

Regulation of *tfdCDEF* by *tfdR* of the 2,4-Dichlorophenoxyacetic Acid Degradation Plasmid pJP4

BRYAN KAPHAMMER, JEROME J. KUKOR, AND RONALD H. OLSEN*

*Department of Microbiology and Immunology, University of Michigan
Medical School, Ann Arbor, Michigan 48109*

Received 18 September 1989/Accepted 19 January 1990

The closely linked structural genes *tfdCDEF* borne on the 2,4-dichlorophenoxyacetic acid (TFD) catabolic plasmid, pRO101, were cloned into vector pRO2321 as a 12.6-kilobase-pair *Bam*HI C fragment and designated pRO2334. The first gene in this cluster, *tfdC*, encodes chlorocatechol 1,2-dioxygenase and was expressed constitutively. Chlorocatechol 1,2-dioxygenase expression by pRO2334 was repressed in *trans* by the negative regulatory element, *tfdR*, on plasmid pRO1949. Derepression of *tfdC* was achieved when *Pseudomonas aeruginosa* PAO4032 containing both plasmids pRO2334 and pRO1949 was grown in minimal glucose medium containing TFD, 2,4-dichlorophenol, or 4-chlorocatechol, suggesting that TFD and other pathway intermediates can act as inducing compounds. Genetic organization of the *tfdCDEF* cluster was established by deletion of the *tfdC* gene, which resulted in the loss of *tfdD* and *tfdE* activity, suggesting that genes *tfdCDEF* are organized in an operon transcribed from the negatively regulated promoter of *tfdC*. Deletion subcloning of pRO1949 was used to localize *tfdR* to a 1.2-kilobase-pair *Bam*HI-*Xho*I region of the *Bam*HI E fragment of plasmid pRO101. The *tfdR* gene product was shown not to regulate the expression of *tfdB*, which encodes 2,4-dichlorophenol hydroxylase.

Bacterial genes which code for the degradation of chlorinated aromatic compounds such as 2,4-dichlorophenoxyacetic acid (TFD) and 3-chlorobenzoate (5, 10, 12) are often plasmid borne. The genetic information on these catabolic plasmids is an excellent source of genetic material for construction of novel pathways for the degradation of more recalcitrant xenobiotics. However, recruitment of genes for the assembly of a novel pathway requires an understanding of the genetic regulation and organization of the structural genes. We are studying the regulation of the TFD pathway encoded by plasmid pJP4 (5, 9, 10, 30). Plasmid pJP4 has been physically characterized (6). The locations of the structural genes of the pathway have been estimated by transposon mutagenesis (7), and recently it has been demonstrated that chloromaleylacetic acid reductase must be recruited from the chromosome for the complete mineralization of TFD (19). The pathway of TFD degradation is shown in Fig. 1.

Genetic manipulation of pJP4 in *Pseudomonas aeruginosa* was facilitated by insertion of transposon Tn1721 into a nonessential region of pJP4 to generate plasmid pRO101 (13) (Fig. 2). Initial studies on the regulatory mechanism of pRO101 led to the isolation of plasmid pRO103, which contained a deletion in pRO101 resulting in the constitutive expression of *tfdA*, which encodes TFD monooxygenase, the first enzyme in the TFD pathway (13) (Fig. 2). When part of the region deleted in pRO103 was supplied in *trans* on a compatible plasmid, regulation of *tfdA* was restored (13). This negative regulatory element of *tfdA* was designated *tfdR* (13).

In this study, we examined the effect of *tfdR* on the expression of the downstream genes of the TFD pathway. The genetic organization of *tfdCDEF* was examined to determine whether it is transcribed as an operon. Regulation of *tfdB*, the structural gene encoding 2,4-dichlorophenol

(DCP) hydroxylase (7), by the gene product of *tfdR* was also investigated.

(A preliminary account for this work was presented previously [B. Kaphammer, J. J. Kukor, and R. H. Olsen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K-1, p. 206].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were routinely maintained on the tryptone-yeast extract-glucose medium (TNA) described elsewhere (24). The growth temperature for all experiments was 37°C. Strains carrying plasmids that conferred trimethoprim resistance was routinely cultured on Vogel-Bonner basal salts medium (34) with 0.5% glucose. Minimal medium for growth of *P. aeruginosa* PAO4032 had methionine added to a final concentration of 0.5 mM. Antibiotics carbenicillin (500 µg/ml) or trimethoprim (600 µg/ml) were added when appropriate to maintain selection for the various plasmids. Where indicated, chlorinated aromatics were added to a final concentration (wt/vol) as follows: TFD to 0.025%, DCP to 0.007%, and 4-chlorocatechol (4CC) to 0.008%.

Cultures for chlorocatechol 1,2-dioxygenase assays were grown by inoculating 25 ml of basal salts medium (MMO [33]) containing 0.5% glucose with the appropriate *P. aeruginosa* strain taken from a TNA plate. Cultures for chloromucate cycloisomerase and *cis*-2-chlorodiene lactone hydroxylase assays were grown by inoculating 25 ml of TNB broth (24) which contained carbenicillin with the appropriate strain of *P. aeruginosa* taken from an overnight TNA-carbenicillin plate. After 14 h of incubation, the cultures were subcultured into 100 ml of fresh medium and incubated until they reached mid-log phase.

Extract preparation and enzyme assays. Cell extracts for chlorocatechol 1,2-dioxygenase assays were prepared by centrifuging mid-log-phase cultures at 8,000 × g for 10 min at room temperature. The cell pellets were washed once with 10 ml of 20 mM Tris hydrochloride (pH 8.0) containing 4 µM

* Corresponding author.

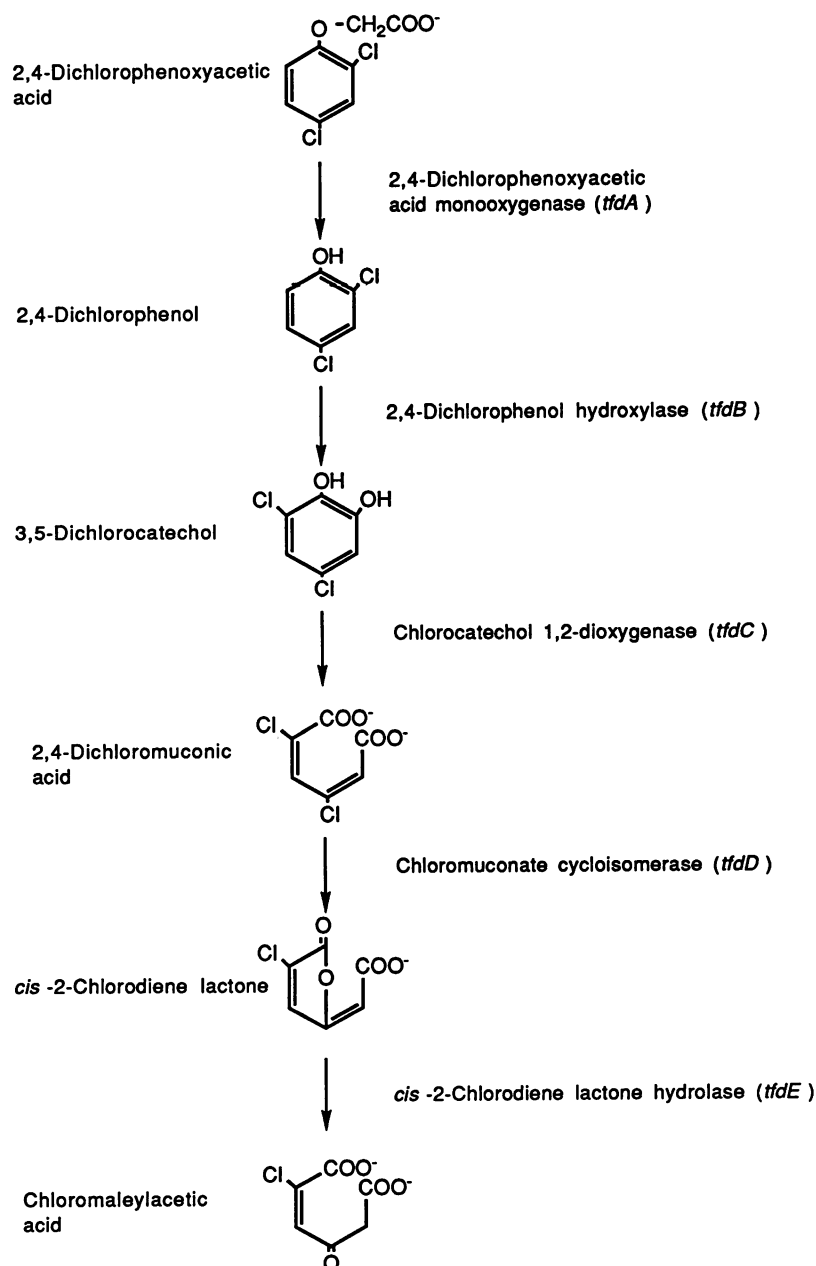


FIG. 1. Pathway of TFD degradation to chloromaleylacetic acid. The gene designation is shown in parentheses for each enzyme.

EDTA at room temperature and resuspended in the same buffer at 4°C. Cells were lysed by sonication, and cell extracts were subjected to ultracentrifugation at $140,000 \times g$ for 30 min at 4°C. The supernatants were collected and used as the source of enzyme in the assays.

Chlorocatechol 1,2-dioxygenase was assayed by a modification of the procedure of Dorn and Knackmuss (8). Each cuvette contained 980 μ l of 30 μ M 4CC in assay buffer (20 mM Tris hydrochloride [pH 8.0], 4 μ M EDTA). The reaction was initiated by adding 20 μ l of cell extract to the sample cuvette. The conversion of 4CC to chloro-*cis,cis*-muconate was observed by monitoring the increase at A_{260} with a Shimadzu UV-160 spectrophotometer. Protein concentrations were determined by the method of Bradford (2) with bovine serum albumin as a standard. The molar absorption

coefficient of chloro-*cis,cis*-muconate was used to calculate the concentration of chloro-*cis,cis*-muconate as described elsewhere (8).

Cell extracts for chloromuconate cycloisomerase assays were prepared as described above except the buffer used was 100 mM Tris hydrochloride (pH 7.5) containing 0.3 mM MnCl₂ (assay buffer). Chloromuconate cycloisomerase assays were performed as described by Pieper et al. (29), except that the assay substrate, chloro-*cis,cis*-muconate, was enzymatically prepared by adding 10 μ l of partially purified chlorocatechol 1,2-dioxygenase (J. J. Kukor and R. H. Olsen, unpublished data) to 950 μ l of assay buffer containing 30 μ M 4CC. The reaction was allowed to continue until there was no further increase at A_{260} , indicating that all the 4CC had been converted to chloromuconate. A

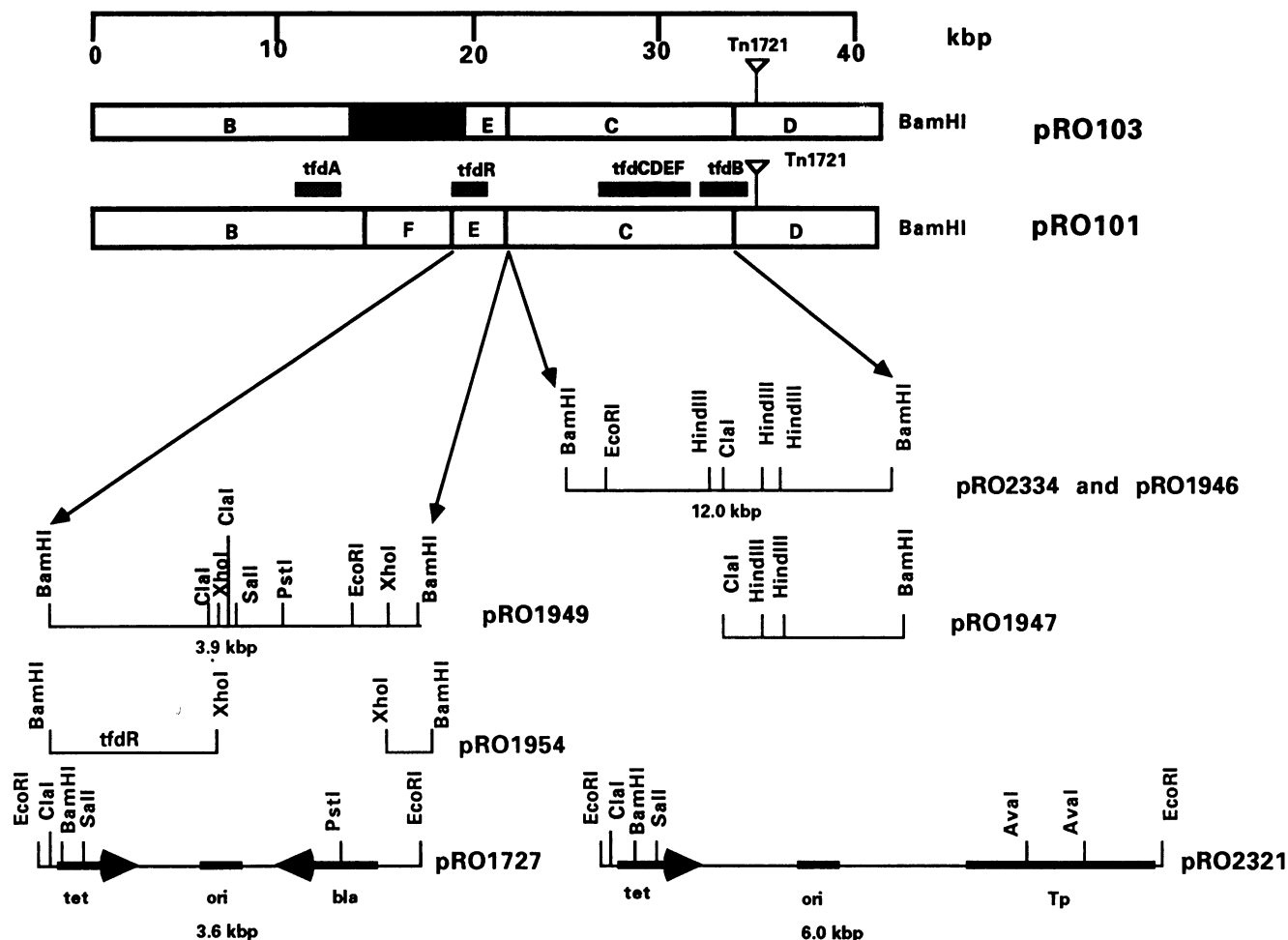


FIG. 2. Restriction map of the catabolic region of plasmid pRO101 and subclones derived from it. The black box in plasmid pRO103 shows the deleted region. The approximate locations of *tfdA*, *tfdR*, *tfdCDEF*, and *tfdB* are shown. Plasmids pRO1727 and pRO2321 are cloning vectors. *tet*, Tetracycline resistance gene; *bla*, β -lactamase resistance gene; *Tp*, trimethoprim resistance gene; *ori*, origin of replication; kbp, kilobase pairs.

20- μ l sample of the cell extract to be assayed was then added to the sample cuvette, and chloromuconate cycloisomerase activity was monitored by observing the decrease at A_{260} , reflecting the disappearance of chloro-*cis,cis*-muconate. The reference cuvette contained everything but the 20 μ l of cell extract being assayed. Enzyme activity was calculated with the absorbance coefficient of Dorn and Knackmuss (8).

Cell extracts for *cis*-2-chlorodiene lactone hydrolase assays were prepared as described above for chloromuconate cycloisomerase. The substrate for these assays was prepared by adding 100 μ l of partially purified chlorocatechol 1,2-dioxygenase (Kukor and Olsen, unpublished data) to 50 ml of 10 mM 4CC in Tris hydrochloride (pH 7.5). This was allowed to react until there was no further increase at A_{260} . This chloromuconate solution was then converted to *cis*-2-chlorodiene lactone by treatment with acid as described by Schmidt and Knackmuss (32). *cis*-2-Chlorodiene lactone hydrolase activity was measured by the method of Schmidt and Knackmuss (32). The 1-ml cuvette contained 880 μ l of 100 mM Tris hydrochloride (pH 6.5) and 20 μ l of cell extract. The reaction was initiated by adding 100 μ l of the prepared *cis*-2-chlorodiene lactone solution. Enzyme activity was calculated with the absorbance coefficient given by Schmidt and Knackmuss (32).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant markers ^a	Source or reference
<i>P. aeruginosa</i>		
PAO4032	<i>met-9020 catA nar-9011 mtuD-9002 dcu-9013</i>	H. Matsumoto ^b
PAO1c	Prototroph	15
Plasmids		
pRO1727	Cb ^r Tc ^r	3
pRO2321	Tp ^r Tc ^r	36
pJP4	Hg ^r <i>tfdR</i> ⁺ <i>tfdA</i> ⁺ <i>tfdB</i> ⁺ <i>tfdCDEF</i> ⁺	6
pRO101	Hg ^r Tc ^r <i>tfdR</i> ⁺ <i>tfdA</i> ⁺ <i>tfdB</i> ⁺ <i>tfdCDEF</i> ⁺	13
pRO103	Hg ^r Tc ^r <i>tfdR</i> <i>tfdA</i> ⁺ <i>tfdB</i> ⁺ <i>tfdCDEF</i> ⁺	13
pRO1946	Cb ^r <i>tfdCDEF</i> ⁺	This study
pRO1947	Cb ^r <i>tfdCDEF</i>	This study
pRO1949	Cb ^r <i>tfdR</i> ⁺	This study
pRO1954	Cb ^r <i>tfdR</i> ⁺	This study
pRO2334	Tp ^r <i>tfdCDEF</i> ⁺	This study

^a Abbreviations: Cb, carbenicillin, Tc, tetracycline, Tp, trimethoprim.

^b Shinshu University, Matsumoto, Japan.

Cell extracts for DCP hydroxylase assays were prepared in the same manner as for chlorocatechol 1,2-dioxygenase assays described above, except the buffer was 100 mM potassium phosphate (pH 7.6) containing 1 mM 2-mercaptoethanol, 0.1 mM EDTA, and 2 μ M flavin adenine dinucleotide. DCP hydroxylase activity was measured by observing the disappearance of the cosubstrate, NADPH, of the enzyme by a modification of the method of Liu and Chapman (20). The 1-ml cuvette contained 960 μ l of buffer, 10 μ l of 10 mM NADPH, and 20 μ l of cell extract. The endogenous oxidation of NADPH was monitored by observing the decrease at A_{340} for 5 min followed by the addition of 10 μ l of 10 mM DCP. DCP hydroxylase activity was calculated with the absorbance coefficient of Liu and Chapman (20).

Genetic techniques. Plasmids pRO1727 (3) and pRO2321 (36) were used as cloning vectors. TFD pathway genes were cloned from plasmid pRO101 (13). Techniques for DNA isolation and purification (23), restriction endonuclease cleavage and ligation of purified DNA (4), and bacterial transformations (22) have been described previously.

Chemicals and reagents. 4CC was purchased from Helix Biotech Ltd., Vancouver, British Columbia, Canada. TFD and DCP were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of the highest quality commercially available. Bradford reagent was purchased from Bio-Rad Laboratories, Richmond, Calif.

Restriction endonucleases and other enzymes involved with DNA manipulations were purchased from International Biotechnologies, Inc., New Haven, Conn., Bethesda Research Laboratories, Inc., Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, Ind.

RESULTS

Cloning of TFD genes from pRO101. A partial library of restriction endonuclease fragments was derived from plasmid pRO101. The restriction maps of these fragments are shown in Fig. 2. Plasmid pRO1946 was constructed by cloning the *Bam*HI C fragment of pRO101 into the *Bam*HI site of the vector plasmid pRO1727. This plasmid contained the structural genes for *tfdC*, *tfdD*, *tfdE*, and *tfdF* (Fig. 2). The same *Bam*HI C fragment was also cloned into vector plasmid pRO2321 to yield plasmid pRO2334. Plasmid pRO1946 was also used as a starting point in the construction of plasmid pRO1947, in which a *Cl*aI fragment that extends from a *Cl*aI site in the vector to a *Cl*aI site in the *Bam*HI C fragment was deleted.

Plasmid pRO1949 (13) contains *tfdR*, the negative regulatory gene of the *tfdA* gene, in vector pRO1727. Plasmid pRO1954 was derived by deletion of the internal *Xho*I fragment from pRO1949 (Fig. 2).

Regulation of *tfdC*. Chlorocatechol 1,2-dioxygenase expression is regulated on plasmid pRO101. When *P. aeruginosa* PAO4032(pRO101) was grown in the absence of TFD, no chlorocatechol 1,2-dioxygenase activity could be detected. However, when the same strain was grown in the presence of TFD, chlorocatechol 1,2-dioxygenase activity was induced (Table 2). *P. aeruginosa* PAO1c carrying plasmid pRO103 (Fig. 2) showed constitutive expression of *tfdC* (Table 2). Regulation was restored in strain *P. aeruginosa* PAO1c which contained both plasmids pRO103 and pRO1949 (Table 2). These data indicated that the gene product of *tfdR* regulates the expression of *tfdC*.

Levels of chlorocatechol 1,2-dioxygenase were similar for strains containing pRO101 (grown in the presence of TFD) and pRO103 (Table 2). This indicated that the genetic

TABLE 2. Regulation of *tfdC* by *tfdR*^a

Strain	Inducer	Chlorocatechol 1,2-dioxygenase activity (mmol/min per mg of protein) ^b
PAO4032(pRO101)	None	0.00
	TFD	0.04
PAO1c(pRO103)	None	0.03
	TFD	0.06
PAO1c(pRO103, pRO1949)	None	0.00
	TFD	0.06
PAO4032(pRO2334)	None	0.02
	TFD	0.02
	DCP	0.03
	4CC	0.05
PAO4032(pRO2334, pRO1949)	None	0.00
	TFD	0.02
	DCP	0.05
	4CC	0.05
PAO4032(pRO1949)	None	0.00
	TFD	0.00
	DCP	0.01
	4CC	0.04
PAO4032(pRO2334, pRO1954)	None	0.00
	TFD	0.01

^a Strains were grown and assayed as described in Materials and Methods.

^b Enzyme activities are characteristic of several independent experiments.

background differences between the two strains, *P. aeruginosa* PAO4032 and PAO1c, do not affect expression of *tfdC*. Also, the catechol 1,2-dioxygenase encoded by the chromosomal *catA* gene of *P. aeruginosa* PAO1c (18), which is absent from strain PAO4032, does not interfere with the assay of chlorocatechol 1,2-dioxygenase, because this enzyme is not induced by TFD (data not shown) and has very little activity on 4CC (17).

P. aeruginosa PAO4032 containing plasmid pRO2334 showed constitutive expression of *tfdC* independent of the presence of TFD, DCP, or 4CC. Cells of *P. aeruginosa* PAO4032 carrying both plasmids pRO2334 and pRO1949 had no detectable chlorocatechol 1,2-dioxygenase activity when grown in the absence of TFD, DCP, or 4CC. Activity was restored to fully induced levels when cells were grown in the presence of TFD, DCP, or 4CC (Table 2). These data indicated that TFD, DCP, and 4CC are effector molecules of *tfdR*, the transcriptional regulator of *tfdC*.

Cells of *P. aeruginosa* PAO4032 carrying plasmid pRO1949 had no detectable chlorocatechol 1,2-dioxygenase activity when grown in the presence or absence of TFD (Table 2). However, chlorocatechol 1,2-dioxygenase activity could be detected when this strain was grown in the presence of 4CC and to a lesser extent when it was grown in the presence of DCP. A region of the *Bam*HI E fragment has been shown to hybridize to a probe from the *tfdC* gene (11). The results presented here showed that this second chlorocatechol 1,2-dioxygenase gene, designated *tfdC1* and carried on plasmid pRO1949, is also induced by 4CC. However, chloromuconate cycloisomerase and *cis*-2 chlorodiene lactone hydrolase activities could not be detected in extracts of this strain (data not shown), indicating that extra *tfdD* and *tfdE* genes are not present on plasmid pRO1949.

TABLE 3. Effects of *tfdC* deletions on expression of *tfdD* and *tfdE*^a

Strain	Enzyme activity (mmol/min per mg of protein) ^b		
	Chlorocatechol 1,2-dioxygenase	Chloromuconate cycloisomerase	<i>cis</i> -2-Chlorodiene lactone hydrolase
PAO1c(pRO1946)	0.16	0.22	0.09
pAO4032(pRO1947)	0.00	0.00	0.00

^a Cells were grown and assayed as described in Materials and Methods.

^b Enzyme activities are characteristic of several independent experiments.

P. aeruginosa PAO4032 containing pRO101 or *P. aeruginosa* PAO1c containing pRO103 had high chlorocatechol 1,2-dioxygenase activity when grown in the presence of TFD compared with strains of *P. aeruginosa* PAO4032 containing the cloned *tfdCDEF* genes on plasmid pRO2334 (Table 2). The enzyme activity in induced strains containing pRO101 or pRO103 reflects the sum of both copies of chlorocatechol 1,2-dioxygenase gene, *tfdC* and *tfdC1*. Clearly, *tfdC1* is not induced by TFD but by an intermediate of the TFD pathway, all of which are present in strains containing plasmid pRO101 or pRO103. When the *Bam*HI E fragment was supplied in *trans* to *P. aeruginosa* PAO4032 containing plasmid pRO2334, the two chlorocatechol 1,2-dioxygenases were present, and these strains could be induced to levels of chlorocatechol 1,2-dioxygenase comparable to those of strains of *P. aeruginosa* containing plasmid pRO101 or plasmid pRO103. This elevation of chlorocatechol 1,2-dioxygenase was only seen when *P. aeruginosa* PAO4032 containing both plasmids pRO2334 and pRO1949 was grown in the presence of DCP or 4CC, indicating that only these compounds induce the second chlorocatechol 1,2-dioxygenase gene, *tfdC1*, on plasmid pRO1949 (Table 2).

The *tfdR*-containing *Bam*HI E fragment of pRO1949 was subcloned by deleting the internal *Xho*I fragment (Fig. 2), creating plasmid pRO1954. *P. aeruginosa* PAO4032 carrying plasmids pRO2334 and pRO1954 and assayed for chlorocatechol 1,2-dioxygenase activity after growth in the presence and absence of TFD showed the same regulatory pattern of repression and induction (Table 2) seen in strain PAO4032(pRO2334, pRO1949). Based on the location of the deletion in plasmid pRO103 (Fig. 2) and also the fact that plasmid pRO1954 negatively regulates *tfdC* (Table 2), the regulatory element for *tfdC* could be localized to the 1.2-kilobase-pair *Bam*HI-*Xho*I fragment of plasmid pRO1949 (Fig. 2).

Effects of deletions in *tfdC* on expression of *tfdD* and *tfdE*. *P. aeruginosa* PAO1c carrying plasmid pRO1946, which encodes a constitutively expressed chlorocatechol 1,2-dioxygenase, exhibited chloromuconate cycloisomerase activity (Table 3), whereas *P. aeruginosa* PAO1c carrying plasmid pRO1947 (Fig. 2), which has a deletion into the *tfdC* structural gene or its promoter region (11, 27) disrupting chlorocatechol 1,2-dioxygenase activity, did not have any detectable chloromuconate cycloisomerase activity (Table 3). The same strains were also assayed for *cis*-2-chlorodiene lactone hydrolase activity, the product of the *tfdE* gene. *P. aeruginosa* PAO1c containing plasmid pRO1946 had *cis*-2-chlorodiene lactone hydrolase activity, whereas the strain containing plasmid pRO1947 had no detectable activity (Table 3). Deletion of *tfdC* or its promoter had a polar effect on *tfdD* and *tfdE*, indicating that *tfdCDEF* is an operon and that only the regulated promoter of *tfdC* is responsible for the transcription of the genes in this operon.

TABLE 4. Regulation of *tfdB* by *tfdR*^a

Strain	Inducer	DCP hydroxylase activity (mmol/min per mg of protein) ^b
PAO4032(pRO101)	None	27.1
	TFD	68.4
PAO1c(pRO103)	None	41.3
	TFD	37.8
PAO4032(pRO101, pRO1949)	None	23.7
	TFD	64.4
PAO1c (pRO103, pRO1949)	None	46.0
	TFD	41.2
PAO4032(pRO1949)	None	0.0
	TFD	0.0

^a Cells were grown and assayed as described in Materials and Methods.

^b Enzyme activities are characteristic of several independent experiments.

Effects of *tfdR* on *tfdB* expression. The expression of DCP hydroxylase encoded by *tfdB* was regulated in *P. aeruginosa* PAO1c carrying plasmid pRO101 (Table 4). Furthermore, *P. aeruginosa* PAO4032(pRO101) showed a threefold increase in DCP hydroxylase activity when grown in the presence of TFD (Table 4). On the other hand, *P. aeruginosa* PAO1c carrying plasmid pRO103, which is a derivative of pRO101 with a deletion in the *Bam*HI-E-*Bam*HI-F region (13) (Fig. 2) expressed *tfdB* independent of the presence of TFD (Table 4), indicating that the regulator of *tfdB* has also been deleted from plasmid pRO103. However, *P. aeruginosa* PAO1c carrying plasmids pRO103 and pRO1949 still expressed *tfdB* independent of the presence of TFD (Table 4). These data suggest that *tfdB* is not regulated by the gene product of *tfdR* and that the regulation of *tfdB* is independent of the regulation of *tfdA* and *tfdCDEF*.

DISCUSSION

The *Bam*HI E fragment from pRO101 contains the negative regulatory gene (*tfdR*) for the *tfdCDEF* operon of the TFD pathway. This negative regulatory gene in *trans* represses the expression of *tfdC*, and *tfdC* is derepressed when cells are grown in media containing one of the inducing compounds TFD, DCP, and 4CC. A subclone of the *Bam*HI E fragment which has a deletion of the internal *Xho*I fragment (Fig. 2) also conferred the same regulatory pattern of *tfdC*. When these data are interpreted with respect to the deletion in plasmid pRO103 being just past the *Bam*HI-F-*Bam*HI-E junction (13), making pRO103 constitutive for *tfdCDEF*, the regulatory gene *tfdR* can be localized to a 1.2-kilobase fragment from the *Bam*HI-F-*Bam*HI-E Junction to the first internal *Xho*I site of the *Bam*HI E fragment as it is drawn in Fig. 2. These data also extend the work of Harker et al. (13); *tfdR*, the negative regulatory element, not only regulates *tfdA*, but also the expression of the *tfdCDEF* operon.

Recently, the promoter regions of the *tfdA* gene and the *tfdCDEF* operon have been sequenced and shown to be approximately 70% homologous (27). This high degree of homology correlates with our data, demonstrating that these two separate genetic regions are regulated by the same regulatory protein encoded by *tfdR*.

The identification of another chlorocatechol 1,2-dioxy-

genase gene on the *Bam*HI E fragment of plasmid pJP4 by hybridization to a *tfdC* probe (11) and our report here that this extra chlorocatechol 1,2-dioxygenase gene is active and regulated is interesting but not unexpected. Duplication of *tfdA* has been observed (28), and plasmid pJP4 has been shown to undergo rearrangements when strains containing it are grown on 3-chlorobenzoate (12).

This extra *tfdC* gene, which we designated *tfdC1*, could be regulated by *tfdR*. Induction experiments with strains of *P. aeruginosa* containing plasmid pRO1949 showed that *tfdC1* is induced by 4CC (Table 2). Therefore, the effector for *tfdR* is either 4CC or chloro-*cis,cis*-muconate, since pRO1949 does not carry active *tfdD* or *tfdE* genes. This is in contrast to the induction pattern of *tfdC*, in which *tfdR* can use TFD, DCP, or 4CC as an effector. This difference in induction pattern could be due to a difference in the binding of *tfdR* to the promoters of *tfdC* or *tfdC1*. If *tfdR* is the regulator of *tfdC1*, the location of *tfdR* near *tfdC1* could be comparable to the organization of other regulatory genes, in which the regulatory gene is transcribed divergently from the promoter of the gene it regulates (14, 31). Sequence analysis of the *Bam*HI E fragment of pJP4 will answer this question.

The *tfdC* operon could be derepressed by several of the TFD pathway intermediates, including TFD, DCP, and 4CC. 3-Chlorobenzoate has also been shown to induce the TFD pathway (7, 12) and may also interact with *tfdR*. 4-Chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid can be used as sole carbon and energy sources by *Alcaligenes eutrophus* JMP134(pJP4) (30), suggesting that they or their methyl-containing metabolites are also inducers of the TFD pathway and interact with *tfdR*.

Pathway intermediates such as chloromuconate or chloromaleylacetic acid may interact with *tfdR* to derepress *tfdC*. Cells are not permeable to these compounds, so it is impossible to assess their effect on the expression of *tfdC* (1, 16, 21, 26). However, the analogous nonchlorinated compound *cis,cis*-muconate is the inducer of chromosomally encoded catechol oxygenase, *cis-cis*-muconate lactonizing enzyme, and muconolactone isomerase of the catechol branch of the beta-ketoadipate pathway in *Pseudomonas putida* and *P. aeruginosa* (16, 25, 35).

Don et al. (7) suggested that genes *tfdCDEF* were organized into an operon on the basis of transposon mutagenesis experiments. However, insertional disruption of *tfdC* or *tfdD* did not have the polar effect on *tfdE* that would be predicted if these genes were part of the same operon. Rather, these insertions resulted in the constitutive expression of *tfdE*. This apparent anomaly may reflect *tfdE* transcription from a promoter in the transposon Tn5 which was inserted into *tfdC* or *tfdD*. If this was true, then the direction of transcription of *tfdE* would be from left to right as diagrammed in Fig. 2, and therefore, if genes *tfdC* through *tfdF* are operonic, transcription would originate from the promoter of the *tfdC* gene. This hypothesis was supported by our findings that deletion of a portion of the *tfdC* gene on the *Bam*HI C fragment resulted in a polar effect on the expression of *tfdD* and *tfdE*. From this physiological evidence it seems apparent that *tfdCDEF* forms an operon, as suggested by Don et al. (7).

The structural gene for DCP hydroxylase, *tfdB*, does not appear to be regulated by *tfdR*. Several observations indicate that *tfdB* has a more complicated regulatory pattern. First, *tfdB* was not expressed constitutively in strains containing pRO103, unlike *tfdA* and *tfdCDEF*. The *tfdB* gene was rendered uninducible in strains of *P. aeruginosa* containing plasmid pRO103, with levels of DCP hydroxylase

that were between baseline levels for strains carrying pRO101 and fully induced levels of these strains. The possibility that *tfdB* is induced by a downstream metabolite such as DCP and not by TFD is eliminated by the observation that pRO103 constitutively expresses *tfdA* (13) and *tfdCDEF*; therefore, TFD would be converted to DCP and subsequently to all the pathway intermediates down to the chloromaleylacetic acid in strains containing pRO103. Second, strains containing both pRO103 and pRO1949 (the *tfdR* plasmid) were still uninducible for *tfdB* independent of the presence of TFD, showing that *tfdR* is not directly involved in the regulation of *tfdB*.

The high-baseline-uninducible phenotype exhibited for *tfdB* expression by strains containing pRO103 suggests that the regulatory gene for *tfdB* is missing or inactive in pRO103. This regulatory gene may be located in the deleted *Bam*HI-E-*Bam*HI-F region of pRO103. Further studies are under way to localize the regulatory element of *tfdB*.

ACKNOWLEDGMENTS

This study was funded by the U.S. Environmental Protection Agency, Cooperative Agreement CR-812679, National Institute of Environmental and Health Sciences grant 1P42ES04911-01, and a fellowship to B.K. from the Dow Chemical Co.

LITERATURE CITED

- Bird, J. A., and R. B. Cain. 1968. *cis,cis*-Muconate, the product inducer of catechol 1,2-oxygenase in *Pseudomonas aeruginosa*. *Biochem. J.* **109**:479-481.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Cuskey, S. M., V. Pecoraro, and R. H. Olsen. 1987. Initial catabolism of aromatic biogenic amines by *Pseudomonas aeruginosa* PAO: pathway description, mapping of mutations, and cloning of essential genes. *J. Bacteriol.* **169**:2398-2404.
- Cuskey, S. M., J. A. Wolff, P. V. Phibbs, Jr., and R. H. Olsen. 1985. Cloning of genes specifying carbohydrate catabolism in *Pseudomonas aeruginosa* and *Pseudomonas putida*. *J. Bacteriol.* **162**:865-871.
- Don, R. H., and J. M. Pemberton. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes eutrophus* and *Alcaligenes paradoxus*. *J. Bacteriol.* **145**:681-686.
- Don, R. H., and J. M. Pemberton. 1985. Genetic and physical map of the 2,4-dichlorophenoxyacetic acid degradative plasmid pJP4. *J. Bacteriol.* **161**:466-468.
- Don, R. H., A. J. Weightman, H.-J. Knackmuss, and K. N. Timmis. 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). *J. Bacteriol.* **161**:85-90.
- Dorn, E., and H.-J. Knackmuss. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. *Biochem. J.* **174**:73-84.
- Evans, W. C., B. S. W. Smith, H. N. Fernley, and J. I. Davies. 1971. Bacterial metabolism of 2,4-dichlorophenoxyacetate. *Biochem. J.* **122**:543-551.
- Fisher, P. R., J. Appleton, and J. M. Pemberton. 1978. Isolation and characterization of the pesticide-degrading plasmid, pJP1, from *Alcaligenes paradoxus*. *J. Bacteriol.* **135**:798-804.
- Ghosal, D., and I.-S. You. 1988. Nucleotide homology and organization of chlorocatechol oxidation genes of plasmids pJP4 and pAC27. *Mol. Gen. Genet.* **211**:113-120.
- Ghosal, D., I.-S. You, D. K. Chatterjee, and A. M. Chakrabarty. 1985. Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4. *Proc. Natl. Acad. Sci. USA* **82**:1638-1642.
- Harker, A. R., R. H. Olsen, and R. J. Seidler. 1989. Phenoxyacetic acid degradation by the 2,4-dichlorophenoxyacetic acid (TFD) pathway of plasmid pJP4: mapping and characterization

- of the TFD regulatory gene *tfdR*. *J. Bacteriol.* **171**:314–320.
14. Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace. 1988. A large family of bacterial activator proteins. *Proc. Natl. Acad. Sci. USA* **85**:6602–6606.
 15. Holloway, B. W., V. Krishnapillai, and A. F. Morgan. 1979. Chromosomal genetics of *Pseudomonas*. *Microbiol. Rev.* **43**:73–102.
 16. Kemp, M. B., and G. D. Hegeman. 1968. Genetic control of the beta-ketoadipate pathway in *Pseudomonas aeruginosa*. *J. Bacteriol.* **96**:1488–1499.
 17. Kojima, Y., H. Fujisawa, A. Nakazawa, T. Nakazawa, F. Kanetsuna, H. Taniuchi, M. Nozaki, and O. Hayaishi. 1967. Studies of pyrochatechase. I. Purification and spectral properties. *J. Biol. Chem.* **242**:3270–3278.
 18. Kukor, J. J., R. H. Olsen, and D. P. Ballou. 1988. Cloning and expression of the *catA* and *catBC* gene clusters for *Pseudomonas aeruginosa* PAO. *J. Bacteriol.* **170**:4458–4465.
 19. Kukor, J. J., R. H. Olsen, and J.-S. Siak. 1989. Recruitment of a chromosomally encoded maleylacetate reductase for degradation of 2,4-dichlorophenoxyacetic acid by plasmid pJP4. *J. Bacteriol.* **171**:3385–3390.
 20. Liu, T., and P. J. Chapman. 1984. Purification and properties of plasmid-encoded 2,4-dichlorophenol hydroxylase. *FEBS Lett.* **173**:314–318.
 21. Meagher, R. B., G. M. McCorkle, M. K. Ornston, and L. N. Ornston. 1972. Inducible uptake system for beta-carboxy-*cis*, *cis*-muconate in a permeability mutant of *Pseudomonas putida*. *J. Bacteriol.* **111**:465–473.
 22. Mercer, A. A., and J. S. Loutit. 1979. Transformation and transfection of *Pseudomonas aeruginosa*. Effects of metal ions. *J. Bacteriol.* **140**:37–42.
 23. Olsen, R. H., G. DeBusscher, and W. R. McCombie. 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J. Bacteriol.* **150**:60–69.
 24. Olsen, R. H., and J. Hansen. 1976. Evolution and utility of a *Pseudomonas aeruginosa* drug resistance factor. *J. Bacteriol.* **125**:837–844.
 25. Ornston, L. N. 1966. The conversion of catechol and protocatechuate to beta-ketoadipate by *Pseudomonas putida*. IV. Regulation. *J. Biol. Chem.* **241**:3800–3810.
 26. Parke, D., and L. N. Ornston. Constitutive synthesis of enzymes of the protocatechuate pathway and of the beta-ketoadipate uptake system in mutant strains of *Pseudomonas putida*. *J. Bacteriol.* **126**:272–281.
 27. Perkins, E. J., G. W. Bolton, M. P. Gordon, and P. F. Lurquin. 1988. Partial nucleotide sequence of the chlorocatechol degradative operon *tfdCDEF* of pJP4 and similarity to promoters of the chlorinated aromatic degradative operons *tfdA* and *clcABC*. *Nucleic Acids Res.* **16**:7200.
 28. Perkins, E. J., and P. F. Lurquin. 1989. Duplication of a 2,4-dichlorophenoxyacetic acid monooxygenase gene in *Alcaligenes eutrophus* JMP134(pJP4). *J. Bacteriol.* **170**:5669–5672.
 29. Pieper, D. H., K.-H. Engesser, and H.-J. Knackmuss. 1989. Regulation of catabolic pathways of phenoxyacetic acids and phenols in *Alcaligenes eutrophus* JMP134. *Arch. Microbiol.* **151**:365–371.
 30. Pieper, D. H., W. Reineke, K.-H. Engesser, and H.-J. Knackmuss. 1988. Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. *Arch. Microbiol.* **150**:95–102.
 31. Schell, M. A., and M. Surkordhaman. 1989. Evidence that the transcription activator encoded by the *Pseudomonas putida* *nahR* gene is evolutionarily related to the transcription activators encoded by the *Rhizobium nodD* genes. *J. Bacteriol.* **171**:1952–1959.
 32. Schmidt, E., and H.-J. Knackmuss. 1980. Chemical structure and biodegradability of halogenated aromatic compounds. Conversion of chlorinated muconic acids into maleylacetic acid. *Biochem. J.* **192**:339–347.
 33. Stanier, R., N. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. *J. Gen. Microbiol.* **43**:159–271.
 34. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* **72**:97–106.
 35. Wheelis, M. L., and L. N. Ornston. 1972. Genetic control of enzyme induction in the beta-ketoadipate pathway of *Pseudomonas putida*: deletion mapping of *cat* mutations. *J. Bacteriol.* **109**:790–795.
 36. Zylstra, G. J., R. H. Olsen, and D. P. Ballou. 1989. Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. *J. Bacteriol.* **171**:5907–5914.