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Substrate Diversity and Expression of the 2,4,5-Trichlorophenoxyacetic Acid Oxygenase from *Burkholderia cepacia* AC1100

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Burkholderia cepacia **AC1100 uses the chlorinated aromatic compound 2,4,5-trichlorophenoxyacetic acid as a sole source of carbon and energy. The genes encoding the proteins involved in the first step (*tftA* and *tftB* [previously designated *tftA1* and *tftA2*, respectively]) have been cloned and sequenced. The oxygenase, TftAB, is capable of converting not only 2,4,5-trichlorophenoxyacetic acid to 2,4,5-trichlorophenol but also a wide range of chlorinated aromatic phenoxyacetates to their corresponding phenolic derivatives, as shown by whole-cell and cell-free assays. The rate of substrate utilization by TftAB depends upon the extent of chlorination of the substrate, the positions of the chlorines, and the phenoxy group. These results indicate a mechanistic similarity between TftAB and the 2,4-dichlorophenoxyacetic acid/α-ketoglutarate-dependent dioxygenase, TfdA, from *Alcaligenes eutrophus* JMP134. The promoter of the oxygenase genes was localized by promoter-probe analysis, and the transcriptional start site was identified by primer extension. The β-galactosidase activity of the construct containing the promoter region cloned upstream of the β-galactosidase gene in the promoter-probe vector pKRZ-1 showed that this construct is constitutively expressed in *Escherichia coli* and in AC1100. The −35 and −10 regions of the oxygenase genes show significant sequence identity to typical *Escherichia coli* σ^{70} promoters.**

The herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) is a component of Agent Orange. This defoliating agent was used in Vietnam and is a suspected carcinogen (9). A bacterial strain which is able to use 2,4,5-T as a sole source of carbon and energy was isolated in our laboratory (20, 21). This strain, *Burkholderia cepacia* AC1100, not only is capable of degrading 2,4,5-T in liquid culture but also is effective in degrading 2,4,5-T in contaminated soil. After treatment of soil containing 20,000 μg of 2,4,5-T per g of soil with AC1100, more than 90% of the compound was degraded (18). *B. cepacia* AC1100 initially converts 2,4,5-T to 2,4,5-trichlorophenol (2,4,5-TCP) (12, 13). The genes for the oxygenase component have been cloned, sequenced, and characterized (3, 12), and the protein products have been purified (10). For complete activity, four polypeptides are necessary, i.e., two polypeptides which compose the oxygenase and two other polypeptides (one which is 15 kDa and another which is 29 kDa) which are believed to constitute a reductase (10). Although the oxygenase component is easily purified, the instability of the reductase components has made purification of the reductase difficult, and therefore complete kinetic studies could not be performed. Although the four polypeptides isolated from AC1100 were necessary for activity, it has been shown that when a segment containing just the *tftAB* genes is transformed into *Pseudomonas aeruginosa* PAO1, this strain is able to convert 2,4,5-T to 2,4,5-TCP. Thus, the reductase components are believed to be endogenous in *P. aeruginosa* PAO1. TftAB confers on *P. aeruginosa* the ability to convert not only 2,4,5-T to 2,4,5-TCP but also 2,4-dichlorophenoxyacetic acid (2,4-D) to 2,4-dichlorophenol and phenoxyacetate to phenol. The derived amino acid sequences of TftAB show significant homology to those of the α and β subunits of the 1,2-dioxygenases of other bacterial degradation pathways (3, 24). Previous studies with whole cells of AC1100 have suggested that the genes encoding the oxygenase are constitutively expressed (17).

In this paper we report on the ability of TftAB to allow utilization of a number of chlorinated phenoxyacetate derivatives, and we delineate the specific structures of these compounds, which may affect the rate of conversion to the corresponding halogenated phenols. Through the use of promoter-probe constructs and primer extension analysis, we also determined the location of and the nature of expression from the *tftAB* promoter.

Effects of chlorine substitution on rates of conversion of the chlorophenoxyacetates to the corresponding chlorophenols. Previous experiments had shown that the 2,4,5-T oxygenase was capable of converting not only 2,4,5-T to its respective phenol but also 2,4-D to 2,4-dichlorophenol and phenoxyacetate to phenol (3). We were therefore interested in determining the extents of utilization of various chlorophenoxyacetates as well as quantitatively determining the rates at which the oxygenase converted each chlorophenoxyacetate to its corresponding chlorophenol. Whole-cell assays for 2,4,5-T oxygenase were carried out by using *P. aeruginosa* PAO1 transformed with the plasmid construct pCD206 as described previously (3). Five hundred microliters of each of the culture supernatants was assayed by high-pressure liquid chromatography and colorimetrically as described previously (13, 22) to confirm the presence of the chloro- or fluorophenols. 2-Chlorophenol, 4-chlorophenol, and 4-fluorophenol were run, and retention times were compared with those of the test samples. Total protein concentrations in the suspensions were measured at the same time points as described by Wackett and Gibson (30).

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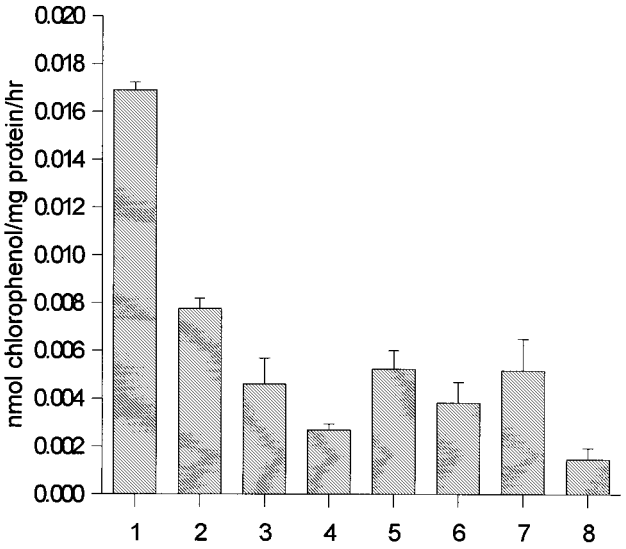


FIG. 1. Results of whole-cell assays showing the rates of conversion of various chlorophenoxyacetates. The numbers below the bar graphs correspond to those of the substrates whose structures are shown in Figure 2. Results with compounds 9 and 10 are not shown but are discussed in the text. Strain PAO1/pCD206 containing the *tftAB* genes under the control of the *lac* promoter, as previously described (3), was grown overnight in basal salts medium plus 1% glucose and 500 μg of carbenicillin per ml (17). Cells were harvested by centrifugation and resuspended in 5 ml of basal salts medium plus 5 mM substrate. Samples were taken at various time points, and the maximal amount of chlorophenol formed was determined.

Assays of crude cell extracts were performed in parallel. Cell extracts were obtained by sonication of an overnight culture, and 20-μg aliquots of the whole-cell lysate were added to a reaction mixture consisting of 0.4% Tween 20, 10 mM substrate, 50 mM KH_2PO_4 (pH 7.8), 0.5 mM $MgSO_4$, 50 μM $FeSO_4$, and 0.5 mM ascorbic acid to a final volume of 100 μl. Protein concentrations were estimated by the method of Bradford (2). The reaction was initiated with the addition of the cell lysate and carried out for 1 h at room temperature. Figure 1 shows a comparison of the different rates of conversion of each substrate to its chlorophenol in whole cells. Figure 2 shows the structures of the compounds tested in the experiments whose results are given in Fig. 1. Incubation with 2,4,5-T (compound 1 [Fig. 2]) gave the highest rate of conversion (0.0169 ± 0.0003 nmol of chlorophenol formed per mg of protein per h). With decreasing chlorination, the rate of conversion of the parent compound to the respective chlorophenol decreases. The activities for 2,4-D (compound 2 [Fig. 2]), and phenoxyacetate (compound 3 [Fig. 2]) were $7.75 \times 10^{-3} \pm 0.0002$ and $4.59 \times 10^{-3} \pm 0.0006$ nmol of chlorophenol per mg of protein per h, respectively. The position of the chlorine atom also appears to affect the rate of conversion. The rate of conversion of *o*-chlorophenoxyacetate (compound 4 [Fig. 2]) was twofold lower than that of *p*-chlorophenoxyacetate (compound 5 [Fig. 2]) in the whole-cell experiment and approximately fourfold lower in crude cell extracts. With all of the compounds tested, no activity was seen in whole-cell experiments with the vector-only PAO1 strain (data not shown). Both whole-cell and crude cell extract assays show basically similar results with respect to the rates of chlorophenol production from the respective phenoxyacetate derivatives.

Effects of alteration in the phenoxy group. Esterification of the phenoxy group does not affect the activity significantly, as shown with the rates of conversion (Fig. 1, bars 5, 6, and 7) of centrophenoxine (compound 6 [Fig. 2]) and *p*-chlorophenoxyacetate methyl ester (compound 7 [Fig. 2]). Substitution at C-2 of the side chain drastically decreases the activity. This is shown by the rate of conversion of (+/−)-2-(*p*-chlorophenoxy)propionate (compound 8 [Fig. 2]), which was 3.6-fold less than the rate of conversion of *p*-chlorophenoxyacetate in both the whole-cell and crude cell extract assays. The addition of another methyl group at this position, with 2-(*p*-chlorophenoxy)-2-methylpropionic acid (compound 9 [Fig. 2]), completely blocks activity (data not shown). To determine the effect of eliminating the ether oxygen atom of the phenoxy group, strain PAO1/pCD206 was tested for the ability to convert benzoate (compound 10 [Fig. 2]) to phenol. No phenolic compound resulted from the whole-cell or crude cell extract assays when the mixtures were incubated with 5 mM benzoate. Since benzoate is normally rapidly metabolized by PAO1 cells without accumulation of catechol or other phenolic compounds, while phenol itself is not metabolized by PAO1, the lack of accumulation of phenol suggests that PAO1/pCD206 cells are unable to convert benzoate to phenol to any significant extent.

Replacement of the chlorine substituent with fluorine. To examine the effect of replacing the chlorine atom with a dif-

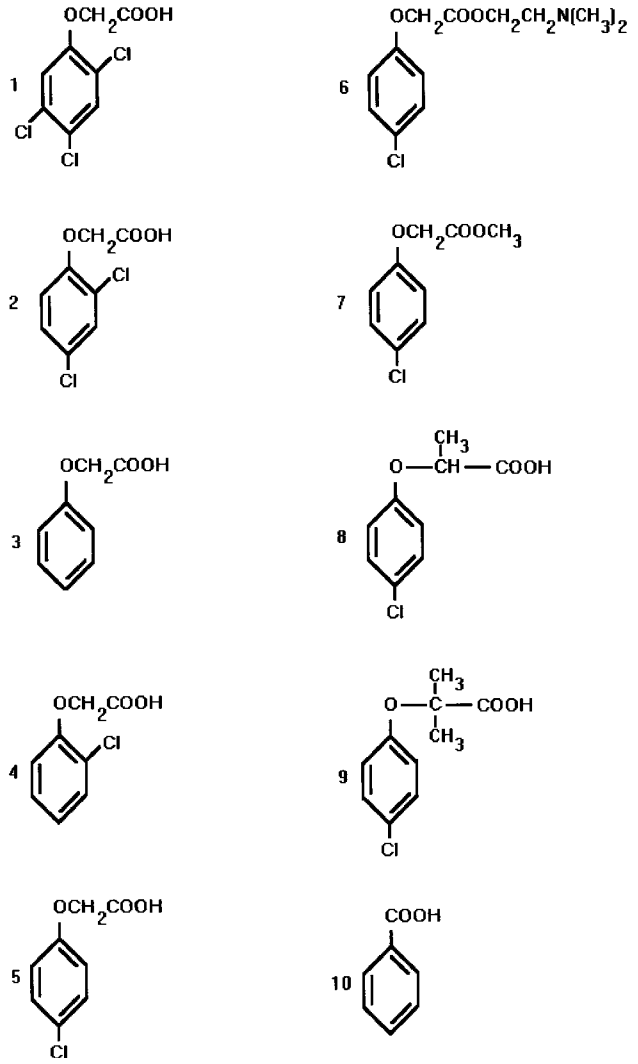


FIG. 2. Summary of the chemical structures of the substrates used in the crude cell extract and whole-cell assays.

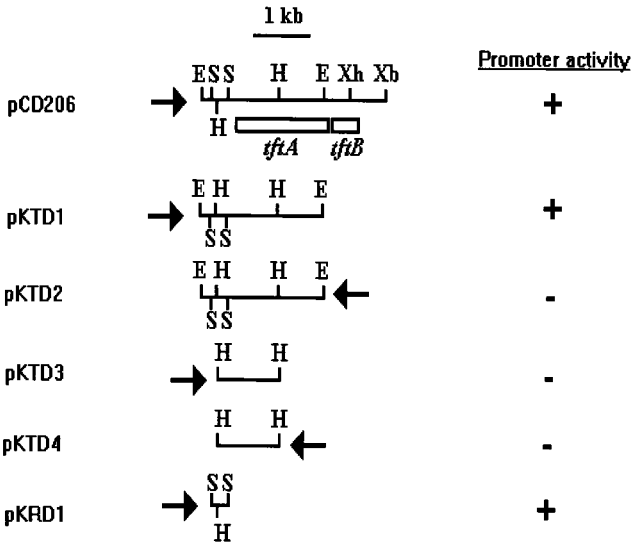


FIG. 3. Restriction maps of the promoter-probe constructs. Constructs pKTD1 to pKTD4 were created from the plasmid pKT240 (1). Inserts were cloned upstream of the promoterless streptomycin phosphotransferase gene in both orientations, as shown by the directions of the arrows. The *E. coli* strain containing pKTD1 to pKTD4 was grown in L broth containing increasing amounts of streptomycin to determine the promoter activity of the DNA fragment. If these strains grew at a streptomycin concentration greater than that at which the vector-only control strain grew, then a promoter is present (+). Construct pKRD1 was created from the plasmid pKRZ-1 (27). E, *Eco*RI, S, *Sal*I, Xh, *Xho*I; Xb, *Xba*I; H, *Hin*dIII.

ferent halogen, the rate of conversion of 4-fluorophenoxyacetic acid to 4-fluorophenol was measured. The whole-cell rate of conversion of 4-fluorophenoxyacetate to 4-fluorophenol was 0.0563 ± 0.00326 nmol of 4-fluorophenol formed per mg of protein per h, compared with 0.0363 ± 0.0053 for the rate of conversion of *p*-chlorophenoxyacetate to *p*-chlorophenol. 4-Fluorophenoxyacetate appears to be a more favorable substrate for TftAB than *p*-chlorophenoxyacetate. This may be due to the stabilizing effect of the fluoride atom on the aromatic ring, since F is the most electronegative of all the halogens. It had previously been shown that AC1100 could dechlorinate a number of different chlorophenols (18, 19). This strain also has the ability to remove different halogen atoms from the aromatic ring in the following decreasing order: F > Cl > Br > I (18). These results are similar to those found by Reineke and Knackmuss in studies of dioxygenation of halogenated benzoic acids (26).

Promoter localization and expression of *tftAB*. The *tftAB* genes were cloned under the transcriptional control of the *lac* promoter, and this construct was used for the substrate specificity experiments described above. We therefore wanted to define the natural promoter of these genes and determine if they are transcriptionally regulated. A series of constructs was made by subcloning DNA fragments from pCD206 (3) and cloning them into the broad-host-range vector pKT240 (1) (Fig. 3). This promoter-probe vector contains a promoterless streptomycin phosphotransferase gene downstream of a multiple cloning site. Therefore, any DNA fragment cloned into the multiple cloning site which contains a promoter sequence would confer steptomycin resistance to the host organism. The promoter-probe constructs were transformed into *Escherichia coli* TG1 by $CaCl_2$ treatment as described by Sambrook et al. (28) and plated on Luria agar plates containing 70 μg of ampicillin per ml or 50 μg of kanamycin per ml. The same constructs were then introduced into AC1100 by the triparental filter mating technique (5). The promoter activity of each construct was determined by growth of the host in a streptomycin concentration above the vector control background level. Only construct pKTD1 allowed growth of *E. coli* at a streptomycin concentration of at least 100 μg/ml. In contrast, the strain containing the vector control, pKT240, grew at a streptomycin concentration of up to 20 μg/ml. To further quantify the activity of the promoter, the region of DNA between the *Eco*RI site and just downstream of the *Hin*dIII site was subcloned as a 0.5-kb *Sal*I fragment in another promoter-probe vector, pKRZ-1 (27), to make construct pKRD1. This broad-host-range vector contains a promoterless *lacZ* gene downstream of a polylinker sequence. Any promoter-containing DNA sequence cloned into this region would then allow the expression of *lacZ*, resulting in the formation of β-galactosidase. When TG1 transformed with this construct was plated on L agar plates containing the chromogenic reagent X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), these cells turned intensely blue overnight. pKRD1 was introduced into strain AC1100 and assayed for β-galactosidase activity at different stages of growth and in the presence and absence of 2,4,5-T (Table 1). The highest level of activity occurred when cells were grown in 1% glucose in the absence of 2,4,5-T. Thus, the expression of *tftAB* is independent of the presence of 2,4,5-T.

Primer extension analysis. AC1100 cells were grown in basal salts medium with 1% glucose and 4 mM 2,4,5-T to late log phase. The cells were pelleted by centrifugation. Cell lysis and RNA extraction were achieved with the Trizol reagent (Gibco BRL, Gaithersburg, Md.) as described by the manufacturer. Eighty micrograms of RNA was added to 5 pmol of [γ-^{32}P] ATP-labeled oligonucleotide primer complementary to the mRNA. Hybridization and the reverse transcriptase reaction was performed by a modification of the procedure described by Hendrickson and Misra (14), using reverse transcriptase purchased from Promega (Madison, Wis.). The same primer used for the reverse transcription reaction was used to generate the sequence upstream of the *tftA* ATG codon in the 0.5-kb *Sal*I fragment of construct pKRD1. Five microliters of this suspension was loaded onto a 6% polyacrylamide sequencing gel next to the corresponding sequence ladder. The DNA sequencing ladder was generated by double-stranded PCR sequencing with Sequitherm polymerase (Epicentre Technologies, Madison, Wis.). The gel was dried, exposed to Kodak X-Omat film, and developed after 3 days of exposure at −70°C. The transcriptional start site was determined to be a G residue (Fig.

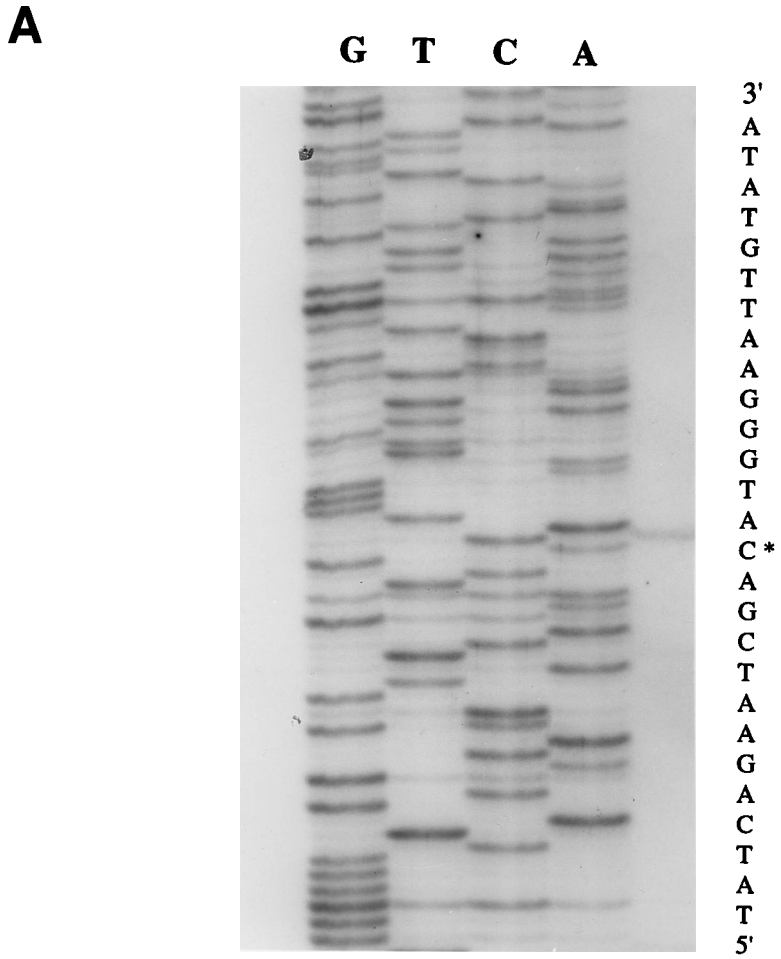
TABLE 1. β-Galactosidase activities of the *tftA* promoter-probe construct in *B. cepacia* AC1100

Promoter-probe/growth medium[a]	β-Galactosidase sp act (nmol of *o*-nitrophenol/min/mg of protein) at the following growth phase[b]:		
	Mid-log	Late log	Stationary
pKRZ-1/BSG	<10	<10	<10
pKRZ-1/BSTG	<10	<10	<10
pKRD1/BSG	190 ± 67	667 ± 62	643 ± 25
pKRD1/BSTG	96 ± 20	398 ± 19	487 ± 19
pKRD1/BST	ND[c]	ND	149 ± 20

[a] Abbreviations: BSG, basal salts medium plus 1% glucose; BSTG, basal salts medium plus 4 mM 2,4,5-T plus 1% glucose; BST, basal salts medium plus 4 mM 2,4,5-T.

[b] Cells were harvested at the indicated growth stages. Mid-log, 23 h; late log, 31 h; stationary, 48 h.

[c] ND, not determined.



B

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                -35                          -10          +1
TGCTCGTACACCAGTCTTGACTATATCTCTCAGGCGATTATACAATTCCCATGTCGATTCTGATACGGCTG
TCGCTAGCCCCCCATTCGAGCAAGCTGGCACAAATCTGTGAGCCAAT
                        tftA
CAAACTTCGGAAGATATT  ACT ATG AAC ACT ACT ATG AAC ACA CCT GTC CCC AGT TAC GTC
AAT GAC GTC AGT CAT CGC GGG CTG GTC GAC GAT CGA GCA ACG GAC GGA ATT TTT CGT
GTG CAC CGC GAG GCA TTT CTC GAT CCA CGT ATC TTT GAA CTA GAA ATG TCG CGC ATA
TTC GAG TCG ACG TGG GTG TTT ATC GGG CTC GAA AGC CAG GTC GCC AAG CCC CAT GAT
TTC ATC ACT GCG GAT ATT GGG CGT
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FIG. 4. (A) Determination of the *tftAB* transcriptional start site. RNA isolated from a stationary-phase AC1100 culture was used for a reverse transcriptase reaction. The DNA sequence shown on the right corresponds to the sequencing ladder on the left. The C residue of the sequencing ladder corresponds to the complementary nucleotide at which transcription initiation occurs and is indicated by an asterisk. (B) Upstream sequence of *tftA* showing the promoter region. This 5′-to-3′ DNA sequence represents part of the open reading frame of *tftA* and the −35/−10 region. The promoter region and the transcriptional start site are identified by boldface type. The underlined residues represent the sequence which is complementary to the synthetic oligonucleotide used for both the sequencing reaction and the primer extension reaction.

4A) which is 87 bp upstream of the ATG codon of *tftA*. The promoter region is a clearly defined −35 and −10 σ^{70} *E. coli*-type promoter, TATAAT/TTGACA (Fig. 4B). This finding is consistent with the result that β-galactosidase activity is constitutively expressed in *E. coli* as well as in *B. cepacia*. These results confirm those of earlier studies which showed that the enzyme necessary for converting 2,4,5-T to 2,4,5-TCP did not require 2,4,5-T as an inducer for activity (17).

Previous work has shown the ability of AC100 to convert not only 2,4,5-T to 2,4,5-TCP but also 2,4-D to 2,4-DCP and phenoxyacetate to phenol (3). Tomasi et al. (29) have demonstrated the capability of this strain to convert pentachlorophenol to tetrachlorohydroquinone. This property of diverse substrate ranges has been observed with other oxygenases (31). 2,4,5-T oxygenase can utilize various chlorophenoxyacetates as substrates, but the degree of conversion to the corresponding chlorophenols depends upon the degree of chlorination, the position of the chlorine atom, the type of halogen on the ring, and alteration of the phenoxy group. These observations are similar to those found for substrate utilization by the 2,4-D/α-ketoglutarate-dependent dioxygenase, TfdA (7, 8). Two notable similarities were that (i) TfdA appears to prefer chlorinated substrates to unsubstituted ones and (ii) propionic acids were the least favorable substrates. Moreover, C-2 of the phe-

noxy group appears to be critical for conversion to the phenol. It is interesting that although 2,4,5-T oxygenase and TfdA have some of the same properties with respect to substrate utilization, there is no significant homology between them at either the DNA or amino acid level.

Regulation of the initial step of aromatic compound degradation is a common attribute of many systems. The plasmid-borne genes *xylXYZ* (encoding toluate dioxygenase) and *xylL* (encoding dihydroxycyclohexadiene carboxylate dehydrogenase) of *Pseudomonas putida* require both an aromatic acid and the positively acting *xylS* regulatory gene product for expression (6, 15, 23, 25). It has also been shown that BenR cloned from *P. aeruginosa* PAO1 can functionally replace *xylS* in benzoate-dependent induction of the TOL lower pathway (16). The localization of the *tftAB* promoter was previously unknown; therefore, we wished to delineate this region and determine whether these genes were regulated. The β-galactosidase assays indicate that this promoter is expressed throughout log- and stationary-phase growth in AC1100 and that the presence of 2,4,5-T in fact decreases the activity. This observation is explainable by the fact that 2,4,5-T is converted to 2,4,5-TCP, which has been shown to be toxic to AC1100 cells (17). TftAB is mechanistically similar to the 2,4-D/dioxygenase, TfdA; however, it is not regulated. TfdA has been reported to be negatively regulated by TfdR (11). Although the *tftAB* genes are constitutively expressed, the genes encoding the 2,4,5-TCP-degrading enzyme(s) appear to be induced by 2,4,5-TCP or an intermediate derived from it (17). Other experiments suggest that the genes encoding enzymes involved in the degradation of 5-chlorohydroxyquinol, an intermediate of the 2,4,5-T pathway, are also positively regulated (4). We are currently cloning the gene(s) responsible for 2,4,5-TCP degradation to look at the possible regulatory elements involved in its expression.

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