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Identification and Characterization of a New Plasmid Carrying Genes for Degradation of 2,4-Dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90

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***Pseudomonas cepacia* CSV90 is able to utilize 2,4-dichlorophenoxyacetate (2,4-D) and 2-methyl-4-chlorophenoxyacetate as sole sources of carbon and energy. Mutants of the strain CSV90 which had lost this ability appeared spontaneously on a nonselective medium. The wild-type strain harbored a 90-kb plasmid, pMAB1, whereas 2,4-D-negative mutants either lost the plasmid or had a 70-kb plasmid, pMAB2. The plasmid pMAB2 was found to have undergone a deletion of a 20-kb fragment of pMAB1. The plasmid-free mutants regained the ability to degrade 2,4-D after introduction of purified pMAB1 by electroporation. Cloning in *Escherichia coli* of a 10-kb *Bam*HI fragment from pMAB1, the region absent in pMAB2, resulted in the expression of the gene *tfdC* encoding 3,5-dichlorocatechol 1,2-dioxygenase. After subcloning, the *tfdC* gene was located in a 1.6-kb *Hin*dIII fragment. The nucleotide sequence of the *tfdC* gene and the restriction map of its contiguous region are identical to those of the well-characterized 2,4-D-degradative plasmid pJP4 of *Alcaligenes eutrophus*, whereas the overall restriction maps of the two plasmids are different. The N-terminal 44-amino-acid sequence of the enzyme purified from the strain CSV90 confirmed the reading frame in the DNA sequence for *tfdC* and indicated that the initiation codon GUG is read as methionine instead of valine.**

Developments in the fields of agriculture and industry have introduced new organic pollutants which find use as refrigerants, fire retardants, paints, solvents, herbicides, and pesticides. Most of these compounds are recalcitrant to biodegradation, and their entry into the biosphere poses many challenges to the existing microflora and even to life on this planet in general (26). Some of these compounds are too toxic to be degraded by microorganisms (20); however, others are degraded and utilized as carbon sources. Several bacteria have been isolated that are able to degrade a wide range of compounds, including chlorinated aromatic compounds such as chlorinated benzoic acids (5), chlorinated phenols (28), chlorinated benzenes (30), and chlorinated biphenyls (1, 14). Recent reviews by Reineke and Knackmuss (25) and Chaudhry and Chapalamadugu (6) present a detailed and up-to-date account of the degradation of halogenated aromatic compounds by various microorganisms.

In most of these organisms, the degradative-pathway genes are carried on plasmids. The roles of these plasmids and their characteristics have been detailed by Chakrabarty (4). Some of the well-characterized plasmids are the xylene- and toluene-degradative plasmid TOL and the naphthalene-degradative plasmid NAH7 (11, 15, 32).

One chlorinated compound, 2,4-dichlorophenoxyacetate (2,4-D), has been widely used as a herbicide and has become a substantial environmental pollutant. This compound is degraded by microorganisms and utilized as a carbon source (12). 2,4-D-degrading bacteria from different genera have been reported, and some of these bacteria carry plasmids which code for 2,4-D metabolic genes (27). The idea of plasmid involvement in the degradation of 2,4-D comes from the report of Pemberton and Fisher (21). Further work by Pemberton et al. and others resulted in the detailed characterization of the 2,4-D-degradative plasmid pJP4 from *Alcaligenes eutrophus* JMP134, encoding resistance to $HgCl_2$ as well. The plasmid has a broad host range, is self-transmissible, and belongs to the *incP* group of plasmids. This 80-kb plasmid has been studied in detail, and several genes on the plasmid have been cloned and sequenced previously (9, 10, 22, 29). Recently, Chaudhry and Huang (7) reported a *Flavobacterium* sp. carrying 2,4-D-degrading genes on a 45-kb plasmid, pRC10, which is smaller than pJP4.

The degradation of 2,4-D in pseudomonads has not been well studied in terms of plasmid characterization, e.g., whether the same degradative plasmids are transferred among the various bacterial populations which are closely related or far from each other in their evolutionary background. The study of the nature of these plasmids, which are similar in function but differ in their hosts, would help us to understand the origin of these plasmids and their distribution within the microbial populations and the arrangement of various genes on these plasmids.

In a recent paper, we have reported purification of 3,5-dichlorocatechol 1,2-dioxygenase from *Pseudomonas cepacia* CSV90, which utilizes 2,4-D as the sole source of carbon and energy (2, 23, 24). We report here the isolation and characterization of a new plasmid, pMAB1 (90 kb), from *P. cepacia* CSV90. This plasmid was different from that of the well-characterized plasmid pJP4 in restriction fragment analysis. The physical maps of the plasmid pMAB1 and its deletion derivative pMAB2 and cloning and sequencing of the gene *tfdC* encoding 3,5-dichlorocatechol 1,2-dioxygenase are reported.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Bacterial strains		
P. cepacia CSV90	2,4-D$^+$, Hg^s, 3CB$^-$, pMAB1 carrier	2, 23, 24, this study
P. cepacia MAB1	2,4-D$^-$, derivative of CSV90, plasmid free	This study
P. cepacia MAB2	2,4-D$^-$, derivative of CSV90, pMAB2 carrier	This study
A. eutrophus JMP134	2,4-D$^+$, Hg^r, 3CB$^+$, pJP4 carrier	9
E. coli HB101	*hsdS20 recA13 ara-14 proA2 lacY1 galK2 rspL20*	19
E. coli RB791	*lacI*^{q}, W3100	3
Plasmids		
pMAB1	2,4-D$^+$	This study
pMAB2	2,4-D$^-$, pMAB1 derivative	This study
pJP4	2,4-D$^+$	9
pUC119	Ap^r	31
pKK223-3	Ap^r	Pharmacia, Inc.

[a] Abbreviations: 2,4-D$^+$ and 2,4-D$^-$, presence or absence of 2,4-D utilization, respectively; 3CB$^+$ and 3CB$^-$, presence or absence of 3-chlorobenzoate utilization, respectively; Hg^s, $HgCl_2$ sensitive; Hg^r, $HgCl_2$ resistant; Ap^r, ampicillin resistant.

MATERIALS AND METHODS

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

A 2,4-D-degrading organism was isolated from soil samples collected from an industrial effluent contaminated with 2,4-D, located near Bangalore in Karnataka State, India, by the enrichment culture technique (23, 24). The bacterium was characterized by conducting morphological, physiological, and biochemical tests and was found to be *Pseudomonas* species. Further identification at the species level was carried out by the German Collection of Microorganisms and Cell Cultures, Ltd., Braunschweig, Germany. The Hewlett-Packard Microbial Identification System identified this strain among 500 species as *P. cepacia* because of its typical fatty acid pattern. This strain has been designated *P. cepacia* CSV90.

Media and growth conditions. Agar plates containing 0.2% 2,4-D in a basal salts medium, pH 7.2 (D agar) (24), was used for utilization experiments and for maintenance of the strain CSV90. For utilization of other compounds, 2,4-D in D agar was replaced by other compounds at the same concentration. The ability to degrade 2,4-D was also determined by using 2,4-D indicator plates (18). L broth (19) was used as a liquid medium and was solidified with 1.5% agar to make L agar. Liquid cultures were grown routinely at 28°C for *P. cepacia* and *A. eutrophus* or at 37°C for *Escherichia coli* strains.

Curing of the strain CSV90. To test spontaneous curing, the cells of *P. cepacia* CSV90 from a single colony on D agar were inoculated in L broth, grown overnight with shaking, spread on L agar plates after appropriate dilution, and incubated. The resulting colonies were replica plated on D agar, and 2,4-D-negative clones were analyzed for the plasmid. To test mitomycin curing, a 0.1-ml sample from an overnight culture of CSV90 in L broth was inoculated into 5 ml of L broth containing 25 or 50 μg of mitomycin per ml. After incubation for 24 or 48 h, an aliquot was removed, diluted, and spread on L agar plates. The resulting colonies were picked to 2,4-D indicator plates to check the 2,4-D-degrading ability, and 2,4-D-negative clones were analyzed for plasmids.

Identification and isolation of plasmid DNA. For detection of any plasmid from *P. cepacia* and for large-scale preparation of plasmids from *P. cepacia* and *A. eutrophus*, the procedures described by Kado and Liu (16) and Kao et al. (17) were employed, with some modifications.

Electrotransformation of the cured strain of *P. cepacia*. The purified DNA of pMAB1 was used to transform *P. cepacia* MAB1, a cured strain of CSV90, using Gene Pulser (Bio-Rad, Richmond, Calif.). Electrocompetent cells prepared by extensive washing with a 10% glycerol solution were mixed with the plasmid pMAB1 and were electrically pulsed at 200 Ω, 25 μF, and 2.5 kV according to the supplier's instructions. The mixture was immediately transferred to 1 ml of ice-cold L broth containing 10% glycerol, incubated overnight, and spread on D agar.

DNA manipulations. Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Kyoto, Japan), and Bethesda Research Laboratories (Gaithersburg, Md.). All digestions were carried out according to the specifications of the suppliers. Restriction fragments were purified from agarose gels by using Gene Clean (Bio 101, Inc., La Jolla, Calif.). Mapping of the plasmids pMAB1 and pMAB2 was carried out by single and double digestions, and fragment sizes were calculated by the method described by Danna (8) with bacteriophage λ DNA cleaved with *Hin*dIII and *Eco*RI-*Hin*dIII as standard size markers. High-molecular-weight DNA markers from Bethesda Research Laboratories were also used as a standard.

For isolation of recombinant plasmids, restriction analysis, ligation, and transformation, established procedures were employed (19).

Enzyme assays. Strain CSV90 was grown in 100 ml of 0.2% 2,4-D in basal salts medium, pH 7.2. The *P. cepacia* strains MAB1 and MAB2 were grown in L broth containing 0.1% 2,4-D. *E. coli* strains carrying recombinant plasmids were grown in L broth containing appropriate antibiotics and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested, washed with 50 mM potassium phosphate buffer (pH 7.2), suspended in the same buffer, and disrupted by sonication. After centrifugation, the supernatant served as the crude extract for enzyme assays. The oxygenases were assayed by monitoring the oxygen consumption on an Oxygraph, model 53 (Yellow Springs). 3,5-Dichlorocatechol 1,2-dioxygenase, the *tfdC* gene product, was also assayed spectrophotometrically in 50 mM Tris-HCl buffer (pH 8.0) by monitoring the increase in the A_{268} due to the formation of 2,4-dichloromuconic acid from 3,5-dichlorocatechol. One unit of the enzyme activity was defined as the amount of enzyme that produces 1 μmol of 2,4-dichloromuconic acid per min under the standard assay conditions (2).

Nucleotide sequencing. The nucleotide sequences of both strands were determined with an ABI 373A automated DNA sequencer (Applied Biosystems Inc., Foster City, Calif.). The sequencing reactions were carried out according to the instructions of the supplier. The sequencing primers used were M13 primer M3 and M13 primer RV.

Chemicals. Phenoxyacetic acid, 2,4-dichlorophenoxyacetic acid, 3-chlorobenzoic acid, and $HgCl_2$ were purchased from Nakarai Tesque (Kyoto, Japan), 2-methyl-4-chlorophenoxyacetic acid was purchased from Tokyo Kasei (Tokyo, Japan), and mitomycin was purchased from Sigma. All other chemicals were of commercially available analytical grade.

Nucleotide sequence accession number. The sequence data reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under the accession number D16356.

RESULTS

Characterization of 2,4-D-degrading strain CSV90. Strain CSV90 could grow on 2,4-D up to a concentration of 0.25% as the sole source of carbon, and its metabolic pathway is proposed to be that diagrammed in Fig. 1. This strain could not grow on L broth containing 5 μg of $HgCl_2$ per ml and failed to utilize 3-chlorobenzoate but was able to grow on 2-methyl-4-chlorophenoxyacetate when used as the sole source of carbon (Table 1). Thus, these characteristics are different from those of *A. eutrophus* JMP134 in terms of $HgCl_2$ resistance and 3-chlorobenzoate utilization. Strain CSV90 was found to harbor a high-molecular-weight plasmid, designated pMAB1. In the case of *A. eutrophus* JMP134 harboring the plasmid pJP4, 2,4-D degradation and $HgCl_2$ resistance have been shown to be plasmid encoded.

Curing of the strain CSV90. The ability of the strain CSV90 to grow on D agar was very unstable and was lost irreversibly. When the cells from a single colony on D agar were grown in L broth overnight, 8% of the resultant clones could not grow on D agar plates. Twenty-four such clones were examined for their plasmid contents, and 15 clones showed complete loss of the plasmid. One of the cured clones was designated *P. cepacia* MAB1 and was used for further studies. The remaining nine clones were indistinguishable, carrying a smaller plasmid termed pMAB2. One of these clones was designated MAB2. Strains MAB1 and MAB2 were also not able to utilize 2-methyl-4-chlorophenoxyacetate. Treatment of strain CSV90 with mitomycin resulted in the generation of 2,4-D-negative-segregants with a frequency of 20 to 50%. In such derivatives, no plasmid corresponding to pMAB1 or pMAB2 was detected.

Both strains CSV90 and MAB1 grew poorly on L agar at 37°C, with plating efficiencies of less than 20% of those at 28°C. Strain CSV90 could grow on D agar at 28°C but not at 37°C.

Electrotransformation of the cured strain. Strain MAB1 was transformed with purified pMAB1 by electroporation, which enabled this strain to grow normally on D agar plates like *P. cepacia* CSV90. A plasmid similar to pMAB1 was recovered from the transformants. The efficiency of transformation was approximately 10^4 transformants per μg of pMAB1 DNA. No differences in growth pattern between *P. cepacia* CSV90 and *P. cepacia* MAB1 transformed with pMAB1 were observed.

Physical maps of pMAB1 and pMAB2. The physical maps of the plasmids pMAB1 and pMAB2 were constructed by single and double digestion with *Bam*HI and *Hin*dIII, and fragments were cloned to obtain more precise maps (Fig. 2).

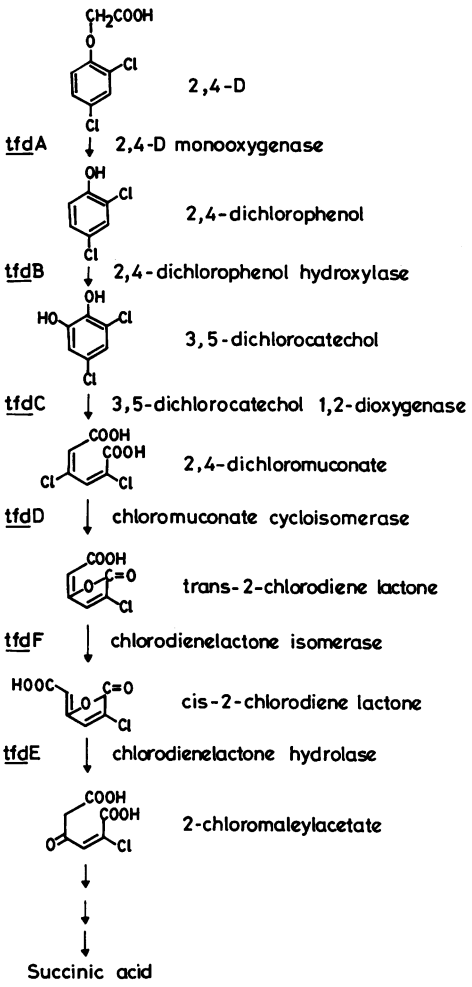

FIG. 1. The proposed degradative pathway of 2,4-D, with structural genes coding for the respective enzymes (adapted from references 2, 6, 9–12, and 21–23).

Restriction analysis of plasmids pMAB1 and pMAB2 revealed that a portion of approximately 20 kb had been deleted from pMAB1, with a loss in 2,4-D-degrading ability. The molecular sizes of the plasmids pMAB1 and pMAB2 were estimated to be 90 and 70 kb, respectively, on the basis of sum totals of *Eco*RI and *Bgl*II restriction digestion fragments, since all of the restriction fragments were well within the range of the size markers (data not shown).

When the physical map of pMAB1 was compared with that of pJP4, overall similarity was not obvious. However, a striking similarity was found in a region deleted from pMAB2 (Fig. 2). The detailed physical map of the 5.8-kb region from the left end of the *Hin*dIII F fragment to the right *Pvu*II site in the *Hin*dIII D fragment of pMAB1 was indistinguishable from that of the region from the *Hin*dIII G fragment to the *Hin*dIII C fragment of pJP4 (22).

Cloning and location of *tfd* genes. *Bam*HI restriction digestion of plasmids pMAB1 and pMAB2 revealed loss of *Bam*HI fragment B (10 kb) and *Bam*HI fragment C (6.1 kb) in pMAB2. Since strain MAB2 carrying pMAB2 was not able to degrade 2,4-D, we anticipated that these two fragments were carrying some of the genes for the degradation pathway. We cloned *Bam*HI fragment B and *Bam*HI frag-

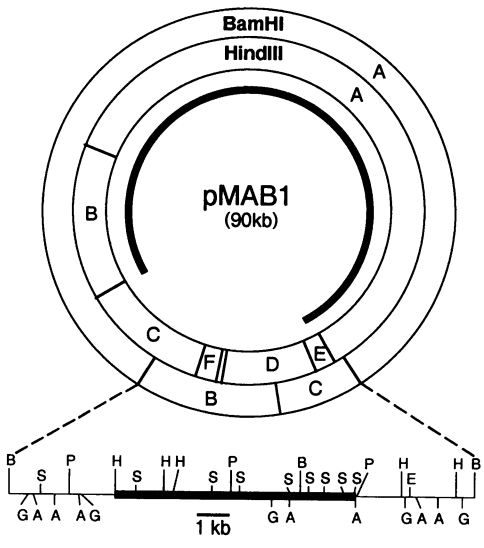

FIG. 2. Physical map of pMAB1 from *P. cepacia* CSV90. The thick arc represents the pMAB2 DNA (70 kb), whereas the thick line in the detailed map shows the DNA fragment, which reveals the restriction map to be indistinguishable from that of pJP4 containing the *tfdCDEF* operon and *tfdB* (22). Abbreviations for restriction sites: A, *Sac*I; B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; P, *Pvu*II; S, *Sal*I.

ment C in pUC119, and the recombinant clones were designated pMAB10 and pMAB4, respectively (Fig. 3). The *E. coli* cells carrying pMAB10 and pMAB4 were grown in the presence of IPTG and were assayed for 2,4-D monooxygenase (*tfdA* product), 2,4-dichlorophenol hydroxylase (*tfdB* product), and 3,5-dichlorocatechol 1,2-dioxygenase (*tfdC* product). The activity of the *tfdC* product alone was observed in the pMAB10-carrying clones. 2,4-D monooxygenase (*tfdA*) and 2,4-dichlorophenol hydroxylase (*tfdB*) activities were not observed. The clones carrying pMAB4 did not show any of the enzyme activities.

The plasmid pMAB10 was digested with *Bam*HI and *Hin*dIII, and the resulting three fragments were subcloned on pUC119 to produce pMAB786, pMAB787, and pMAB788 (Fig. 3). Among these plasmids, only pMAB786 containing the 1.6-kb *Hin*dIII F fragment showed high levels of activity

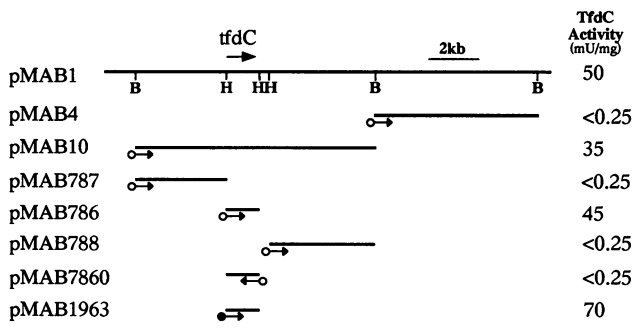

FIG. 3. Cloning of the *tfdC* gene from pMAB1 in *E. coli*. The lines represent inserts, the arrows show the directions of transcription from the vector promoters, and the open and closed circles represent *lac* and *tac* promoters, respectively. TfdC (3,5-dichlorocatechol 1,2-dioxygenase) activity was measured spectrophotometrically as described in Materials and Methods.

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                                                AAGCTTTGCCCCAATATCGCGTTCCAAGGCCTGGAT   36
CTGACGAGTAAGCGGTGGCTGCGATATGTGCATCCTCTGTGCCGCTGTTCCGAAACCTCC   96
CGCCTCCGCGACCGCGACGAAGTATTTCAACTGTCTTATTTCCATGTTCCGTCCCGTTGC  156
GCTGCCTGTTACAAGGCGATTGTGAGCAAGCACCTGCGGTCTCGCAACTAGGGAAAGACA  216
TACCGAAACCGTATGGTCTAGGCGGAGAAAAGGTATTGGACGGTATGGGATTGCCGTCTC  276
TATTATGACTGTGCGGGGCAATTGTCGCCCACGTGTTCATGTTTTCATGACGGAGGCAAA  336

 M  N  K  R  V  K  D  V  V  D  A  I  V  A  A  V  Q  R  V  L   20
GTGAACAAAAGAGTCAAGGATGTTGTCGATGCGATCGTCGCGGCGGTACAGCGGGTGCTC  396
➥tfdC
 D  Q  K  E  V  T  E  A  E  Y  R  T  A  V  H  Y  L  M  Q  V   40
GACCAGAAGGAGGTTACTGAGGCGGAATACCGAACTGCGGTTCATTACCTCATGCAAGTA  456

 A  E  Q  R  E  T  A  L  L  C  D  V  F  F  N  S  T  V  A  A   60
GCCGAACAGCGTGAAACCGCTCTTCTTTGCGATGTTTTTTTCAATAGCACCGTGGCTGCG  516

 T  K  A  R  I  S  E  G  S  T  P  A  I  E  G  P  Y  Y  R  D   80
ACGAAGGCTCGTATTAGCGAAGGTTCAACGCCTGCTATCGAGGGACCCTACTATCGCGAC  576

 D  A  P  L  V  D  D  R  L  K  T  Y  D  T  D  D  H  K  P  L  100
GACGCCCCTCTGGTCGATGACCGGCTCAAGACTTACGACACGGACGACCACAAGCCTTTG  636

 L  I  Q  G  T  V  K  A  V  D  G  S  V  V  E  D  V  T  I  D  120
CTTATCCAGGGAACGGTCAAAGCGGTCGACGGGAGCGTTGTCGAGGACGTGACGATTGAT  696

 V  W  H  S  T  P  D  G  K  Y  S  G  F  H  D  D  I  P  T  D  140
GTCTGGCATTCGACGCCCGATGGGAAGTACAGCGGTTTCCATGACGACATCCCGACTGAT  756

 F  Y  R  G  K  L  R  V  G  T  D  G  S  F  R  V  R  T  T  M  160
TTTTATCGAGGGAAGCTCAGGGTGGGCACCGATGGCAGCTTCCGCGTGCGCACAACGATG  816

 P  V  P  Y  Q  I  P  D  Q  G  P  T  G  A  L  L  E  T  M  G  180
CCGGTGCCGTATCAGATCCCGGATCAGGGTCCCACGGGCGCATTGCTCGAAACCATGGGT  876

 G  H  S  W  R  P  A  H  V  H  F  K  V  K  A  P  G  Y  E  T  200
GGTCACTCGTGGCGTCCCGCTCATGTACATTTCAAGGTGAAGGCGCCGGGATATGAAACG  936

 L  T  T  Q  Y  Y  F  E  G  G  D  W  I  T  D  D  C  C  N  G  220
TTGACCACGCAGTACTACTTCGAAGGTGGAGATTGGATCACGGACGACTGTTGCAACGGC  996

 V  Q  S  S  L  I  T  P  D  I  V  E  E  G  V  R  L  M  N  I  240
GTCCAGTCTAGTCTGATTACTCCCGATATTGTGGAAGAGGGTGTCCGCCTGATGAACATC 1056

 N  F  V  I  E  P  A  R  A  Q  A  G  A  N  P                255
AACTTCGTTATCGAGCCCCGCGCGTGCGCAGGCGGGGGCAAACCCGTGAAGATTGACGCGA 1116
                                  M  K  I  D  A                5
                                 ➥tfdD
TTGAAGCAGTGATCGTGGATGTGCCGACCAAGCGGCCGATCCAGATGTCGATCACTACCG 1176
I  E  A  V  I  V  D  V  P  T  K  R  P  I  Q  M  S  I  T  T    25

TGCACCAGCAGAGCTACGTTATCGTCCGGGTGTATTCGGAGGGGCTCGTTGGTGTCGGCG 1236
V  H  Q  Q  S  Y  V  I  V  R  V  Y  S  E  G  L  V  G  V  G    45

AGGGTGGAAGCGTTGGTGGTCCCGTCTGGAGCGCAGAGTGTGCGGAGACGATCAAGATCA 1296
E  G  G  S  V  G  G  P  V  W  S  A  E  C  A  E  T  I  K  I    65

TCGTGGAACGGTATCTCGCGCCCCACCTCCTCGGAACTGATGCGTTCAACGTTTCAGGTG 1356
I  V  E  R  Y  L  A  P  H  L  L  G  T  D  A  F  N  V  S  G    85

CACTGCAAACCATGGCGCGTGCCGTCAGCGGAAACGCCTCTGCAAAGGCTGCGGTCGAGA 1416
A  L  Q  T  M  A  R  A  V  S  G  N  A  S  A  K  A  A  V  E   105

TGGCGTTACTGGATCTCAAAGCTCGAGCGTTAGGCGTATCGATCGCCGAGTTACTTGGCG 1476
M  A  L  L  D  L  K  A  R  A  L  G  V  S  I  A  E  L  L  G   125

GGCCGTTGCGCAGTGCGATTCCGATTGCCTGGACATTGGCGAGCGGAGATACGAAACGCG 1536
G  P  L  R  S  A  I  P  I  A  W  T  L  A  S  G  D  T  K  R   145

ATCTCGATTCTGCCGTCGAGATGATTGAAAGACGACGACACAATCGCTTCAAAGTCAAGC 1596
D  L  D  S  A  V  E  M  I  E  R  R  R  H  N  R  F  K  V  K   165

TT                                                           1598
L                                                             166
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FIG. 4. Nucleotide and deduced amino acid sequences of 3,5-dichlorocatechol 1,2-dioxygenase (*tfdC*) and the putative dichloromuconate cycloisomerase (*tfdD*) on a 1.6-kb *Hin*dIII fragment of pMAB1. Bases are numbered relative to the initial *Hin*dIII site. The potential ribosome-binding site is underlined. Amino acid sequence determined from the purified enzyme (2) is italicized. The closed circle (at 1384) represents the nucleotide which can distinguish pMAB1 from pJP4 (22).

for the *tfdC* gene product. Cells carrying pMAB7860, a plasmid containing the same *Hin*dIII insert as that in pMAB786 but in the opposite orientation with respect to the vector promoter, did not produce the enzyme. Therefore, the expression of *tfdC* in pMAB786 was due to a read-through transcription from the vector promoter. The enzyme activities of the *tfdA* and *tfdB* gene products were not detected with any of the plasmids.

The 1.6-kb *Hin*dIII fragment was also cloned in pKK223-3, and the recombinant plasmid, pMAB1963, produced high levels of the gene product by the read-through transcription from the *tac* promoter.

Nucleotide sequencing and primary structure of *tfdC*. The nucleotide sequence of the 1.6-kb *Hin*dIII fragment and the deduced amino acid sequence of the *tfdC* gene product are presented in Fig. 4. The nucleotide sequence indicated an open reading frame of 765 nucleotides, beginning with a GUG codon at sequence coordinates 337 to 339, which is capable of specifying 255 amino acids with the predicted

molecular weight of 28,300. We have recently purified 3,5-dichlorocatechol 1,2-dioxygenase to homogeneity and have shown that it is a homodimer with a subunit molecular weight of 28,000, as established by various physical methods (2). This is in good agreement with the predicted molecular weight. We have also determined the N-terminal amino acid sequence of this enzyme up to 44 amino acid residues. This amino acid sequence was in perfect agreement with that deduced from the nucleotide sequence. This agreement established that this is indeed the sequence of *tfdC*, the structural gene for 3,5-dichlorocatechol 1,2-dioxygenase. The determined and predicted amino acid compositions for *tfdC* also showed perfect agreement. It should be noted that the codon GUG served as an initiator specifying methionine, since the N-terminal amino acid residue from the protein sequence was methionine. Several bases upstream from the initiation codon, a typical Shine-Dalgarno sequence, GGAGG, was found.

The nucleotide sequence of the *tfdC* gene of pMAB1 from *P. cepacia* CSV90 was identical to that of pJP4 from *A. eutrophus*. In addition, the 5′ flanking sequence of 336 nucleotides was exactly the same.

Nucleotide sequence and partial primary structure of *tfdD*. The nucleotide sequence following the *tfdC* gene shows another open reading frame of more than 498 nucleotides beginning with a GUG codon at sequence coordinates 1101 to 1103 (Fig. 4). This sequence was also identical to that of the *tfdD* gene of pJP4, with the exception of one nucleotide; i.e., nucleotide 1384 was G for Ser in pMAB1 instead of C for Thr in pJP4 (22).

DISCUSSION

We have isolated a new 2,4-D-degradative plasmid, pMAB1, from *P. cepacia* CSV90. The role of pMAB1 in metabolizing 2,4-D was elucidated by curing and transformation experiments. This plasmid was rather unstable. Incubation of the cells in nonselective medium resulted in either complete loss of the plasmid or specific deletion of a 20-kb fragment from the original plasmid pMAB1. The deletant plasmid, designated pMAB2, did not confer 2,4-D-degrading ability, indicating that the 20-kb region was involved in 2,4-D metabolism. Reintroduction of pMAB1 into the cured strain resulted in the production of 2,4-D-positive clones. The plasmid pMAB1 was also found to control 2-methyl-4-chlorophenoxyacetate degradation, since the cured strains could not utilize the compound. The failure of strain CSV90 to degrade 3-chlorobenzoate may be due to lack of enzymes converting it to 3-chlorocatechol and not to lack of enzymes degrading 3-chlorocatechol, since we have previously reported that 3-chlorocatechol could serve as a good substrate for 3,5-dichlorocatechol 1,2-dioxygenase (2). The genes for degradation of 3-chlorobenzoate in *A. eutrophus* JMP134 are chromosomally encoded (10) and might be absent from the *P. cepacia* CSV90 chromosome.

The whole physical map of pMAB1 was different from those of the reported plasmids such as pJP4 and pRC10 which encode 2,4-D metabolic genes (6, 22). On the other hand, the fine map of pMAB10 and pMAB4 showed a similarity to that of the *tfdCDEF* operon of pJP4 (22). To our surprise, the 1,598-base sequence of pMAB1 carrying the *tfdC* gene and its flanking regions was almost identical to that of pJP4 carrying the *tfdC* gene and a part of the *tfdD* gene. Therefore, it is highly probable that pMAB1 has a *tfdCDEF* operon similar to that of pJP4. The expression of *tfdC* on pMAB10 in *E. coli* was dependent on the vector promoter, probably because a regulatory gene activating gene expression that was identified in pJP4 was absent in pMAB10.

Plasmid pJP4 has the *tfdB* gene encoding 2,4-dichlorophenol hydroxylase downstream from the *tfdCDEF* operon. We could not detect the activity of the *tfdB* product with pMAB10 in *E. coli*. Restriction map analysis of pMAB10 revealed that the *tfdB* region of pJP4 seems to be similar to that of pMAB10. If this is the case, the coding sequence of *tfdB* in pMAB10 is incomplete and misses the C-terminal region, which might explain why we could not detect this enzyme activity with pMAB10 and were unable to locate the *tfdA* gene on pMAB1.

The majority of the chlorinated aromatic compounds are degraded via respective chlorocatechol intermediates, and the chlorocatechol dioxygenases act as key enzymes in the degradation of these compounds. The chlorocatechol oxygenases form part of an operon, the chlorocatechol oxidative operon: *tfdCDEF* in the case of pJP4 (22), *clcABD* in the case of pAC27 (13), and *tcbCDEF* in the case of p51 (30). These operons show considerable sequence homology and are closely related, leading researchers to propose a common ancestral origin for these catabolic operons (30). The chlorocatechol dioxygenase enzymes show a level of homology to each other of up to 60%. It is surprising that the *tfdC* gene sequence of pJP4 is completely similar to that of pMAB1, although the origins of these two plasmids and their hosts are different. It would be interesting to see whether the *tfdC* gene on the plasmid pRC10 from a *Flavobacterium* sp. also has the same sequence and how related these two plasmids are at the operon level and at the DNA sequence level. Thus, comparison of nucleotide sequences of the plasmid-borne *tfd* structural and regulatory genes among the related plasmids specifying catabolic activities against chloroaromatics will throw considerable light on the mode of evolution of new degradative functions against new synthetic compounds. This would also give a clue about the various ways of transfer of resistance between soil microorganisms. Hybridization analysis among these related plasmids will further clarify their homology, their relatedness, and their modes of distribution.

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