the remaining *tfd* genes are also present on the same fragment requires further investigation. It would be interesting to determine differences in structure and expression of the gene (i.e., *tfdA*) coding for an identical function from the two different bacterial sources.

Plasmid pRC10 did not hybridize with regions of pJP4 (fragments *Eco*RI-A and *Hind*III-A) which have been reported to harbor the conjugative, incompatibility, replication, and maintenance functions (9). Unlike pJP4, the *tfd* genes on pRC10 were present in a region with fewer restriction sites (Table 2 and Fig. 3). This suggests that pRC10 may be different from pJP4 not only in the functions listed above, but also in the restriction map of its *tfd* genes. Interestingly, the cloning results support such an hypothesis. The DNA fragment encoding *tfdA* from pRC10 hybridized with the part of *tfdA* from pJP4 used as a probe. However, this fragment also had a different restriction map (manuscript in preparation) from that of the gene recently reported from pJP4 (29).

Degradative plasmids such as TOL, NAH, SAL, etc., of *Pseudomonas* spp. demonstrate appreciable homology with one another, suggesting evolutionary interrelationships (13, 17, 19). More recently, TOL genes have also been localized on the chromosome of a strain of *P. putida* (24). The chromosomal DNA segment conferring the degradative genes was reported to be almost identical to the same region found in the TOL plasmid pWW0. Relationships among the other isofunctional plasmids, such as those involved in the degradation of 2,4-D, evolved in different microbial backgrounds, are not well understood. A comparative study of pRC10 and pJP4 should further our understanding of the unique differences and evolutionary relationships between such plasmids.

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