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Nucleotide Sequence and Functional Analysis of the Genes Encoding 2,4,5-Trichlorophenoxyacetic Acid Oxygenase in *Pseudomonas cepacia* AC1100

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Pseudomonas cepacia* AC1100 is able to use the chlorinated aromatic compound 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as the sole source of carbon and energy. One of the early steps in this pathway is the conversion of 2,4,5-T to 2,4,5-trichlorophenol (2,4,5-TCP). 2,4,5-TCP accumulates in the culture medium when AC1100 is grown in the presence of 2,4,5-T. A DNA region from the AC1100 genome has been subcloned as a 2.7-kb *Sst*I-*Xba*I DNA fragment, which on transfer to *Pseudomonas aeruginosa* PAO1 allows the conversion of 2,4,5-T to 2,4,5-TCP. We have determined the directions of transcription of these genes as well as the complete nucleotide sequences of the genes and the number and sizes of the polypeptides synthesized by pulse-labeling experiments. This 2.7-kb DNA fragment encodes two polypeptides with calculated molecular masses of 51 and 18 kDa. Proteins of similar sizes were seen in the T7 pulse-labeling experiment in *Escherichia coli*. We have designated the genes for these proteins *tftA1* (which encodes the 51-kDa protein) and *tftA2* (which encodes the 18-kDa protein). TftA1 and TftA2 have strong amino acid sequence homology to BenA and BenB from the benzoate 1,2-dioxygenase system of *Acinetobacter calcoaceticus*, as well as to XylX and XylY from the toluate 1,2-dioxygenase system of *Pseudomonas putida*. The *Pseudomonas aeruginosa* PAO1 strain containing the 2.7-kb *Sst*I-*Xba*I fragment was able to convert not only 2,4,5-T to 2,4,5-TCP but also 2,4-dichlorophenoxyacetic acid to 2,4-dichlorophenol and phenoxyacetate to phenol.

Pseudomonas cepacia has the unique ability to utilize a wide variety of carbon sources (22). Its versatility in adapting to alternative sources of energy has been attributed to the acquisition of foreign genes mediated by insertion elements (23). As such, this property provides an opportunity to study the evolution of novel catabolic pathways as well as providing an alternative means to detoxify the growing number of synthetic environmental pollutants. One such compound, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), was used extensively and in massive amounts in many countries. 2,4,5-T is a defoliating herbicide which was used for the control of poison ivy, poison oak, and various broadleaf weeds (5). This compound has been classified as carcinogenic (9). A strain of *P. cepacia*, designated AC1100, was isolated in a chemostat environment which was capable of utilizing 2,4,5-T as the sole source of carbon and energy (18). Previous studies of 2,4,5-T biodegradation by *P. cepacia* AC1100 have shown that one of the intermediates formed during the degradative process is 2,4,5-trichlorophenol (2,4,5-TCP) (17). One clone from a genomic library of AC1100 was able to complement a mutant that was unable to grow on 2,4,5-T as the sole source of carbon and did not accumulate any known intermediate, suggesting that the mutation was in the early step of the pathway. This construct (pRHC89) was isolated and transformed into *Pseudomonas aeruginosa* PAO1. This transformant was able to convert 2,4,5-T to 2,4,5-TCP and 2,4-dichlorophenoxyacetic acid (2,4-D) to 2,4-dichlorophenol (2,4-DCP) in whole-cell experiments (15). In this report, we present data which demonstrate that the oxygenase activity is present on a 2.7-kb *Sst*I-*Xba*I DNA fragment. The sequence of this DNA fragment indicates that there are two open reading frames (ORFs) with significant amino acid homology to the BenA and BenB and XylX and XylY proteins, as well as other proteins composing biodegradative dioxygenase complexes (28–30). The enzymes encoded by the two ORFs, designated TftA1 and TftA2, have a relaxed substrate specificity, allowing the conversion of 2,4,5-T to 2,4,5-TCP, 2,4-D to 2,4-DCP, and phenoxyacetate to phenol.

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MATERIALS AND METHODS

Bacterial strains and media. The *Escherichia coli* strains used in this study were MV1184 [*ara* Δ(*lac-pro*) *strA thi* (φ80 Δ*lacIZ*ΔM15) Δ(*srl-recA*)*306*::Tn*10*(Tc^r) F′ *traD36 proAB lacI*^{q}ZΔM15] (38), TG1 [K-12 Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15] (purchased from Amersham Corp.), and K-38 [HfrC(λ)] (34). Luria broth (LB) medium (Difco Laboratories) was used for normal culturing of these strains at 37°C. For production of single-stranded DNA, 2× YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) medium was used. When antibiotic selection was necessary, ampicillin was used at a concentration of 50 μg/ml for *E. coli* strains. For whole-cell assay experiments, *P. aeruginosa* PAO1 was used. PAO1 strains were maintained on Pseudomonas Isolation agar (PIA) plates (Difco Laboratories) with 500 μg of carbenicillin per ml purchased from Research Organics Corp.

Culture conditions for whole-cell assay experiments. *P. aeruginosa* PAO1 cells were cultured in basal salts medium (BSM) (17) with 2% glucose and 500 μg of carbenicillin per ml, when harboring the appropriate plasmids, at 30°C on a

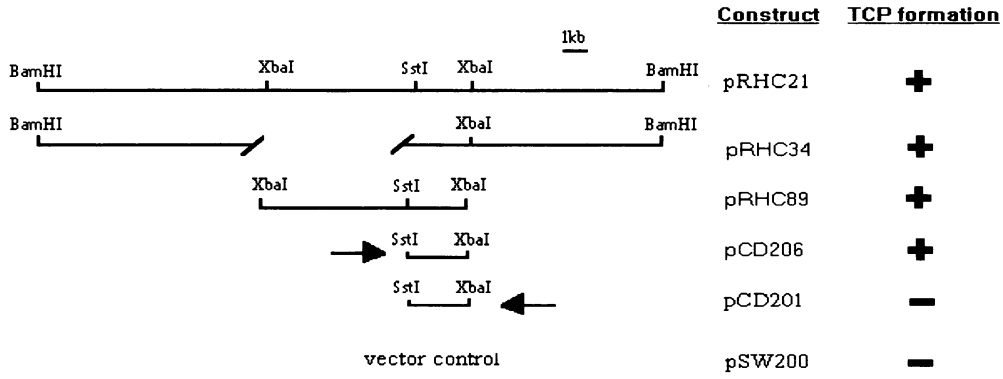

FIG. 1. Restriction map and localization of the genes encoding TftA1 and TftA2 oxygenase. pRHC89 was subcloned into the broad-host-range vectors pSW200 and pSW201 to create pCD206 and pCD201, respectively. The arrows indicate the orientation of the *lac* promoter. All constructs shown were transformed into *P. aeruginosa* PAO1 and assayed for TCP formation (+ [TCP formed] and − [TCP not formed]) by HPLC and colorimetric analysis of the culture supernatants of cells grown in BSM containing glucose and 2,4,5-T. The diagonal lines in pRHC34 indicate a deleted region in this construct (15).

shaker at 250 rpm in a volume of 100 ml. For TCP accumulation experiments, cells were washed twice in BSM and resuspended in 5 ml of BSM with 4 mM 2,4,5-T, 2,4-D, or phenoxyacetate. The medium also contained 0.2% Casamino Acids (Difco Laboratories). Cells were then incubated on a shaker at 100 rpm at 30°C for up to 48 h.

Analytical methods. A modification of the 4-aminoantipyrine assay (19) to determine the concentrations of 2,4,5-TCP, 2,4-DCP, and phenol was used. The absorption maxima were determined for each compound being analyzed, and standard curves were drawn on the basis of spectrophotometric readings at the absorption maxima. Absorption maxima were 502, 509, and 505 nm for the phenolic derivatives of 2,4,5-TCP, 2,4-DCP, and phenol, respectively. To monitor the accumulation of the phenolic derivatives in the culture medium, 0.1 to 0.3 ml of culture supernatant was used in the 4-aminoantipyrine assay. Reagents were made fresh before each assay. High-performance liquid chromatography (HPLC) was performed as described previously to confirm the identity of each phenolic derivative (16).

DNA preparation, restriction endonuclease cleavage, ligation, and transformation. Large-scale purification of cosmids for use in subcloning experiments was performed through the use of plasmid-preparative columns as described by Quiagen Corporation. Minipreparations of plasmid DNA from *E. coli* were obtained by using a modification of the protocol by Majumdar and Williams (24). Restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Labora-

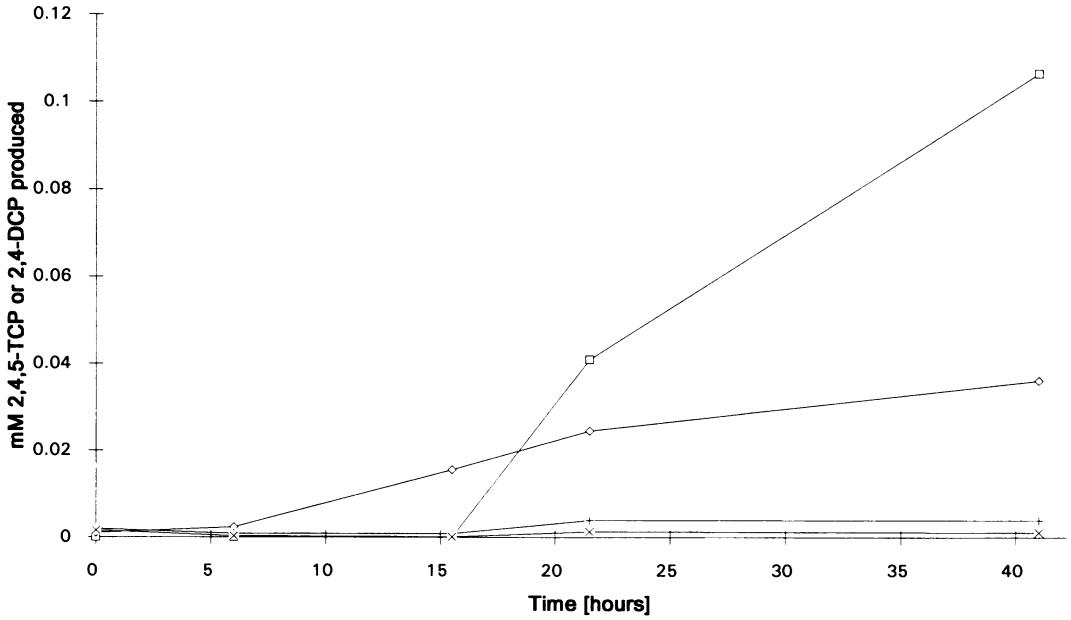

FIG. 2. Whole-cell assays showing the conversion of 2,4,5-T to 2,4,5-TCP and 2,4-D to 2,4-DCP by the PAO1 strain containing construct pCD206, which encodes the oxygenase components, TftA1 and TftA2. PAO1/pCD206 grown in the presence of 2,4,5-T (□) or 2,4-D (◇) and PAO1/pSW200 (vector control) grown in the presence of 2,4-D (×) or 2,4,5-T (+) are shown. PAO1/pCD201 (no formation of 2,4,5-TCP or 2,4-DCP) is not shown.

tories (Gaithersburg, Md.). Restriction enzyme digestion, transformation, and cloning techniques were performed as previously described by Maniatis et al. (25). For expression of TftA1 and TftA2 in *P. aeruginosa*, the DNA fragment encoding these genes was cloned into the *E. coli-Pseudomonas* shuttle vectors, pSW200 (8) and pSW201 (39). Both of these vectors are derivatives of pUC18 and pUC19 (27), respectively, which have the multiple cloning sites in opposite orientations. They contain a 1.8-kbp *Pst*I DNA fragment from plasmid pRO1614, which encodes a *P. aeruginosa* origin of replication (31). The *P. aeruginosa* cells were transformed by electroporation, using the Bio-Rad Gene Pulser model 1652102 (Bio-Rad Laboratories, Richmond, Calif.). The cells (5 ml) were grown overnight in LB medium. The cells were then washed twice in ice-cold 0.3 M sucrose. The cells were suspended in 0.25 ml of this ice-cold buffer. One to five microliters of DNA suspension was added to 50 μl of the cell suspension and kept on ice for 5 min. The DNA-containing cell suspension was then loaded into an electroporation cuvette and pulsed at 2.5 kV. LB was added immediately to the cuvette, and the suspension was then transferred to a sterile Eppendorf tube, which was incubated at 30°C for at least 2 h. The cells were spread on PIA plates and incubated at 30°C overnight.

DNA sequencing. Nucleotide sequence determination of both strands was accomplished by the dideoxy-chain termination method of Sanger et al. (35), using single-stranded DNA templates synthesized from phagemid pUC118 or pUC119 (27) after transformation into *E. coli* MV1184 and TG1 and infection with the helper phage M13K07, as previously described by Vieira and Messing (38). Sequenase version 2.0 (United States Biochemical Corporation) was used. [α-^{35}S]dCTP was purchased from Amersham Corporation. A PCR-based double-stranded sequencing protocol was used for regions of DNA where sequence information was difficult to obtain by the single-stranded method described above. Sequitherm kit (Epicentre Corporation, Madison, Wis.) was employed for this protocol. Primers were end labeled according to manufacturer's instructions with [γ-^{32}P]dATP supplied by Amersham Corporation.

DNA sequence analysis. Computer-assisted analysis was achieved with FASTA protein search and comparison program for the VAX developed by Pearson (32). Multiple amino acid sequence alignment was done with MACAW version 2.0.2 for Windows 3.1 on an algorithm from the National Center for Biotechnology Information.

Pulse-chase labeling of translation products. The DNA fragment encoding the oxygenase activity was subcloned into the vectors T7-5 and T7-6 (37), which contain the multiple cloning sites in opposite orientations, and expressed in *E. coli* K-38. The cells were then grown and pulsed with [^{35}S]methionine as described by Tabor and Richardson (37). Portions (50 μl) of the cell extracts were then loaded onto a sodium dodecyl sulfate–10% polyacrylamide gel. After electrophoresis, the gel was dried and exposed for 2 days on Kodak X-OMAT film.

Nucleotide sequence accession number. The nucleotide sequence presented here has been reported to GenBank and assigned accession number U11420.

RESULTS

Cloning of the 2,4,5-T oxygenase genes and expression in *P. aeruginosa* PAO1. Previous studies have shown that the initial step in the biodegradation of 2,4,5-T by *P. cepacia* AC1100 is the conversion of 2,4,5-T to 2,4,5-TCP (17). An 8.9-kb *Xba*I DNA fragment containing the genes encoding the 2,4,5-T oxidation enzymes from AC1100 had been previously cloned from an AC1100 genomic DNA library (15). This fragment was able to complement an AC1100 mutant defective in its ability to grow on 2,4,5-T, designated RHC22, for growth on 2,4,5-T. By assaying other constructs for the formation of 2,4,5-TCP in the medium, it was postulated that the DNA region conferring this oxygenase activity to PAO1 was localized to a region of DNA defined by constructs pRHC34 and pRHC89 (15) (Fig. 1). A fragment of approximately 2.7 kb was subcloned from pRHC89 as an *Sst*I-*Xba*I fragment in the *E. coli-Pseudomonas* shuttle vectors pSW200 and pSW201 to create pCD206 and pCD201, respectively. Both constructs, pCD206 and pCD201, were electroporated into *P. aeruginosa* PAO1 cells. Construct pCD206 was shown to harbor the genes functional for the conversion of 2,4,5-T to 2,4,5-TCP in whole-cell assay experiments (Fig. 2). With an initial concentration of 4 mM 2,4,5-T in the medium, TCP was produced to a maximum amount of 0.11 mM over the course of this experiment. The activity was seen only when the fragment was cloned downstream of the *lac* promoter in the direction of *Sst*I to *Xba*I. The presence of the inducer, isopropyl-β-D-thiogalactopyranoside (IPTG), did not significantly increase the whole-cell activity because of the leakiness of the *lac* promoter in PAO1 (data not shown). When cloned in the opposite orientation (pCD201), there was no activity, suggesting that the direction of transcription of these genes was from *Sst*I to *Xba*I. Consistent with previous results, these transformants were able to convert 2,4-D to 2,4-DCP as well, producing a maximum amount of 0.036 mM over the course of this experiment. Furthermore, phenoxyacetate was also converted to phenol (data not shown).

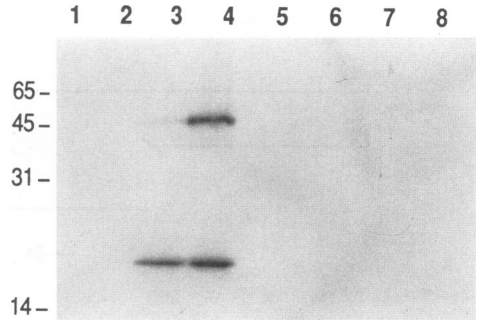

FIG. 3. Autoradiogram of a gel containing TftA1 and TftA2 expressed in *E. coli*. The 2.7-kb *Sst*I-*Xba*I insert encoding TftA1 and TftA2 was cloned into the pT7 vectors which place the cloned genes under the control of the bacteriophage T7 RNA polymerase promoter; the genes present on the insert are then expressed when cells are induced at 42°C. Proteins encoded on the insert are specifically labelled with [^{35}S]methionine. Lanes 1 and 2, extracts from the pT7-5 vector control under noninduced and induced conditions, respectively; lane 3, extract from the pT7-5 construct containing the insert (*Sst*I to *Xba*I) under noninduced conditions; lane 4, the same sample as in lane 3 but under inducing conditions; lanes 5 and 6, extracts from the pT7-6 vector control under noninduced and induced conditions, respectively; lanes 7 and 8, extracts from the construct with the insert cloned in pT7-6 under noninduced and induced conditions, respectively. The positions of molecular mass standards (in kilodaltons) are shown to the left of the gel.

Expression of 2,4,5-T oxygenase genes in *E. coli*. The directions of transcription of these genes were further confirmed in a pulse-labeling experiment in *E. coli* K-38 cells. When cloned in the T7 vector, where the φ10 promoter is situated upstream of the *Sst*I side of the fragment, two polypeptides were specifically labeled: a ca. 51-kDa polypeptide and a ca. 18-kDa polypeptide (Fig. 3). Neither the vector control nor the frag-

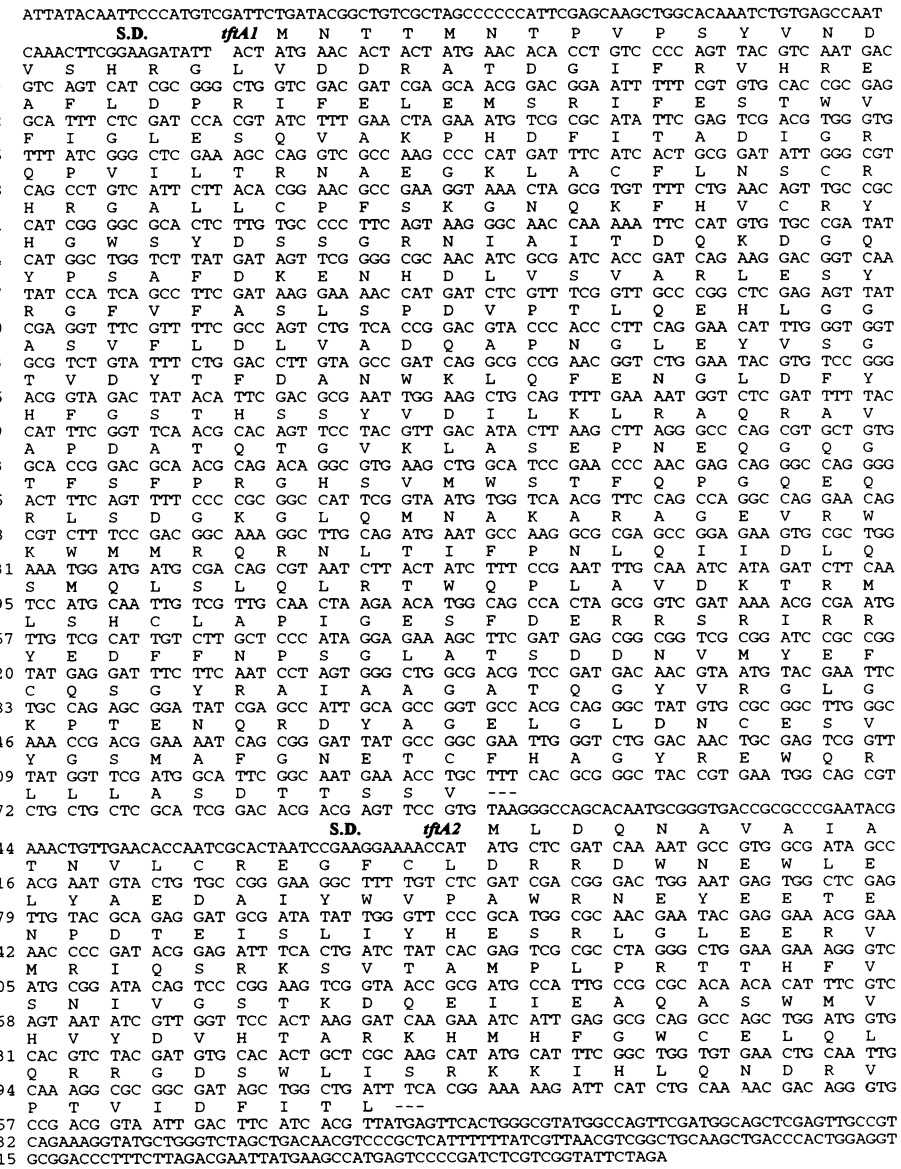

FIG. 4. Nucleotide sequences of *tftA1* and *tftA2*. This sequence represents 2.275 kb of the 2.7-kb *Sst*I-*Xba*I fragment cloned in the construct pCD206. The putative ribosomal binding sites are labeled S.D. (for Shine-Dalgarno sequence).

ment cloned in the opposite orientation produced any labeled polypeptides. The protein band representing TftA2 appears to be induced to a greater degree than TftA1 (lanes 3 and 4). The reason for this is not clear, but it may be due to a greater match between the Shine-Dalgarno sequence of TftA2 and the *E. coli* consensus Shine-Dalgarno sequence.

TftA1 and TftA2 show strong homology to the α- and β-subunits of the 2,3-dioxygenases of other biodegradative pathways. The *Sst*I-*Xba*I fragment was digested with other restriction enzymes, smaller DNA fragments were subcloned into pUC118 and pUC119, and a 2.2-kb DNA fragment was sequenced (Fig. 4). Two ORFs were found. The calculated molecular masses of the predicted proteins produced are 51 and 18 kDa, comparable to the molecular masses of the proteins as seen in the T7 labeling experiment shown in Fig. 3. Ten residues from the N-terminal amino acid sequences from both the purified proteins from AC1100 (TftA1 and TftA2) have been determined and found to match exactly those predicted from the nucleotide sequence (10). The amino acid sequence was compared to other sequences in the PIR International Protein Sequence Database. As shown in Fig. 5, the 51-kDa protein, TftA1, has strong amino acid homology to the α-subunits of the terminal dioxygenases of some biodegradative enzyme systems. TftA1 has 37% sequence identity in a 395-amino-acid overlap to the benzoate dioxygenase α-subunit BenA from *Acinetobacter calcoaceticus* (28), 40% sequence identity in a 413-amino-acid overlap to the toluate dioxygenase α-subunit XylX from *Pseudomonas putida* (29), 28% sequence identity in a 396-amino-acid overlap to the napthalene dioxygenase iron-sulfur protein α-subunit NdoB (21), and 25% sequence identity in a 430-amino-acid overlap to toluene dioxygenase iron-sulfur protein α-subunit TodC1 (41). All of

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                                                    *       *    *   *
TftA1    mnttmntpvpsyvndvshrglvddratdgifrvhreaflDprifelEmsriFestWvfig  60
BenA     mpripvintshldridellvdntetgefkl---hrsvftDqalfdlEmkyiFegnWvyla  57
XylX     mtmhlgldyidslveedenegiyrc--------kremftDprlfdlEmkhiFegnWiyla  52
NdoB     mnynnkilvsesglsq-----------------khlihgDeelfqhElktiFarnWlflt  43
TodC1    mnqtdtspirlrrswntseiealfdehagri--dpriytDedlyqlElervFarsWlllg  58

                *  *                          * * *         **     *
TftA1    lesqvakphDfiTadigrqpviltrnaegklacflnsCrHrGallcpfskGNqkfhvCry 120
BenA     hesqipnnnDyyTtyigrqpiliarnpngelnaminaCsHrGaqllghkrGNkttytCpf 117
XylX     hesqipeknDyyTtqmgrqpifitrnkdgelnafvnaCsHrGatlcrfrsGNkathtCsf 112
NdoB     hdslipapgDyvTakmgidevivsrqndgsiraflnvCrHrGktlvsveaGNakgfvCsy 103
TodC1    hetqirkpgDyiTtymgedpvvvvrqkdasiavflnqCrHrGmricradaGNakaftCsy 118

         ***     *
TftA1    HGWsydssGrniaitdqkdgqypsafdkenhdlvsvarlesyrgfvfaslspdvptlqeh 180
BenA     HGWtfnnsGkllkvkdpsdagysdcfnqdgshdlkkvarfesykgflfgslnpvdpslqe 177
XylX     HGWtfsnsGkllkvkdpkgagypdsfdcdgshdlkkvarfasyrgflfgslredvaplee 172
NdoB     HGWgfgsnGelqsvpfekdlygeslnkkclglkevarvesfhgfiygcfdqeapplmdyl 163
TodC1    HGWaydtaGnlvnvpyeaesfaclnkkewsplkarvetykglifanwdenavdldtylge 178


TftA1    lggasvfldlvadqapngleyvsgtvdytfdanwklqfengldfyhfgsthssyvdilkl 240
BenA     flgettkiidmivgqsdqglevlrgvstytyegnwkltaengadgyhvsavhwnyaattq 237
XylX     flgesrkvidmvvdqspeglevlrgsstyvyegnwkvqvengadgyhvstvhwnyaatqq 232
NdoB     gdaawylepmfkhsgglelvgppgkvvikanwkapaenfvgdayhvgwthasslrsgesi 223
TodC1    akfymdhmldrteagteaipgvqkwvipcnwkfaaeqfcsdmyhagttshlsgilaglpe 238
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FIG. 5. Amino acid comparison of TftA1 with α-subunits of four multicomponent aromatic ring dioxygenases. Asterisks indicate positions at which amino acid residues are identical. Those residues which are in boldface type indicate those which may bind a Rieske-type [2Fe-2S] center. Dashes represent gaps created to optimize alignment.

these proteins have two conserved [cysteine-histidine] pairs. This motif is also conserved among Rieske iron-sulfur proteins (33). These residues may bind a [2Fe-2S] cluster (11).

The 18-kDa protein, TftA2, has amino acid residues that are conserved among β-subunits of the terminal dioxygenase components as shown in Fig. 6. TftA2 has a 36% amino acid identity in a 151-amino-acid overlap to toluate dioxygenase β-subunit XylY (29), a 36% amino acid identity in a 163-residue overlap to benzoate dioxygenase β-subunit, BenB (28), a 26% amino acid identity over a 113-residue overlap to toluene dioxygenase iron sulfur protein β-subunit, TodC2 (41), and a 23% identity over a 111-amino-acid sequence overlap to napthalene dioxygenase iron sulfur protein β-subunit, NdoC (21).

Deletion analysis. To determine whether both of the proteins encoded on this fragment were necessary for activity, deletion constructs were made and transformed into PAO1. These strains were then assayed for 2,4,5-TCP formation as described above. Figure 7 shows that both of the proteins, TftA1 and TftA2, are needed for the conversion of 2,4,5-T to 2,4,5-TCP, 2,4-D to 2,4-DCP, and phenoxyacetate to phenol. Eliminating either one of them completely abolishes the activity.

DISCUSSION

The genes encoding the 2,4,5-T oxygenase have been cloned and sequenced. The proteins, designated TftA1 and TftA2, are able to allow *P. aeruginosa* PAO1 to catalyze the conversion of 2,4,5-T to 2,4,5-TCP, 2,4-D to 2,4-DCP, and phenoxyacetate to phenol. The genes *tftA1* and *tftA2* encode proteins of approximately 51 and 18 kDa, respectively, on the basis of the nucleotide sequence. Pulse-labeling experiments demonstrate that two proteins of these sizes are synthesized in *E. coli*. Deletions in either of these ORFs appear to eliminate activity as shown by whole-cell assays (Fig. 7), suggesting that both proteins are needed for oxygenase activity.

Analyses of the deduced amino acid sequences of TftA1 and

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                                                  *   *       *
TftA2    mld-----------------------qnavaiatnvlcrEgfcLdrrdwneWlelyaed  36
XylY     m-------------------------tisyeavrdflyrEaryLddkqwesWlemyapd  34
BenB     mnatalld------------------tisieqisqflysEarfLddeqwddWlecyapq  41
TodC2    midsanradvflrkpapva--------pelqheveqfyywEaklLndrrfeeWfallaed  52
NdoC     mminiqedklvsahdaeeilrffnchdsalqqeattlltqEahlLdiqayraWlehcvgs  60

                                              *           *       *
TftA2    aiywvpawrneyeetenpdteisliyhesrlgleeRvmriqsrksvtamPlprtthfvsN  96
XylY     atfwmpawddrdqltedpqsqisliwygnrsgledRvfrikterssatiPdtrtshnisN  94
BenB     asfwmpawddndqltenpqteisliyypdrqgledRvfrikterssatmPdtrtahnisN 101
TodC2    ihyfmpirttrisrleysgsreyahfdddatmmkgRlrkitsdvswsenPasrtrhlvsN 112
NdoC     evyqvisreraaserryklneamnvynenfqqlkvRvehqldpqnwgnsPklrftrfitN 120


TftA2    ivgstkdqeiieaqaswmvhvydvhtarkhmhfgwcelqlqrrgdswlisrkkihlqndr 156
XylY     lelleqsdgvcklrynwhtmnyryktvdhffgtnfctldtcgetplitakkvvlkndyir 154
BenB     ievesrdglqitvrfnwntlsfryknsysyfgmsryvidfsgeqpkilskyvmlkndyin 161
TodC2    vmivgaeaegeyeissafivyrnrlerqldifa---------------------------- 145
NdoC     vqaavndkellhirsnvilh------------------------------------------ 140
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FIG. 6. Amino acid comparison of TftA2 with the β-subunits of four multicomponent aromatic ring dioxygenases. Positions at which amino acid residues are identical are indicated by asterisks. Dashes represent gaps created to optimize alignment.

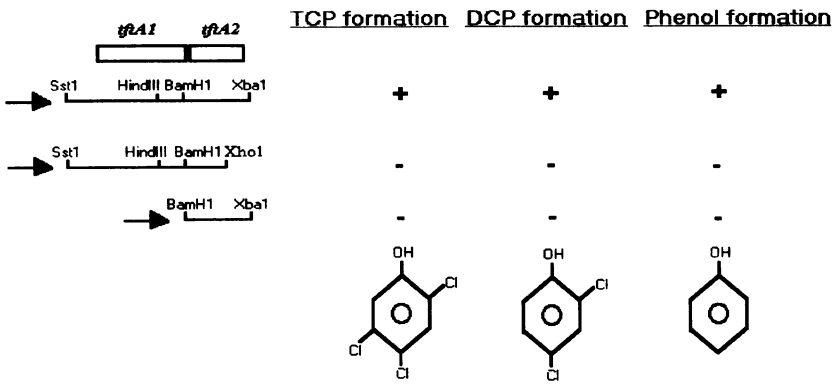

FIG. 7. Deletion analysis of TftA1 and TftA2. Deletion constructs were made by subcloning fragments of the insert from pCD206 in vector pSW200. Constructs were then transformed into PAO1 and assayed for chlorophenol or phenol formation (+ [formed] and − [not formed]) during growth with BSM containing glucose and 2,4,5-T, 2,4-D, or phenoxyacetate as described in Materials and Methods.

TftA2 show that these proteins show homology to class IB, IIB, and III terminal oxygenases of multicomponent dioxygenases (2). These dioxygenases are composed of two components, an oxygenase and a reductase. Neidle et al. (28) reported the conservation of two cysteine-histidine pairs in BenA and the large iron-sulfur subunits from other multicomponent dioxygenases of biodegradative systems and Rieske iron-sulfur proteins. This motif also appears within TftA1, which suggests that these residues may bind a [2Fe-2S] center. Unlike the other members of the multicomponent dioxygenases, the reaction catalyzed by TftA1 and TftA2 is the conversion of 2,4,5-T to its phenolic derivative, 2,4,5-TCP. The other dioxygenases convert their respective substrates to the *cis*-diol derivatives. The reaction catalyzed by TftA1 and TftA2 is similar to that catalyzed by the *Alcaligenes eutrophus* enzyme, TfdA, which converts 2,4-D to 2,4-DCP (3). This enzyme was first classified as a monooxygenase because of the fact that it catalyzed the conversion of its substrate to the corresponding phenolic derivative. Previous work with other monooxygenases demonstrated that the reaction they catalyze depends upon the presence of reducing compounds such as NADH or NADPH (3, 13). The conversion of 2,4-D to 2,4-DCP is dependent upon the oxidation of α-ketoglutarate to form succinate and CO_2 and does not require NADPH or NADH (7). This enzyme has been redesignated as 2,4-D-α-ketoglutarate-dependent dioxygenase. Although TftA1 and TftA2 are functionally similar to TfdA, there is no protein or nucleotide sequence homology among these genes. The homology among the oxygenase components is greatest among the α-subunits (28). Although conserved amino acid residues exist among the β-subunits, the overall homology is much less. The roles of each subunit in some terminal oxygenases have been investigated. There have been studies showing the importance of both subunits in substrate recognition. Site-directed mutagenesis of the *bphA* gene from the polychlorinated biphenyl-degrading *Pseudomonas* sp. strain LB400 resulted in an enzyme with increased activity toward several polychlorinated biphenyl congeners (4). Harayama et al. (12) demonstrated that a specific mutation in XylY changes the substrate specificity of the dioxygenase activity. Site-directed mutagenesis of residues in both TftA1 and TftA2 and substrate activity analysis should elucidate the function of both of these subunits in the conversion of 2,4,5-T to 2,4,5-TCP.

Many microorganisms have acquired the ability to degrade xenobiotic compounds through plasmid-encoded enzymes. Thus, the evolution of some novel biodegradative pathways in the environment has been achieved through plasmid-mediated (1) or transposon-mediated (40) recruitment of novel genes from different bacteria. Some biodegradative enzymes encoded on plasmids have a broad substrate specificity. Examples include toluate 1,2-dioxygenase (6) and enzymes degrading anthracene and phenanthrene to naphthoic acids (26, 36). TftA1A2, which also exhibits a relaxed substrate specificity, is chromosomally encoded (15), and transposable elements have been implicated in the recruitment of these genes (14). With 1,2-dioxygenase systems, at least three proteins are necessary for complete activity, an oxygenase component, which is composed of two protein subunits, and a reductase component. This is also the case with the oxygenase activity from AC1100. Gray and Xun have shown that at least three proteins purified from AC1100 are necessary for the conversion of 2,4,5-T to 2,4,5-TCP (10). Although a reductase component has not been cloned from AC1100, the conversion of 2,4,5-T to 2,4,5-TCP in *P. aeruginosa* PAO1 proceeds when transformed solely with the oxygenase components, TftA1 and TftA2. PAO1 may be providing a functional chromosomally encoded reductase analog, allowing the conversion to occur. PAO1 contains the genes encoding the proteins necessary for the conversion of benzoate to 2-hydro-1,2-dihydroxybenzoate (20). It is thus possible that TftA1 and TftA2 can utilize the BenC protein or some other oxidoreductase of PAO1 as an alternative electron transfer protein in the reaction converting 2,4,5-T to 2,4,5-TCP. The purification of the reductase is currently in progress, which is expected to provide insights into the mechanism of conversion of 2,4,5-T to 2,4,5-TCP. This information could then be used to determine to which class of multicomponent oxygenases the TftA1-TftA2 complex belongs.

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