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Sequence Analysis of a Gene Cluster Involved in Metabolism of 2,4,5-Trichlorophenoxyacetic Acid by *Burkholderia cepacia* AC1100

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Burkholderia cepacia **AC1100 utilizes 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a sole source of carbon and energy. PT88 is a chromosomal deletion mutant of *B. cepacia* AC1100 and is unable to grow on 2,4,5-T. The nucleotide sequence of a 5.5-kb chromosomal fragment from *B. cepacia* AC1100 which complemented PT88 for growth on 2,4,5-T was determined. The sequence revealed the presence of six open reading frames, designated ORF1 to ORF6. Five polypeptides were produced when this DNA region was under control of the T7 promoter in *Escherichia coli*; however, no polypeptide was produced from the fourth open reading frame, ORF4. Homology searches of protein sequence databases were performed to determine if the proteins involved in 2,4,5-T metabolism were similar to other biodegradative enzymes. In addition, complementation studies were used to determine which genes were essential for the metabolism of 2,4,5-T. The first gene of the cluster, ORF1, encoded a 37-kDa polypeptide which was essential for complementation of PT88 and showed significant homology to putative *trans*-chlorodienelactone isomerases. The next gene, ORF2, was necessary for complementation and encoded a 47-kDa protein which showed homology to glutathione reductases. ORF3 was not essential for complementation; however, both the 23-kDa protein encoded by ORF3 and the predicted amino acid sequence of ORF4 showed homology to glutathione S-transferases. ORF5, which encoded an 11-kDa polypeptide, was essential for growth on 2,4,5-T, but the amino acid sequence did not show homology to those of any known proteins. The last gene of the cluster, ORF6, was necessary for complementation of PT88, and the 32-kDa protein encoded by this gene showed homology to catechol and chlorocatechol-1,2-dioxygenases.**

During the past several years, bacteria capable of using chlorinated aromatic compounds as sole sources of carbon and energy have been isolated from the environment. Many of these microorganisms degrade chlorinated benzoic acids, chlorinated phenols, chlorinated phenoxyacetic acids, and chlorinated benzenes through a common chlorocatechol intermediate that is further converted by the *ortho*-cleavage pathway to β-ketoadipate (50). The metabolic pathways for the degradation of 3-chlorobenzoate by *Pseudomonas* sp. strain B13 (18) and *Pseudomonas putida*(pAC27) (5), the degradation of 2,4-dichlorophenoxyacetic acid by *Alcaligenes eutrophus* JMP134 (pJP4) (17), and the degradation of 1,2,4-trichlorobenzene by *Pseudomonas* sp. strain P51 (61) are some typical examples. Many of the catabolic enzymes of these pathways are similar, and the gene clusters that encode them are organized in similar fashions (11).

Burkholderia cepacia AC1100 (formerly *Pseudomonas cepacia* AC1100) is known for its ability to utilize the recalcitrant herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a sole source of carbon and energy (36). This microorganism was isolated from a chemostat after several months of selection in the presence of 2,4,5-T (35). In order to gain an understanding of the organization and regulation of the genes for 2,4,5-T degradation, we initiated a study on the cloning and characterization of some of these genes. Previous studies have shown that the degradation of 2,4,5-T proceeds through the formation of 2,4,5-trichlorophenol (32). The genes involved in the conversion of 2,4,5-T to 2,4,5-trichlorophenol have been cloned, sequenced, and expressed in vivo (9, 29). The *tftA1* and *tftA2* genes encode two subunits of the oxygenase enzyme responsible for this first step of the pathway (9). The gene products showed homology to two multicomponent dioxygenases involved in benzoate and toluate degradation by *Acinetobacter calcoaceticus* (*benAB*) (44) and *P. putida* (*xylXY*) (27), respectively.

The next few steps of the pathway are not well understood. However, it has been shown that the lower part of the pathway proceeds through the formation of an intermediate, 5-chloro-1,2,4-trihydroxybenzene (4). A 2,4,5-T-negative mutant, harboring a chromosomal deletion of at least 4.0 kb of DNA, accumulated this intermediate when grown in the presence of glucose and 2,4,5-T (52). The mutant, PT88, was complemented for growth on 2,4,5-T by an 8.0-kb chromosomal DNA fragment from *B. cepacia* AC1100 (52). Further subcloning of this fragment until complementation was eliminated resulted in a 5.5-kb chromosomal DNA fragment which was essential for PT88 to grow on 2,4,5-T as a sole source of carbon. Nucleotide sequencing of this fragment was performed in order to determine the number of genes present on this fragment and the essential nature of such genes. In this report, we present the nucleotide sequence of the 5.5-kb chromosomal DNA fragment, in addition to complementation data for each of the genes present. The predicted amino acid sequences of the genes present on this DNA fragment are compared with those of the gene products of the other degradative pathways mentioned above. The results show that some of the gene products

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TABLE 1. Bacterial strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Relevant characteristics	Reference(s)
Strains		
E. coli		
AC80	*thr leu met hsdR hsdM*	2
MV1184	*ara* Δ(*lac-proAB*) *rpsL thi* (ϕ80 *lacZ* Δ*M15*) Δ(*srl-recA*)*306*::Tn*10* (Tc^r) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	63
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	40
K-38	HfrC (λ)	56
RB404	*dam-3 dcm-6 metB1 galK2 galT22 lacY thi tonAB tsx-78 mtl-1 supE44*	55
B. cepacia		
AC1100	Wild type Tft$^+$ Nal^r	36
PT88	Tft$^-$ Nal^r AC1100 (ΔORF123456)	52, 57
Plasmids		
pCP13	IncP1 *mob*$^+$ *tra*$^-$ *cos*$^+$ Tc^r Km^r	10
pVDZ′2	IncP1 *mob*$^+$ *tra*$^-$ *lacZ* Tc^r	14
pRK415	IncP1 *mob*$^+$ *tra*$^-$ P*lac* Tc^r	34
pMMB66HE	IncQ *rrnB mob*$^+$ *lacI* P*tac* Ap^r (RSF1010)	22
pMMB66EH	pMMB66HE reversed multicloning site, Ap^r	22
pRK2013	ColE1 *mob*$^+$ *tra*$^+$ Km^r (RK2)	20
pUC119	*lacZ′*, M13 intergenic region, Ap^r	62
pGP1-2	Contains T7 RNA polymerase gene, Ap^r	56
pT7-5	T7 promoter expression vector, Ap^r	56
pT7-6	pT7-5 reversed multicloning site, Ap^r	56
pKS100	4.2-kb *Pst*I-*Bam*HI fragment from pUS1028 in pUC119	This study
pKS200	4.2-kb *Hin*dIII-*Bam*HI fragment from pKS200 in pUC119 blunt ended into the *Hin*cII site	This study
pDD1Q	4.2-kb *Pst*I-*Bam*HI fragment from pKS100 in pT7-5	This study
pDD2Q	4.2-kb *Pst*I-*Bam*HI fragment from pKS100 in pT7-6	This study
pDD2QΔ1	1.3-kb *Pst*I-*Xho*I fragment from pKS100 in pT7-6 *Pst*I-*Sal*I sites	This study
pDD2QΔ2	1.8-kb *Pst*I-*Eco*RI fragment from pKS100 in pT7-6	This study
pDD2QΔ3	2.4-kb *Pst*I-*Eco*RI fragment from pKS100 in pT7-6	This study
pDD2QΔ4	3.3-kb *Pst*I-*Cla*I fragment from pKS100 in pT7-5	This study
pDD2QΔ5	4.0-kb *Pst*I-*Eco*RV fragment from pKS100 in pT7-6 *Pst*I-*Sma*I sites	This study
pDD2QΔ7	1.6-kb *Bgl*II-*Bam*HI fragment from pKS100 in pT7-6 *Bam*HI site	This study
pCDH1E	3.0-kb *Eco*RI fragment from pUS1016 in pT7-6	This study
pCDH2E	3.0-kb *Eco*RI fragment from pUS1016 in pT7-6 reverse orientation of pCDH1E	This study
pUS105	8.0-kb *Hin*dIII-*Bam*HI fragment in pCP13	52
pUS1014	6.5-kb *Hin*dIII-*Bgl*II fragment in pVDZ′2	52
pUS1016	5.6-kb *Eco*RI-*Bam*HI fragment in pCP13	52
pUS1028	pUS105 (Δ[1.3-kb *Pst*I fragment])	52
pMMD0	1.6-kb *Eco*RI-*Hin*dIII PCR fragment of pMMD01 in pMMB66EH	This study
pMMD01	3.0-kb *Eco*RI fragment from pUS1016 in pMMB66HE	This study
pMMD1234	4.2-kb *Pst*I-*Bam*HI fragment from pKS100 in pMMB66HE	This study
pMMD34	1.6-kb *Bgl*II-*Bam*HI fragment from pKS100 in pMMB66HE *Bam*HI site	This study
pMMD3	pMMD34 (Δ[0.5-kb *Eco*RI fragment])	This study
pMMD4	1.1-kb *Bcl*I-*Bam*HI fragment from pKS100 in pMMB66HE	This study
pRKD1234	4.2-kb *Pst*I-*Bam*HI fragment from pKS100 in pRK415	This study
pRKD234	2.7-kb *Hin*dIII-*Bam*HI fragment from pKS100 in pRK415 (Δ[1.5-kb *Sph*I fragment])	This study
pRKD34	1.6-kb *Hin*dIII-*Bam*HI fragment from pMMD34 in pRK415	This study
Phage M13K07	M13 Km^r	63

are partially homologous to other degradative enzymes; however, the gene organization of this cluster is unique.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria broth (Difco Laboratories) or on Luria agar plates supplemented with 75 μg of ampicillin per ml, 50 μg of kanamycin per ml, and 30 μg of tetracycline per ml for plasmid selection. For single-stranded DNA production, *E. coli* MV1184 was grown in 2XYT medium (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl). *B. cepacia* AC1100 and *B. cepacia* PT88 were grown at 30°C in basal salts medium supplemented with 1 mg of 2,4,5-T (Aldrich) per ml or 0.2% glucose as the sole carbon source, as previously described (36). For solid basal salts medium, Gelrite (Adams Scientific) was added at a concentration of 1.0%. *Pseudomonas* isolation agar (Difco Laboratories) was used to maintain transconjugants of *B. cepacia*. Antibiotic concentrations used for selection of *B. cepacia* transconjugants were 100 μg of tetracycline per ml and 200 μg of carbenicillin (Research Organics Corp.) per ml.

DNA preparation and manipulation. Plasmid DNA was isolated by either the alkaline lysis or the Holmes-Quigley rapid boiling method (31, 40). DNA restriction digestions were performed by standard procedures for restriction endonucleases purchased from Bethesda Research Laboratories (Gaithersburg, Md.). *E. coli* RB404 was used to prepare unmethylated DNA for digestion with the restriction endonuclease *Bcl*I, which cuts the DNA only when it is not methylated. Preparation of competent cells and transformations were performed according to standard procedures described by Maniatis et al. (40). Ligation mixtures were incubated at 16°C for 24 h or at room temperature for 3 h with T4 DNA ligase purchased from New England Biolabs (Beverly, Mass.). Before ligations were performed, vector DNA was treated with calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, Ind.).

Cloning of open reading frame 1 (ORF1) by itself into the vector pMMB66EH was accomplished by PCR. The GeneAmp PCR Core Reagents kit with Amplitaq DNA polymerase from Perkin-Elmer Cetus (Norwalk, Conn.) was used for PCR. Clone pMMD01, which contained ORF1, was used as the template DNA. Oligonucleotides synthesized by Genosys Biotechnologies (The Woodlands, Tex.) were used as primers for PCR. Each primer had a restriction endonuclease site created at the 5′ end, in order to facilitate cloning of the PCR product into the vector.

DNA sequencing and analysis. The 4.2-kb *Pst*I-*Bam*HI fragment was cloned into the pUC119 vector in both orientations, resulting in plasmids pKS100 and pKS200 (Table 1), which were used for sequencing both DNA strands. Successive unidirectional digestions of both plasmids were made with the exonuclease III mung bean nuclease deletion kit purchased from Stratagene (La Jolla, Calif.). Small fragments of the 1.3-kb *Pst*I-*Pst*I fragment upstream of the 4.2-kb *Pst*I-*Bam*HI fragment were subcloned into pUC119 for sequencing of both strands of the DNA. In addition to exonuclease III digestion and subcloning, oligonucleotide primers synthesized by Operon Technologies (Alameda, Calif.) were used to sequence some DNA regions. Single-stranded DNA templates of each resulting subclone were prepared in *E. coli* MV1184 with the helper phage M13K07 (63). The dideoxy chain termination method of sequencing described by Sanger et al. was performed at 37°C using the enzyme Sequenase, version 2.0, purchased from United States Biochemical Corp. (Cleveland, Ohio) (51). The deoxy- and dideoxynucleotides were purchased from Pharmacia (Piscataway, N.J.), and the radioactive nucleotide [α-^{35}S]dCTP was purchased from Amersham Corp. (Arlington Heights, Ill.). In order to reduce sequence compressions, 7-deaza-dGTP was substituted for dGTP (42). In addition, some difficult regions were sequenced at 65°C with the *Bst* DNA polymerase sequencing kit from Bio-Rad Laboratories (Richmond, Calif.) with [α-^{35}S]dATP (Amersham Corp.).

Computer analysis of the DNA sequence and the predicted amino acid sequences was accomplished through the use of the FASTA database searching program for the VAX developed by Pearson (47). The default values for the search program were used.

Detection of gene products in vivo. In order to detect polypeptides encoded by the predicted ORFs, the T7 expression assay system of Tabor and Richardson was used (56). Various fragments from the 5.5-kb DNA region were subcloned into T7 expression vectors pT7-5 and pT7-6. These constructs are shown in Fig. 2 and are described in detail in Table 1. The pT7-5 and pT7-6 vectors allow the selective expression of genes from the T7 promoter. The resulting clones were transformed into *E. coli* K-38 containing plasmid pGP1-2, which has a heat-inducible T7 RNA polymerase gene. The genes present on these fragments were expressed from the T7 promoter by induction of the T7 RNA polymerase at 42°C in the presence of rifampin. The cells were then pulsed with L-[^{35}S]methionine (Amersham Corp.), and whole-cell lysates were loaded onto a gel for sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS-PAGE). The radioactive proteins were visualized by autoradiography. The molecular weights of the proteins were estimated by comparison with the ^{14}C-labeled protein molecular mass markers run on the same polyacrylamide gel. The data presented show only the cells induced at 42°C and not the control cells grown at 30°C. Also, the results from the pDD1Q plasmid are not presented because no polypeptides were produced from this construct.

Complementation analysis. In order to complement PT88 for growth on 2,4,5-T, broad-host-range recombinant plasmids containing the gene cluster were triparentally mated into PT88 by using the helper plasmid pRK2013 (20). Triparental matings were performed by pelleting, washing, and mixing 0.5 ml of a 5.0-ml overnight culture of *E. coli* MV1184 or *E. coli* AC80 containing the plasmid to be transferred and 0.5 ml of a 5.0-ml overnight culture of *E. coli* HB101/pRK2013 with a 2-day 5.0-ml culture of PT88 grown on basal salts medium with glucose and 2,4,5-T. This mixture of cells was pelleted and placed on a Luria agar plate at 30°C for 24 h. For selection of transconjugants, the cells were resuspended in saline and dilutions were plated on *Pseudomonas* isolation agar plates with the appropriate antibiotic. Complementation was assayed for by plating undiluted cells on solid basal salts medium containing 1 mg of 2,4,5-T per ml and incubating them at 30°C for 10 to 15 days. When two plasmids were necessary for complementation, the plasmids were transferred into PT88 through two subsequent triparental mating procedures. The vectors alone without the genes were used as negative controls for complementation. When fragments were cloned under the *tac* promoter as in vectors pMMB66HE and pMMB66EH or the *lac* promoter as in vector pRK415, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was spread on the plates to induce expression from these promoters.

Nucleotide sequence accession number. The nucleotide sequence presented here was reported to GenBank and was assigned the accesion number U19883.

RESULTS

Nucleotide sequence analysis and expression of the genes for 2,4,5-T degradation. The complete nucleotide sequence of the 5,471-bp region and the derived amino acid sequences are shown in Fig. 1. In order to determine the number of polypeptides produced from this DNA region, various fragments were cloned under the T7 promoter for the selective expression and labeling of proteins in *E. coli*, as shown in Fig. 2. The sequence revealed the presence of six ORFs, and five of these were confirmed by the T7 expression assay as shown in Fig. 3. The first ORF, ORF1, had a possible valine (GTG) start codon at position 295 with a putative ribosome binding sequence, GGACCGG, at 7 to 13 bp upstream of the start. This ORF encoded a protein of 352 amino acids with a calculated molecular mass of 36.8 kDa. A 39-kDa polypeptide and a 49-kDa polypeptide were observed when the pCDH1E construct was used in the T7 expression assay (Fig. 3, lane 1). No polypeptides from the pCDH2E construct, which contained the same DNA region in the reverse orientation of the T7 promoter (data not shown), were observed. In addition, the pDD2QΔ2 construct, which had a deletion of the *Eco*RI-*Pst*I region of pCDH1E, did not produce a 39-kDa protein but still produced a 49-kDa protein (Fig. 3, lane 3). Therefore, the 39-kDa polypeptide was a product of this DNA region and the size reflected that of the calculated ORF1 gene product.

The 49-kDa protein produced from the pDD2QΔ2 construct, as mentioned above, corresponded to ORF2. This ORF had an ATG start at position 1393 preceded by a putative ribosome binding sequence, AGCGGGT, 5 to 11 bp upstream. The 449 amino acid residues of the ORF2 gene product gave a predicted molecular mass of 47.5-kDa. This prediction corresponded to the size of the polypeptide produced from all the constructs that contained the 1.5-kb *Pst*I-*Eco*RI DNA fragment of pDD2QΔ2. In addition, the pDD2QΔ1 construct, which had an *Xho*I-*Eco*RI deletion at the 3′ end of pDD2QΔ2, was expected to produce a truncated ORF2 gene product of 28.8 kDa. The polypeptide produced closely matched, with a size of about 32 kDa (Fig. 3, lane 2).

Downstream of ORF2, between bp 2872 and 3489, was a third ORF, ORF3. This ORF encoded a protein of 205 amino acids with a calculated mass of 23.3 kDa. The presumed ATG start codon was preceded by a putative ribosome binding site, GGAGCAG, 6 to 12 bp upstream. The construct pDD2QΔ4, which contained this region of DNA, produced a labeled protein of 25 kDa when used in the T7 expression assay (Fig. 3, lane 5). Also, an 18-kDa truncated form of this protein was produced from the pDD2QΔ3 construct (Fig. 3, lane 4). This protein closely matched the expected size of 17.1 kDa encoded by the region of ORF3 remaining in the pDD2QΔ3 construct. Thus, the polypeptides produced confirmed the presence of the ORF3 gene.

Next, the TGA stop codon of ORF3 overlapped with the ATG start codon of a fourth ORF at position 3486. A putative ribosome binding sequence, GGGGGT, was found 7 to 12 bp upstream of this start codon. A protein of 164 amino acids could be encoded by this ORF, and the predicted molecular mass was 18.7-kDa. The pDD2QΔ4 construct mentioned above contained this fourth ORF in its entirety; however, no polypeptide of 19 kDa was observed (Fig. 3, lane 5). The only labeled proteins produced were the 49-kDa protein from ORF2 and the 25-kDa protein from ORF3. The 18-kDa polypeptide in Fig. 3, lane 4, was not a product of ORF4, because it was produced from the pDD2QΔ3 construct, which lacked the *Eco*RI-*Cla*I fragment containing the ORF4 region. The reason for which this ORF was not expressed may be the absence of an ideal ribosome binding site, since GGGGGT is not a good ribosome binding sequence.

Two polypeptides, 12 and 16 kDa, in addition to the 49- and the 25-kDa polypeptides, were produced from the pDD2QΔ5 construct (Fig. 3, lane 6). The 12-kDa polypeptide was consistently produced from two other constructs, pDD2Q and pDD2QΔ7 (Fig. 3, lanes 7 and 8). This polypeptide reflected the calculated mass of an 11.2-kDa protein encoded by a fifth ORF, ORF5. This ORF had an ATG start codon at position 4199 preceded by a putative ribosome binding site, AGGAG, found 6 to 10 bp upstream. The entire ORF5 was present in all three constructs mentioned. Therefore, the ORF5 gene product of 100 amino acids corresponded to the 12-kDa polypeptide that was produced.

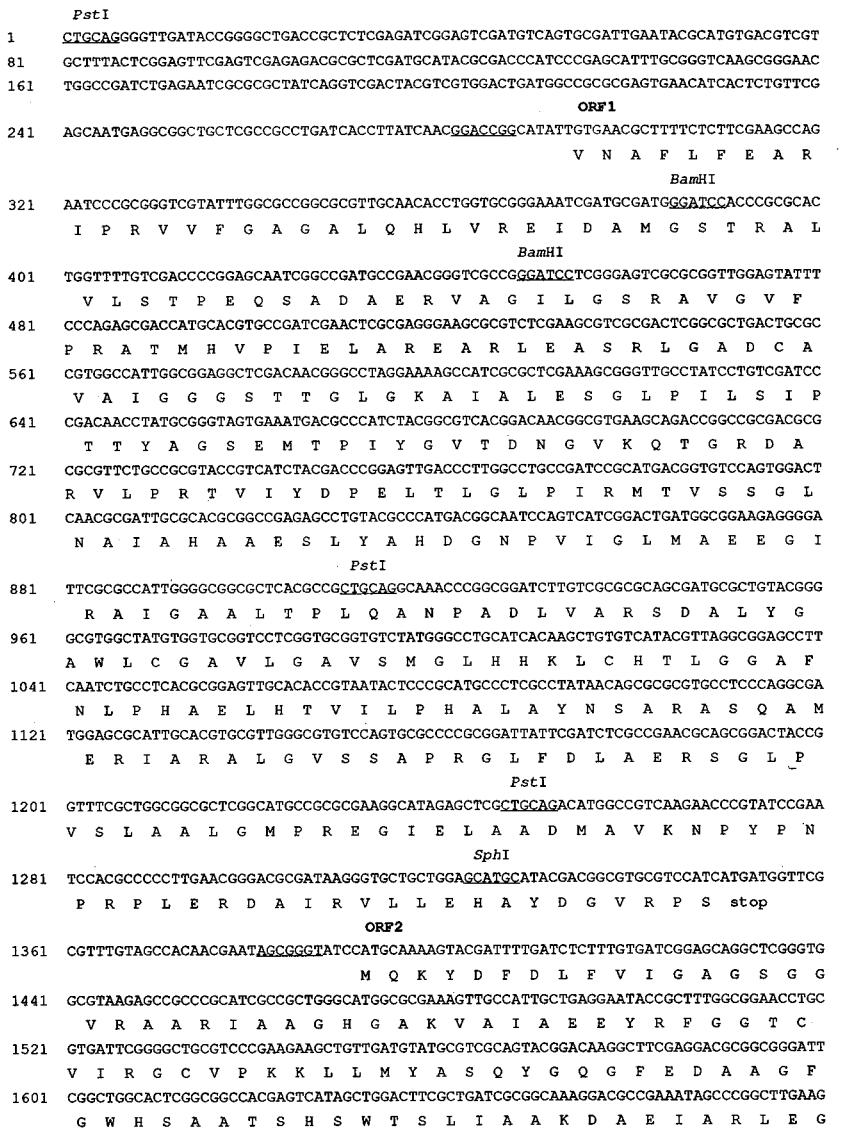

FIG. 1. Nucleotide sequence of the 5.471-kb *Pst*I-*Bam*HI fragment containing the 2,4,5-T gene cluster. The deduced amino acid sequences are shown below each of the ORFs. Relevant restriction sites are labeled and underlined. In addition, putative ribosome binding sites for each ORF are underlined.

The last ORF of the cluster, ORF6, encoded a protein of 293 amino acids with a predicted molecular mass of 32.2 kDa. The ATG start codon at position 4579 was preceded by a consensus *E. coli* ribosome binding sequence, AGGAGGT, at 6 to 12 bp upstream. Both the constructs pDD2Q and pDD2QΔ7 had a full copy of ORF6, and when they were used in the T7 assay, a 35-kDa polypeptide was observed (Fig. 3, lanes 7 and 8). The molecular mass of this polypeptide closely matched the predicted molecular mass of the ORF6 gene product. In addition, the pDD2QΔ5 construct, which had an *Eco*RV-*Bam*HI deletion of pDD2Q, produced a 16-kDa truncated gene product from ORF6 (Fig. 3, lane 6). This truncated protein also closely matched the predicted mass of 16.2 kDa for the partial ORF6 gene present.

Homologies of the 2,4,5-T gene products to other enzymes. The predicted amino acid sequences of the six ORFs were compared with protein sequences in the PIR International Protein Sequence Database. The results of the FASTA database searches are summarized in Table 2. Only sequences with the highest optimal alignment scores were represented; no optimal scores less than 200 were reported. Numerous sequences with identities to ORF1 and ORF2 had optimal scores greater than 200, but only the highest scores were included in Table 2. ORF3 also had several homologous sequences with optimal scores greater than 200. Most of these sequences were isozymes of glutathione *S*-transferase from *Drosophila melanogaster*, and therefore, only the highest optimal sequence alignment with *D. melanogaster* was reported. ORF4 showed 25% amino acid sequence identity to ORF3, with an optimal alignment score of 119. ORF4 also showed identity to many of the same sequences as ORF3; however, only the glutathione *S*-transferase from *Zea mays* had an optimal score greater than 200 with ORF4 (the score with ORF3 was 184). ORF5 did not show significant amino acid identity to any proteins in the database. All optimal alignment scores for ORF5 were less than 100 and thus were considered due to chance. ORF6 had several homologous sequences with optimal scores greater

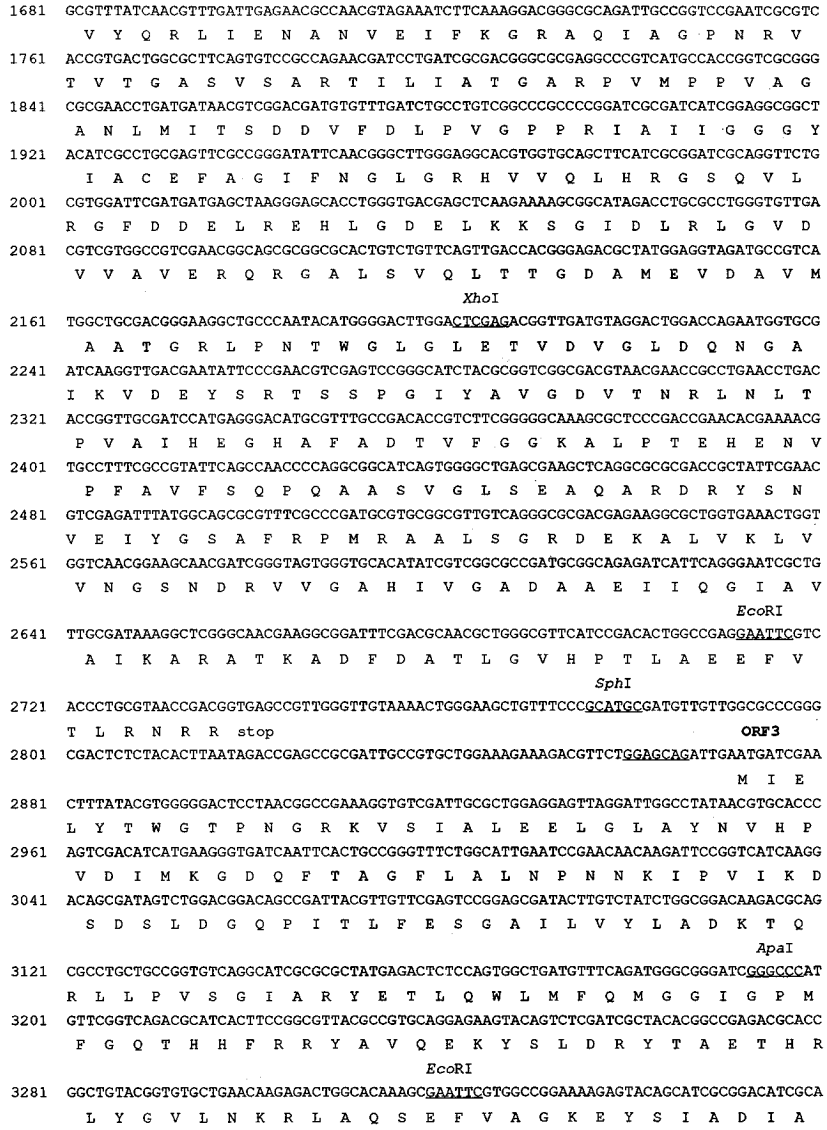

FIG. 1—*Continued.*

than 200. The sequences with the top six optimal scores are presented in Table 2. These sequences constitute a family of enzymes known as the catechol 1,2-dioxygenases, which are involved in the metabolism of chlorinated or nonchlorinated aromatics.

Complementation analysis. In order to determine which of the ORFs were essential for complementation of the mutant PT88 for growth on 2,4,5-T, various deletion clones were constructed and transferred into the mutant. Figure 4 shows the restriction maps of the constructs used in the complementation experiments. The first two constructs, pUS105 and pUS1016, both contained the entire gene cluster and were able to complement PT88 for growth on 2,4,5-T. The next construct, pUS1028, had a deletion of a 1.3-kb *Pst*I fragment that encompassed ORF1 and did not allow PT88 to grow on 2,4,5-T. However, when either of the constructs, pMMD01 or pMMD0, was transferred into PT88 in addition to pUS1028 (but not alone), PT88 was complemented. Thus, ORF1 present in pMMD01 and pMMD0 was essential for PT88 to grow on 2,4,5-T as the sole carbon source.

The next construct, pUS1014, had a *Bgl*II-*Bam*HI deletion at its 3′ end and did not complement PT88. This deletion encompassed ORF5 and ORF6. When construct pMMD34, which contained both of these ORFs under the *tac* promoter, was transferred into PT88 along with pUS1014, PT88 was able to grow on 2,4,5-T (only when IPTG was added to the media). To determine if ORF5 and ORF6 together were responsible for the complementation, two more constructs, pMMD3 and pMMD4, were separately transferred into PT88 containing pUS1014. Neither pMMD3, which contained ORF5 under the *tac* promoter, nor pMMD4, which contained ORF6 under the *tac* promoter, was able to complement PT88 while it contained pUS1014. Thus, ORF5 and ORF6 together were essential for PT88 to grow on 2,4,5-T.

In order to determine if ORF3 was essential for the metabolism of 2,4,5-T, two sets of constructs were transferred into PT88. The first set, pMMD01 and pRKD234, which together contained the entire gene cluster, was able to complement PT88. The second set of constructs, pMMD01 and pRKD34, also complemented the mutant although ORF3 and ORF4

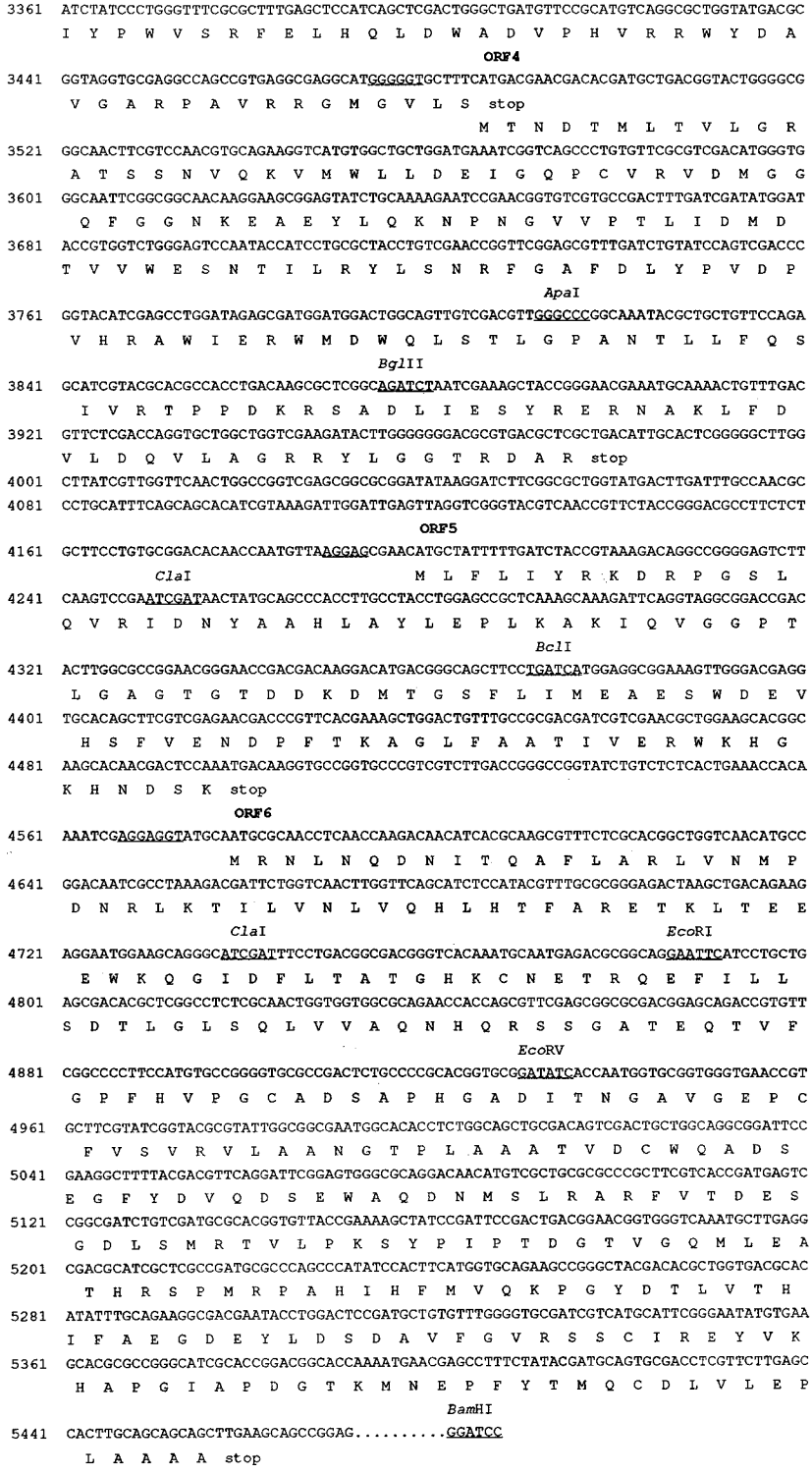

FIG. 1—*Continued.*

were deleted. Therefore, ORF3 and ORF4 were not essential for the complementation of PT88.

Similarly, two sets of constructs were transferred into PT88 to determine if ORF2 was essential for complementation. Constructs pMMD0 and pRKD1234 together contained the entire gene cluster and complemented PT88. However, constructs pMMD0 and pRKD234 had a deletion of ORF2 and did not allow PT88 to grow on 2,4,5-T. Thus, ORF2 was essential for the complementation of PT88. In summary, ORF1, ORF2, ORF5, and ORF6 were essential for the deletion mutant PT88 to grow on 2,4,5-T as the sole source of carbon; however, ORF3 and ORF4 were not.

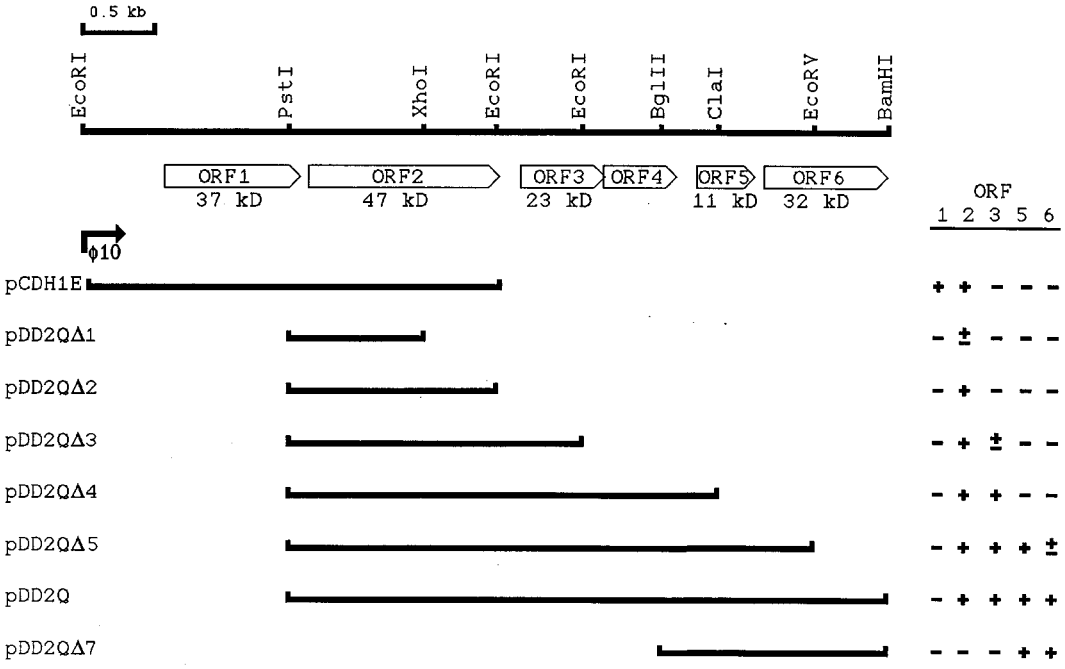

FIG. 2. Restriction maps of the constructs used in the T7 expression assay. The position of each of the ORFs is shown below the restriction map by an open rectangle. The molecular mass calculated from the amino acid sequence of each of these proteins is shown below each ORF. DNA fragments were cloned into the T7 expression vectors containing the ϕ10 promoter (designated by an arrow) upstream of the inserts. A chart to the right of the constructs shows which proteins are produced from each DNA fragment (+, full protein produced; −, no protein produced; ±, truncated protein produced).

DISCUSSION

Sequence analysis of the 5.5-kb DNA fragment revealed the presence of six ORFs preceded by reasonable ribosomal binding sites, except ORF4 (Fig. 1). Five polypeptides were produced in the *E. coli* T7 expression assay, and the molecular masses of these polypeptides corresponded to the molecular masses of the predicted amino acid sequences of the five ORFs (ORF1, ORF2, ORF3, ORF5, and ORF6) (Fig. 2 and 3). No polypeptide was produced from ORF4 in the T7 assay. ORF1, ORF2, ORF5, and ORF6 were essential for the complementation of PT88 for growth on 2,4,5-T; however, ORF3 and ORF4 were not. All of the predicted amino acid sequences of the gene products showed significant homology to known enzymes, except for that of ORF5 (Table 2). Based on the protein comparisons, biochemical assays, and the intermediate PT88 accumulated, a tentative pathway for 2,4,5-T degradation is presented in Fig. 5.

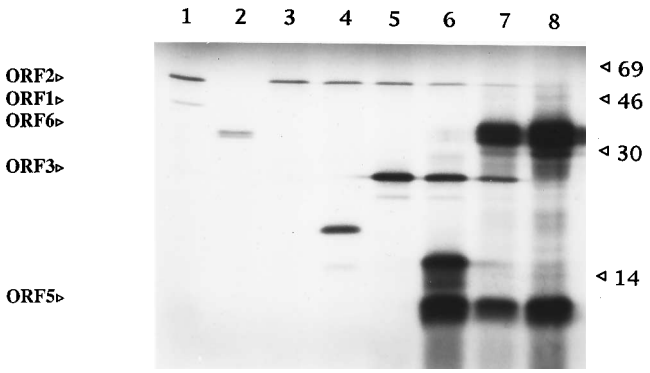

FIG. 3. Autoradiogram of the [^{35}S]methionine-labeled proteins produced in the T7 expression assay. The autoradiogram of the SDS-PAGE gel shows where each of the proteins migrates (indicated on the left). Lanes 1 through 8 correspond to the constructs pCDH1E through pDD2QΔ7 as listed in Fig. 2. Protein molecular mass markers (in kilodaltons) are on the right.

The mutant PT88 accumulated the intermediate 5-chloro-1,2,4-trihydroxybenzene (or 5-chlorohydroxyquinol) when grown in the presence of glucose and 2,4,5-T (4). In addition, Sangodkar et al. showed that PT88 was able to oxidatively dechlorinate the compound 4,6-dichlororesorcinol to 5-chloro-1,2,4-trihydroxybenzene (52). Recently, Tomasi et al. have shown that resting cells of *B. cepacia* AC1100 converted, 2,4,5-trichlorophenol to 2,5-dichlorohydroquinone by hydroxylating 2,4,5-trichlorophenol at the *para* position; subsequently, 2,5-dichlorohydroquinone was converted to 5-chloro-1,2,4-trihydroxybenzene (58a). The presence of trihydroxylated intermediates in aromatic acid degradation has been reported for bacteria, yeasts, and fungi. For example, the metabolism of resorcinol by *P. putida* (3) and *Trichosporon cutaneum* (23) was shown to proceed through the formation of hydroxyquinol. Also, *T. cutaneum* metabolized L-tyrosine via a hydroxyquinol intermediate (54). *Nocardioides simplex* 3E, which degraded both 2,4,5-T and 2,4-dichlorophenoxyacetic acid, converted 2,4-dichlorophenoxyacetic acid to 3,5-dichlorocatechol in a manner similar to that of *A. eutrophus* JMP134 (38). However, instead of cleaving the 3,5-dichlorocatechol intermediate to the corresponding muconic acid, *N. simplex* hydroxylated the 3,5-dichlorocatechol to 3,5-dichloro-1,2,4-trihydroxybenzene (38). Metabolism of the trihydroxylated intermediate is catalyzed by a hydroxyquinol 1,2-dioxygenase. This enzymatic activity has been detected in *N. simplex* 3E (38), in addition to *P. putida* (3), *T. cutaneum* (23), and the white rot fungus *Sporotrichum pulverulentum* (1). Recently, a 6-chlorohydroxyquinol 1,2-dioxygenase was purified from *Streptomyces rochei* 303 and compared with a similar enzyme purified from *Azotobacter* sp. strain GP1 which degrades 2,4,6-trichlorophenol to 6-chlorohydroxyquinol (64). The hydroxyquinol 1,2-dioxygenase cata-

TABLE 2. Amino acid sequence comparisons

ORF	PIR accession no.	Function (gene/organism)	% Identity	Score	Reference(s)
ORF1	E43673	*trans*-Chlorodienelactone isomerase (*tcbF*/*Pseudomonas* sp. strain P51)	56	996	60
ORF1	D35255	*trans*-Chlorodienelactone isomerase (*tfdF*/*Alcaligenes eutrophus* JMP134)	52	912	48
ORF2	S38908	Glutathione reductase (*Nicotiana tabacum*)	50	1,127	None
ORF2	S18973	Glutathione reductase (*Pisum sativum*)	49	1,122	8
ORF2	S15236	Glutathione reductase (*Pseudomonas aeruginosa*)	50	1,124	49
ORF3	A39609	Glutathione *S*-transferase (*Saccharomyces cerevisiae*)	35	250	7
ORF3	A46681	Glutathione *S*-transferase (*Drosophila melanogaster*)	32	245	59
ORF3	S29772	Glutathione *S*-transferase (*Proteus mirabilis*)	26	202	41
ORF4	S00717	Glutathione *S*-transferase (*Zea mays*)	33	205	26
ORF6	M23245[a]	Catechol 1,2-dioxygenase (*catA*/*Acinetobacter calcoaceticus*)	28	352	43
ORF6	A43673	Catechol 1,2-dioxygenase (*tcbC*/*Pseudomonas* sp. strain P51)	28	291	60
ORF6	A27058	Chlorocatechol 1,2-dioxygenase (*clcA*/*Pseudomonas putida*)	28	289	21
ORF6	JN0143	Catechol 1,2-dioxygenase (*pheB*/*Pseudomonas* sp. strain EST1001)	28	267	37
ORF6	JT0613	Catechol 1,2-dioxygenase (*catA*/*Arthrobacter* sp. strain mA3)	25	262	19
ORF6	S06866	Chlorocatechol 1,2-dioxygenase (*tfdC*/*Alcaligenes eutrophus* JMP134)	26	261	25, 48

[a] Accession number from EMBL database.

lyzes the *ortho* cleavage of the trihydroxylated intermediate but not the catechol intermediate (1, 3, 38, 64).

The product of the last gene in the 2,4,5-T gene cluster, ORF6, showed homology to a family of catechol or chlorocatechol 1,2-dioxygenase enzymes encoded by the *catA*, *tcbC*, *clcA*, *pheB*, and *tfdC* genes listed in Table 2. Although the amino acid sequence identity of the ORF6 gene product compared to this family of enzymes is relatively low (about 30%, whereas the identity within this family of enzymes is 50 to 60%), the amino acid residues involved in the binding of ferric ion by the protocatechuate 3,4-dioxygenase from *P. putida* (45) were conserved. Thus, it is likely that ORF6 encodes a catechol

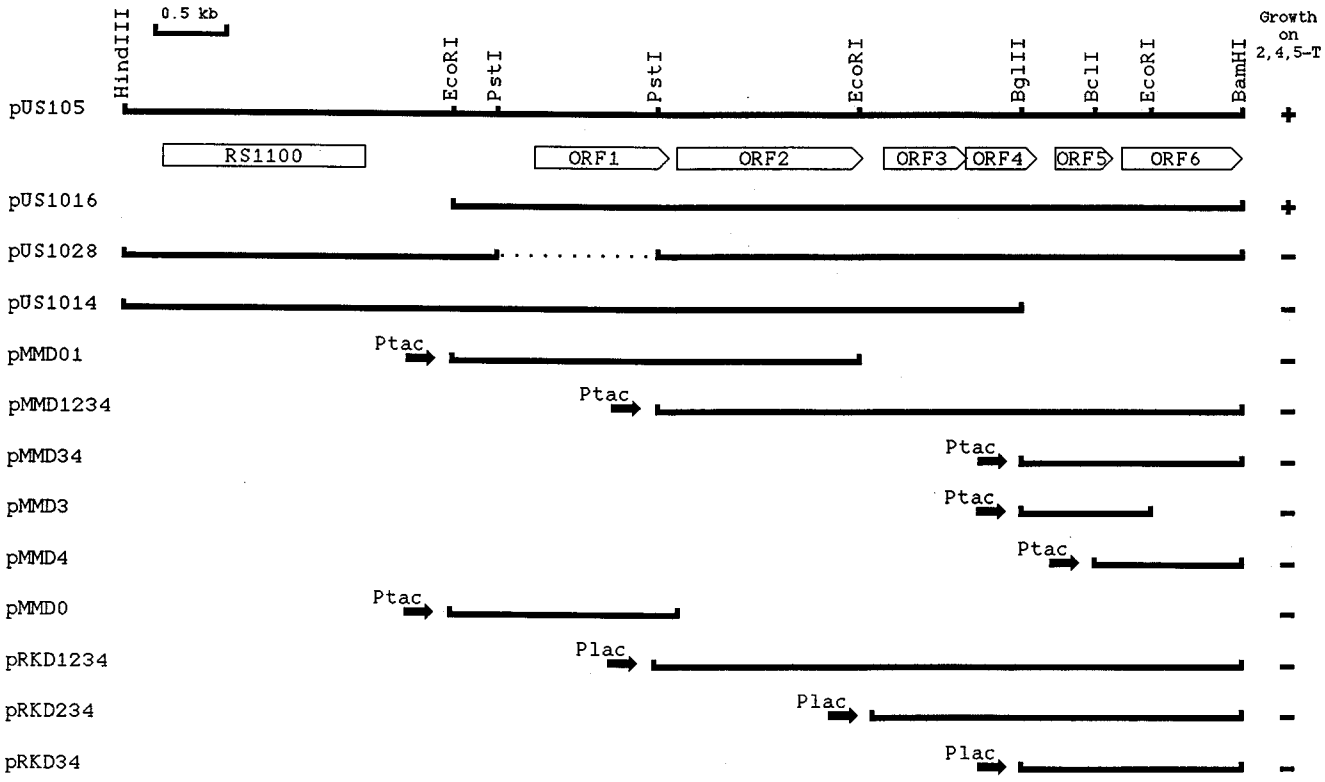

FIG. 4. Restriction maps of the constructs used in the complementation analysis of the 2,4,5-T gene cluster. The position of each of the ORFs is shown below the restriction map by an open rectangle. RS1100, upstream of ORF1, is a transposable element (Tn*931*) which has been shown to promote gene expression at the site of insertion (28, 58). The constructs are labeled on the left. Solid lines represent the regions of DNA included in the constructs, and dotted lines or blank spaces represent a deletion of that region. The arrows represent inducible promoters upstream of the cloned DNA fragment. +, PT88 complemented for growth on 2,4,5-T; −, PT88 not complemented for growth on 2,4,5-T. Complementation by more than one construct is discussed in Results.

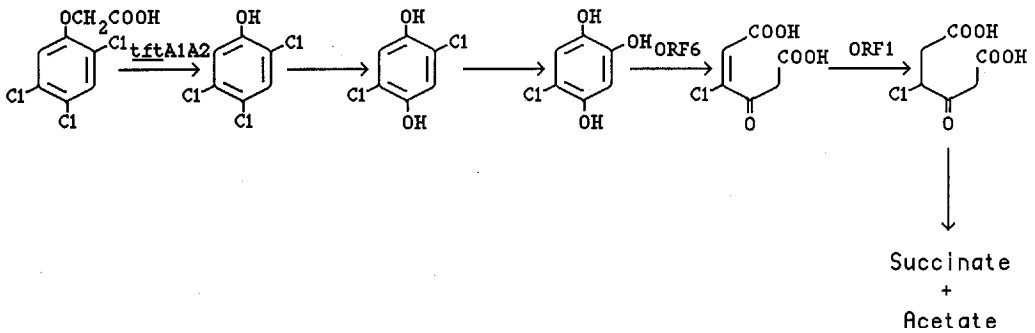

FIG. 5. Proposed pathway of 2,4,5-T degradation. The ORF6 gene product, which is similar to chloro-hydroxyquinol-1,2-dioxygenases, may catalyze the ring cleavage of the 5-chloro-1,2,4-trihydroxybenzene intermediate. ORF1, which encodes a maleylacetate reductase enzyme, could then catalyze the conversion of the 3-chloromaleylacetate to 3-chloro-4-oxoadipate.

1,2-dioxygenase-like enzyme which binds iron. The low identity of this gene product to the known catechol 1,2-dioxygenases could be explained by the possibility that the ORF6 gene product has a different substrate specificity for a compound with a hydroxyl group at the *para* position, i.e., a hydroxyquinol. Further support for this hypothesis is based on the comparison of the NH_2-terminal sequence of the recently purified 6-chlorohydroxyquinol 1,2-dioxygenase enzyme from *Azotobacter* sp. strain GP1 (64) to the predicted NH_2-terminal sequence of ORF6. This comparison revealed 12 identical amino acids of 21 comparable residues (57% identity). If conservative replacements for amino acids are considered, the sequences would be 76% identical. In addition, the *Azotobacter* 6-chlorohydroxyquinol 1,2-dioxygenase enzyme was found to be a homodimer with a subunit size of 34.2 kDa (64). The polypeptide produced from ORF6 in the T7 assay had a molecular mass of 35 kDa, and the sequence data predict a polypeptide of 32 kDa. Thus, the higher identity of the ORF6 gene product to the NH_2-terminal sequence of the 6-chloroydroxyquinol 1,2-dioxygenase enzyme compared with that of the catechol 1,2-dioxygenase enzymes suggests that the ORF6 gene product is a hydroxyquinol 1,2-dioxygenase enzyme. However, to our knowledge, none of the hydroxyquinol 1,2-dioxygenase genes have been sequenced, and thus, full amino acid sequence comparisons can be made only with the catechol 1,2-dioxygenase family of enzymes.

ortho cleavage of the trihydroxybenzene intermediate by hydroxyquinol 1,2-dioxygenase mentioned above yields maleylacetate (3). The maleylacetate is reduced to 3-oxoadipate by a maleylacetate reductase enzyme detected in bacteria (3, 38), yeasts (23, 24, 54), and fungi (1). A maleylacetate reductase enzyme has been purified from *A. eutrophus* JMP134 (53) and *Pseudomonas* sp. strain B13 (33). The NH_2-terminal sequence of the maleylacetate reductase from *A. eutrophus* JMP134 shows identity (36%) to the predicted NH_2-terminal sequence of the ORF1 gene product. Recently, we have overexpressed the ORF1 gene product in *E. coli* and assayed for maleylacetate reductase activity by the standard procedure of Kaschabek and Reineke (33). A fourfold increase in maleylacetate reductase activity was detected in induced cells harboring the gene under the *tac* promoter compared with the activity in uninduced cells or cells harboring the vector alone. Therefore, ORF1 encodes a maleylacetate reductase enzyme. In the metabolism of 2,4,5-T, *ortho* cleavage of the 5-chloro-1,2,4-trihydroxybenzene intermediate would yield 3-chloromaleylacetate. The 3-chloromaleylacetate would in turn be reduced by the maleylacetate reductase enzyme encoded by ORF1 to yield 3-chloro-4-oxoadipate (Fig. 5), which would be converted to tricarboxylic acid cycle intermediates.

A role for the other genes in the metabolism of 2,4,5-T is difficult to explain at present. The ORF5 gene product, which was essential for complementation of PT88 for growth on 2,4,5-T, did not show homology to any known enzymes. Although PT88 still grew on 2,4,5-T when both ORF3 and ORF4 were completely deleted, they showed identity to an important family of enzymes, the glutathione *S*-transferases. Several glutathione *S*-transferases have been identified in the degradation of chlorinated compounds, for example, the glutathione *S*-transferase encoded by the *bphK* gene from *Pseudomonas* sp. strain LB400 which degrades biphenyls and polychlorinated biphenyls (30). ORF3 showed 25% identity to *bphK* with an optimal alignment score of 169. In addition, the *dcmA* gene product was identified as a glutathione-dependent dichloromethane dehalogenase from *Methylobacterium* sp. strain DM4 (39). ORF3 showed 23% identity to *dcmA*, with an optimal alignment score of 92. Also, ORF3 showed 18% identity, with an optimal alignment score of 97, to the *pcpC* gene, which encoded a glutathione-dependent tetrachloro-*p*-hydroquinone dehalogenase, from the pentachlorophenol-degrading *Flavobacterium* sp. strain ATCC 39723 (46). It is possible that the ORF3 gene product is involved in 2,4,5-T metabolism and in dehalogenation of one of the intermediates. Although ORF3 was not essential for the organism to metabolize 2,4,5-T, it is possible that there is an alternate glutathione *S*-transferase enzyme or another enzyme encoded elsewhere which has the ability to perform the same function as the deleted ORF3. Indeed, it is common for glutathione *S*-transferases to occur as isozymes within many organisms (59). For example, three forms of glutathione *S*-transferase were purified from *Proteus mirabilis* (16), and four forms were identified in *Serratia marcescens* (15).

ORF2 was essential for the complementation of PT88 for growth on 2,4,5-T. The predicted amino acid sequence of ORF2 showed high identity to glutathione reductases from many organisms (Table 2). The active site of this family of enzymes with its redox-active cysteines was well conserved within the predicted amino acid sequence of ORF2 (49). It has been shown that overexpression of this gene produced an active glutathione reductase (12), assayed for by the standard procedure described by Davis et al. (13). The function of this enzyme may be to provide reduced glutathione to the glutathione *S*-transferase enzyme, thus suggesting a role for the glutathione *S*-transferase in 2,4,5-T metabolism. However, it may also be necessary to maintain a reduced environment, either for the function of one of the enzymes or for the maintenance of a reduced form of a metabolic intermediate.

Finally, the 2,4,5-T gene cluster of *B. cepacia* AC1100 is a unique cluster of genes involved in the degradation of chlori-

nated phenols. Although it has two genes (ORF1 and ORF6) which are homologous to genes in the *tfdCDEF* (*tfdF* and *tfdC*) (48) and *tcbCDEF* (*tcbF* and *tcbC*) (60) gene clusters, the organization of the genes is different. In the *tfd* and the *tcb* gene clusters, the catechol 1,2-dioxygenase genes (*tfdC* and *tcbC*) are found at the 5′ end of the cluster and the putative *trans*-chlorodienelactone isomerase genes (*tfdF* and *tcbF*) are found at the 3′ end of the cluster. In the 2,4,5-T gene cluster the genes are reversed, with the catechol 1,2-dioxygenase gene (ORF6) at the 3′ end of the cluster and the maleylacetate reductase gene (ORF1), which is homologous to the *tfdF* and *tcbF* genes (Table 2), at the 5′ end of the cluster. Also, these genes in the 2,4,5-T gene cluster are separated by four ORFs, whereas in the other gene clusters there are two or three ORFs in between. The other ORFs in the 2,4,5-T gene cluster encode unique enzymes compared with the cycloisomerase (TfdD and TcbD) and hydrolase (TfdE and TcbE) encoding genes of the *tfd* and *tcb* gene clusters (60). ORF3 and ORF4 are homologous to glutathione *S*-transferases which have been identified in other degradative pathways. However, the genes encoding these other glutathione *S*-transferases have not been associated with a cluster of genes nor a gene encoding a glutathione reductase enzyme (ORF2). Therefore, the 2,4,5-T gene cluster is a unique cluster of genes involved in the degradation of a chlorinated aromatic compound which is not related to other gene clusters described so far. On the basis of identification of at least three chromosomes in *B. cepacia* (6) and also in *B. cepacia* AC1100 (31a), it would be interesting to determine the chromosomal location and the genetic linkage of the *tftA1A2* genes in relation to the gene cluster described here.

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