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Duplication of a 2,4-Dichlorophenoxyacetic Acid Monooxygenase Gene in *Alcaligenes eutrophus* JMP134(pJP4)

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The *Alcaligenes eutrophus* JMP134 plasmid pJP4 contains genes necessary for the complete degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3-chlorobenzoic acid. *tfdA* encodes 2,4-D monooxygenase, the initial enzyme in the 2,4-D catabolic pathway. The *tfdA* locus has recently been localized to a region on pJP4 13 kilobases away from a cluster of five genes, *tfdB* to *tfdF*, which encode the enzymes responsible for the further degradation of 2,4-D to chloromaleylacetic acid (W. R. Streber, K. N. Timmis, and M. H. Zenk, J. Bacteriol. 169:2950–2955, 1987). A second, dissimilar locus on pJP4, *tfdAII*, has been observed which encodes 2,4-D monooxygenase activity. Gas chromatographic analysis of the 2,4-D metabolites of *A. eutrophus* harboring pJP4 or subclones thereof localized *tfdAII* to within a 9-kilobase *Sst*I fragment of pJP4 which also carries the genes *tfdBCDEF*. This fragment was further characterized in *Escherichia coli* by deletion and subcloning analysis. A region of 2.5 kilobases, adjacent to *tfdC*, enabled *E. coli* extracts to degrade 2,4-D to 2,4-dichlorophenol. Hybridization under low-stringency conditions was observed between *tfdA* and *tfdAII*, signifying that the 2,4-D monooxygenase gene was present as two related copies on pJP4.

Halogenated aromatics have become increasingly prevalent in our environment as a result of industrial pollution and herbicide use in agriculture. Several genera of soil bacteria are known to degrade and utilize a variety of these compounds via the presence of partial or complete catabolic pathways encoded by large plasmids. The enzymes responsible for catalyzing the initial steps in the degradative pathways are of particular interest because of their potential use in the development of bacteria and plants capable of degrading and detoxifying xenobiotic compounds (10, 17, 20).

The plasmid pJP4 of *Alcaligenes eutrophus* JMP134 encodes enzymes for the catabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) and 3-chlorobenzoate (3-CBA) (5, 7). The first enzyme in the 2,4-D catabolic pathway cleaves the ether linkage of 2,4-D to produce glyoxylate and 2,4-dichlorophenol (2,4-DCP). Five genes, *tfdB* to *tfdF*, converting 2,4-DCP to chloromaleylacetic acid, are located on a 6.3-kilobase (kb) region of *Eco*RI fragment B (7) (Fig. 1). A gene encoding 2,4-D monooxygenase, *tfdA*, has been localized to a region 13 kb distant from the *tfdBCDEF* genes (19) (Fig. 1).

When grown on 3-chlorobenzoate as a sole carbon source, pJP4 can undergo a wide variety of genetic rearrangements (10). This may reflect a capacity of the bacterium to adapt itself to grow more efficiently under different environmental conditions. Such rearrangements include deletions and duplications of specific regions of the plasmid. We have found a second locus which encodes an additional 2,4-D monooxygenase activity in pJP4. We have designated the enzyme 2,4-D monooxygenase II and the gene *tfdAII*. This locus was found adjacent to the gene encoding chlorocatechol 1,2-dioxygenase, *tfdC*. The *tfdAII* locus is clustered with genes *tfdBCDEF* on plasmid pJP4.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction enzymes were purchased from Bethesda Research Laboratories, International Biotechnologies Inc., and New England BioLabs. 2,4-D, 2,4-DCP, and antibiotics were purchased from Sigma Chemical Co. Pesticide-grade hexanes were purchased from Fisher Chemical Co.

Strains and plasmids. *A. eutrophus* JMP134 contains plasmid pJP4 and degrades 2,4-D and 3-CBA (6). Strain JMP228r, a rifampin-resistant derivative of JMP134 that has been cured of pJP4, was provided by J. M. Pemberton. *Escherichia coli* DH1 (12) was used for cloning experiments. *E. coli* HB101 (2) was used as a host for pJP4 subclones to allow gas chromatographic analysis of 2,4-D monooxygenase activity in *E. coli*. Plasmids pUC19 (21) and Bluescript M13 phagemid (Stratagene) were used for cloning in *E. coli*, while pRK404 (4) and pKT231 (1) were used as mobilizable broad-host-range vectors. *E. coli* RR1 harbored the mobilizing plasmid pRK2013 (4). Plasmid pRD25, which carries the genes necessary for growth on 3-CBA, contains a 9.0-kb *Sst*I DNA fragment from *Eco*RI fragment B of pJP4 (7). Plasmid pRE2.1C contains the 1.5-kb *Bam*HI-*Eco*RI fragment from *Eco*RI fragment E of pJP4 cloned into pUC19.

Media and culture conditions. Bacteria were grown in Luria broth (LB) (14). Solid medium contained 1.5% agar. *A. eutrophus* strains were grown at 29°C. *E. coli* strains were grown at 37°C. Antibiotic concentrations for selection were: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; rifampin, 70 μg/ml; and tetracycline, 15 μg/ml for *E. coli* and 50 μg/ml for *A. eutrophus* strains.

DNA manipulation. Restriction endonuclease cleavages, ligations, DNA isolation from bacteria, and agarose gel electrophoresis were performed as described by Maniatis et al. (14). Transformation of *E. coli* was performed by the method of Hanahan (12).

Conjugative transfer. To mobilize plasmids into *A. eutrophus* JMP228r, a sterile filter disk was inoculated with 50 μl each of overnight cultures of *A. eutrophus* JMP228r, *E. coli* DH1 containing the desired plasmid(s), and *E. coli* RR1(pRK2013). The filter disk was then incubated for 24 h on an LB agar plate at 29°C. Transconjugant *A. eutrophus* JMP228r cells were selected on LB agar with rifampin (70 μg/ml) and tetracycline or kanamycin (50 μg/ml).

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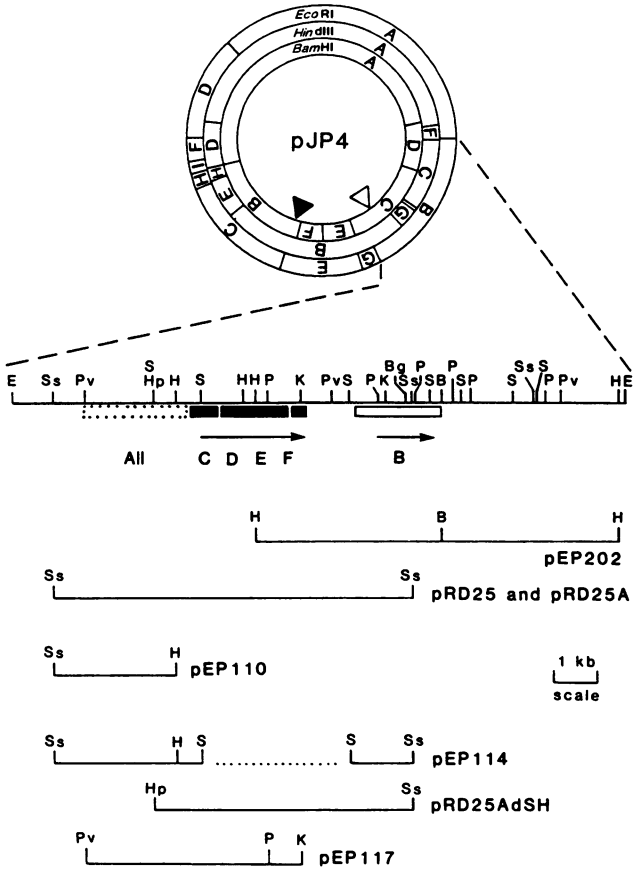

FIG. 1. Physical map of pJP4 from *A. eutrophus* JMP134. The solid arrowhead indicates the location of *tfdA*, and the open arrowhead shows the position of *tfdAII*. Also shown is the restriction map of *Eco*RI fragment B from pJP4 and the subclones used in the localization of *tfdAII*. The broken line in clone pEP114 indicates the *Sal*I fragment that has been deleted. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Ss, *Sst*I. The broken-line box indicates the location of *tfdAII*, the solid boxes indicate the locations of *tfdC*, *tfdD*, *tfdE*, and *tfdF*, and the open box shows the location of *tfdB* (7; this study). Directions of transcription are indicated by the arrows (7; E. Perkins, unpublished data).

DNA-DNA hybridization. DNA restriction fragments were transferred from agarose gels to Magna Nylon66 paper (MSI, Honeoye Falls, N.Y.) by the method of Southern (18). DNA restriction fragments were purified by three passages of the fragments through 0.7% low-melting-point agarose. DNA fragments used for the generation of probe were isolated from low-melting-point agarose in an NACS column (Bethesda Research Laboratories) according to the manufacturer's instructions. Radiolabeled probe was generated by hexamer labeling (8) of the isolated fragment. Probe was hybridized to the filter in 7% sodium dodecyl sulfate–0.25 M $NaPO_4$ (pH 6.8)–10 mM EDTA and incubated for 3 h at 55°C with shaking. Filters were transferred to a solution containing 15 mM NaCl, 1.5 mM sodium citrate, 0.1% sodium dodecyl sulfate, and 1 mM EDTA and then washed for 1 h at 50 or 25°C. Washed blots were then exposed to X-ray film.

Gas chromatographic analysis of 2,4-D monooxygenase activity. For analysis of *tfdA* gene product activity in *A. eutrophus* JMP228r, 1.5-ml overnight cultures were inoculated into 250 ml of LB medium with 1 mM 2,4-D and appropriate antibiotics. Cultures were grown at 29°C on a rotary shaker for 18 to 20 h. Portions of the cultures were diluted and plated on LB agar to determine their viable-cell count. All cultures contained between 1×10^9 and 2×10^9 cells per ml at the time of analysis. Culture samples were centrifuged, and the cell pellet was discarded. The supernatant was divided equally between two 250-ml flasks, and 50 ml of pesticide-grade hexanes was added to each flask and mixed. The aqueous and organic phases were separated by centrifugation at 27,000 × *g* for 10 min. The organic phase was removed, and a second extraction of the aqueous phase was done as described above. The organic phases were pooled and evaporated under a stream of N_2 gas to a volume of 50 ml. To remove the large amount of 2,4-D, which could interfere with analysis, the hexane fraction was extracted twice with 2.5 ml of 0.1 M KOH, The aqueous KOH phases were removed to a separate tube, neutralized with 1 M HCl, and extracted twice with 10 ml of pesticide-grade hexanes. The organic phase was evaporated to 1 ml under nitrogen gas, and 1 μl was used for gas chromatographic analysis.

Analysis was performed on an SBP-5 30-m fused silica column (0.25-mm inner diameter; Supelco) connected to a fused silica precolumn (1 m, 0.25-mm inner diameter) by a capillary butt-end connector (Supelco). Conditions were as follows: initial temperature, 30°C; hold 4 min; ramp rate of 4°C per min to 250°C; then hold at 250°C for 10 min. Underivatized 2,4-DCP was used as a standard (retention time, 20.57 min). The presence of 2,4-DCP was confirmed by analysis of silylated derivatives of both the standard and the extracted samples (data not shown). The amount of 2,4-DCP was quantified by calculating the peak area and comparing it with that of the standard at a known concentration.

Analysis of 2,4-D monooxygenase activity in *E. coli*. Since *E. coli* is not permeable to 2,4-D, a cell-free assay was developed to monitor the activity of 2,4-D monooxygenase II. Each set of assays included samples of HB101 harboring pRD25A as a positive control and HB101 with no plasmid as a negative control. Bacteria from a 1-ml overnight culture in 20 ml of LB with appropriate antibiotics were grown at 37°C to an A_{600} of 0.5 to 0.8. Cells were collected by centrifugation, washed once with 0.5 ml of reaction buffer (10 mM K_2HPO_4-KH_2PO_4, pH 7.2), and suspended in 0.5 ml of reaction buffer. Cells were incubated with 200 μg of lysozyme for 15 to 20 min at 0°C. β-Mercaptoethanol was added to the lysozyme-treated cells to a final concentration of 1 μM, and the suspension was sonicated for 1 min on ice. Cell debris was separated from the soluble extract by centrifugation in an Eppendorf microfuge at 4°C for 15 min. A 450-μl amount of the supernatant was transferred to a fresh tube, and 200 μg of NADPH and 5 μM 2,4-D were added. The reaction mixture was incubated for 16 to 20 h with shaking at 30°C. Incubated samples were extracted three times with an equal volume of pesticide-grade hexanes. Since low amounts of 2,4-D were used and hence would not interfere with analysis, the organic phases were pooled, evaporated under a stream of N_2 gas, and dissolved into 10 or 40 μl of hexanes. From 2 to 4 μl was used for gas chromatographic analysis of 2,4-D metabolites. Analysis was performed on an SBP-5 fused silica column (0.32-mm inner diameter, 30 m; Supelco) connected to a fused silica precolumn (1 m, 0.32-mm inner diameter) by a capillary butt-end connector (Supelco). Conditions were as follows: initial temperature 25°C, hold 4 min, ramp rate of 10°C per min to 250°C, and then hold at 250°C for 10 min. Underivatized 2,4-DCP was used as a standard (retention time, 13.88 min).

All sample analysis was performed on a Perkin-Elmer Sigma 3-B gas chromatograph equipped with an electron

capture detector. Helium, at a flow rate of 22 ml/min, was used as the carrier gas, and make-up gas to the detector was 95% argon and 5% methane. The injector oven temperature was 250°C, and the detector temperature was 300°C.

RESULTS

Subcloning and deletion mapping of *tfdAII*. A detailed restriction map of *Eco*RI fragment B was derived and is presented in Fig. 1 along with a physical map of plasmid pJP4. The *Eco*RI B fragment is sufficient for growth of *A. eutrophus* on 2,4-D as a sole carbon source, and catabolic genes encoding the enzymes able to degrade 2,4-DCP to chloromaleyacetic acid have been localized to this fragment (7) (Fig. 1).

Gas chromatographic analysis of 2,4-D metabolites of *A. eutrophus* JMP134 cured of pJP4, JMP228r, indicated that only 3 ng of 2,4-DCP was produced per ml of culture. *A. eutrophus* JMP134(pJP4) produced 925 ng of 2,4-DCP per ml of culture under the same conditions. This suggests that either a gene encoding a 2,4-D monooxygenase activity or a transcriptional regulatory factor required in *trans* must be located on *Eco*RI fragment B. To localize this activity further, overlapping regions of *Eco*RI fragment B were cloned and assayed for their ability to encode or promote 2,4-D monooxygenase activity.

Initial screening for 2,4-D monooxygenase activity was performed in *A. eutrophus* JMP228r. To determine the general location, pRD25 and pEP202 (Fig. 1) were mobilized into *A. eutrophus* JMP228r and analyzed for 2,4-DCP production. No significant activity was found in *A. eutrophus* JMP228r harboring pEP202. In contrast, 29 ng of 2,4-DCP was produced per ml of culture by *A. eutrophus* JMP228r harboring pRD25—nearly 10 times the background level of 2,4-DCP was produced. *tfdAII* must then reside on the 9.0-kb *Sst*I fragment of *Eco*RI-B near *tfdC* (the gene for chlorocatechol 1,2-dioxygenase) or near *tfdF* (the gene for *trans*-chlorodiene-lactone isomerase) (Fig. 1).

Growth of *A. eutrophus* JMP228r with 2,4-D as a sole carbon source. Our data indicate that pRD25 may contain the gene encoding the second 2,4-D monooxygenase and the genes expressing enzymes required for the catabolism of 3-CBA (7) (Fig. 1). Clone pEP202 should carry a functional 2,4-DCP hydroxylase gene, *tfdB* (7) (Fig. 1). If all of these genes are present and expressed, then coinoculation of a liquid culture containing M-9 salts (14) and 1 mM 2,4-D with *A. eutrophus* JMP228r harboring pRD25 and *A. eutrophus* JMP228r harboring pEP202 should allow growth. In a culture containing 2,4-D as a sole carbon source, growth was observed only when both strains were present. Gas chromatographic analysis of the spent growth medium indicated accumulation of 2,4-DCP (data not shown).

Cloning of *tfdAII* in an *E. coli* background. Most methods of DNA manipulation have been optimized for use in *E. coli*; therefore we developed an in vitro assay for the detection of 2,4-D monooxygenase activity in *E. coli*. Strain HB101 with no plasmid produced 80 pg of 2,4-DCP per ml of culture. 2,4-D monooxygenase activity, although lower than in *A. eutrophus*, was easily detectable in HB101 harboring pRD25A, which produced 2,820 pg of 2,4-DCP per ml of culture (Fig. 2). Presumably this was because the *Sst*I fragment of pRD25 was cloned into the high-copy-number vector pUC19, resulting in amplification of *tfdAII* expression.

Further efforts were concentrated on the 4-kb region adjacent to *tfdC*. Don et al. (7) localized the start of *tfdC* to within approximately 500 bp of the left *Hin*dIII site (Fig. 1). The 3.0-kb *Sst*I-*Hin*dIII fragment next to *tfdC*, when cloned into pUC19 (pEP110), was found to have no detectable 2,4-D monooxygenase activity. An *Hpa*I deletion of pRD25A, pRD25AdSH (Fig. 1), also eliminated 2,4-D monooxygenase activity. This confirmed the location of *tfdAII* near *tfdC*. Cloning of a partial *Sal*I digest of pRD25A resulted in pEP114 (Fig. 1), which retained 2,4-D monooxygenase activity, producing 820 pg of 2,4-DCP per ml of culture. Further subcloning of a 4.7-kb *Pvu*II-*Kpn*I fragment of pRD25A into a Bluescript phagemid vector (pEP117) also retained a functional 2,4-D monooxygenase gene, producing 340 pg of 2,4-DCP per ml of culture. These deletions and subclones defined a 2.5-kb region located immediately upstream of *tfdC* on *Eco*RI fragment B of pJP4 which encodes 2,4-D monooxygenase activity.

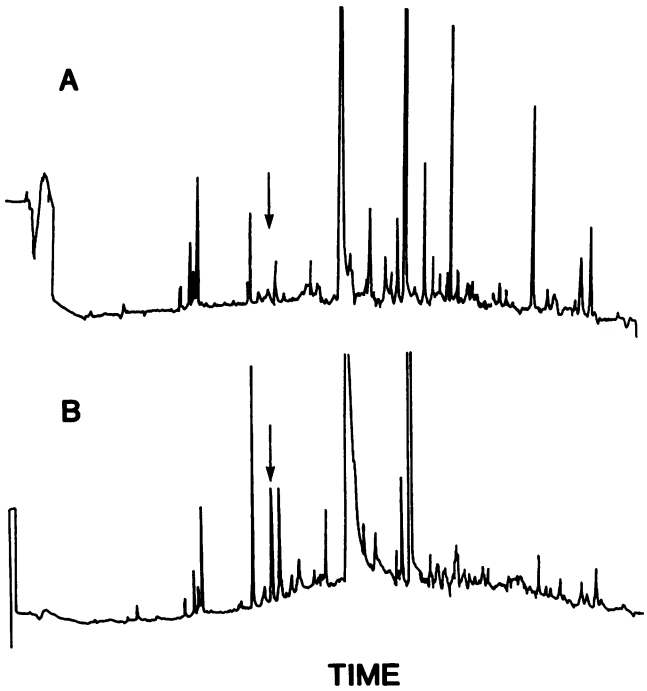

FIG. 2. Chromatographic analysis of 2,4-D monooxygenase expression in *E. coli*. The arrow indicates the position of the peak representing 2,4-DCP. (A) *E. coli* HB101 with no plasmid; (B) *E. coli* HB101 with pRD25A.

DNA-DNA hybridization of *tfdAII* with *tfdA*. The 828-bp *Xba*I-*Eco*RI restriction fragment from pRE2.1C, which carries the 2,4-D monooxygenase I gene, was probed with the 3-kb *Sst*I-*Hin*dIII restriction fragment from pRD25A to determine whether any detectable similarity existed between *tfdA* and *tfdAII*. When probed under moderately stringent conditions, with a wash at 50°C, no hybridization was observed. Only under low-stringency conditions, washing at 25°C, was hybridization observed between *tfdAII* and the fragment containing *tfdA* (Fig. 3). The 640-bp *Bam*HI-*Xba*I fragment of pRE2.1C showed no hybridization. The 2.7-kb band in pRE2.1C and bands in the 1-kb ladder lane were due to pUC19 or homologous sequences.

DISCUSSION

We have described the localization and cloning of a second locus on plasmid pJP4 of *A. eutrophus* JMP134 which encodes a 2,4-D monooxygenase activity. This gene, *tfdAII*,

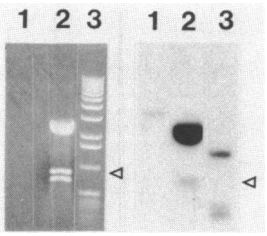

FIG. 3. Southern hybridization of *tfdA* with *tfdAII*. On the left is the agarose gel, which was transferred to nylon filter on the right. Probe was the 3-kb *Sst*I-*Hin*dIII fragment from pRD25A, as described in the text. Lanes: 1, purified 3-kb *Sst*I-*Hin*dIII fragment of pRD25A; 2, *Xba*I-*Eco*RI-digested pRE2.1C; 3, 1-kb ladder (Bethesda Research Laboratories). Arrows indicate the position of the 828-bp fragment containing *tfdA*.

is located 13 kb away from *tfdA* as described by Streber et al. (19) and lies immediately adjacent to the operon of *tfdCDE*. *tfdAII* occupies a region of at most 2.5 kb. Expression of *tfdAII* in *E. coli* results in 2,4-D monooxygenase activity and converts 2,4-D into 2,4-DCP. *tfdAII* has also been expressed in an *E. coli* in vitro transcription-translation system (data not shown). These results suggest that *tfdAII* encodes a 2,4-D monooxygenase rather than a *trans*-acting regulator of a chromosomally encoded enzyme. It is not known to what extent *tfdA* and *tfdAII* are interchangeable. The inability of other workers to inactivate 2,4-D monooxygenase activity with single transposon insertions (7, 19) may reflect the ability of *tfdA* to functionally replace *tfdAII*, and vice versa.

Under severe metabolic pressures, pJP4 can undergo extensive rearrangements (10, 11). These rearrangements include large deletions and duplications. A duplication of the 2,4-D monooxygenase gene could have resulted in the presence of two functional copies. The two genes *tfdA* and *tfdAII* do not appear to be simple duplications of a single gene. Both the restriction enzyme maps of *tfdA* and *tfdAII* and DNA-DNA hybridization experiments indicate a significant degree of difference between the two genes. Recent evidence also suggests that two dissimilar chlorocatechol 1,2-dioxygenase genes are present on pJP4, one encoded by *tfdC* (9). It is not known whether the second gene is expressed. A similar phenomenon has been found in toluene-degradative (TOL) plasmids of *Pseudomonas* spp. Two functional, genetically dissimilar copies of catechol 2,3-oxygenase genes exist on several different TOL plasmids (3, 13). Genes encoding nylon-degrading enzymes have been found to have been duplicated on the nylon oligomer-degradative plasmid pOAD2 (16).

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