Genes for 2,4,5-Trichlorophenoxyacetic Acid Metabolism in *Burkholderia cepacia* AC1100: Characterization of the *tftC* and *tftD* Genes and Locations of the *tft* Operons on Multiple Replicons

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Burkholderia cepacia AC1100 uses the chlorinated aromatic compound 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a sole source of carbon and energy. The enzyme which converts the first intermediate in the pathway, 2,4,5-trichlorophenol, to 5-chlorohydroquinone has been purified and consists of two subunits of 58 and 22 kDa, encoded by the *tftC* and *tftD* genes (48). A degenerate primer was designed from the N terminus of the 58-kDa polypeptide and used to isolate a clone containing the *tftC* and *tftD* genes from a genomic library of AC1100. The derived amino acid sequences of *tftC* and *tftD* show significant homology to the two-component monooxygenases HadA of *Burkholderia pickettii*, HpaBC of *Escherichia coli*, and HpaAH of *Klebsiella pneumoniae*. Expression of the *tftC* and *tftD* genes appeared to be induced when they were grown in the presence of 2,4,5-T, as shown by RNA slot blot and primer extension analyses. Three sets of cloned *tft* genes were used as probes to explore the genomic organization of the pathway. Pulsed-field gel electrophoresis analyses of whole chromosomes of *B. cepacia* AC1100 demonstrated that the genome is comprised of five replicons of 4.0, 2.7, 0.53, 0.34, and 0.15 Mbp, designated I to V, respectively. The *tft* genes are located on the smaller replicons: the *tftAB* cluster is on replicon IV, *tftEFGH* is on replicon III, and copies of the *tftC* and the *tftCD* operons are found on both replicons III and IV. When cells were grown in the absence of 2,4,5-T, the genes were lost at high frequency by chromosomal deletions and rearrangements to produce 2,4,5-T-negative mutants. In one mutant, the *tftA* and *tftB* genes translocated from one replicon to another, with the concomitant loss of *tftEFGH* and one copy of *tftCD*.

Chlorinated phenols and phenoxyacetates are a group of chemical compounds which have been used extensively as pesticides, wood preservatives, and herbicides in the agricultural industry. They are a major group of recalcitrant environmental pollutants (11, 26). *Burkholderia cepacia* AC1100 is a bacterium capable of using the chlorinated aromatic compound 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a sole source of carbon and energy (21). 2,4,5-T is converted to 2,4,5-trichlorophenol (2,4,5-TCP) by 2,4,5-T oxygenase (6, 16, 47). The genes encoding the oxygenase component of this complex, *tftA* and *tftB*, have been cloned and sequenced (6). The *tftA* and *tftB* genes are clustered (6) and their expression is controlled by a constitutive *Escherichia coli* σ^{70} -like fusion promoter created by insertion of IS1490 (5, 19). 2,4,5-TCP is then converted to 5-chloro-2-hydroxy-1,4-benzoquinone (5-CHQ) in a two-step hydroxylation catalysis (48). In resting-cell experiments, Karns et al. (20) showed that when AC1100 was grown on glucose, succinate, or lactate as the sole carbon source, these cells could convert 2,4,5-T to 2,4,5-TCP but were unable to dechlorinate 2,4,5-TCP. Growth in the presence of either 2,4,5-T or 2,4,5-TCP allowed induction of *tftCD*, resulting in dechlorination of 2,4,5-T and 2,4,5-TCP. It was therefore hy-

pothesized that the steps converting 2,4,5-TCP to other pathway intermediates are inducible. Inhibitor studies suggested that a flavin-containing enzyme(s) is responsible for at least one of the two 2,4,5-TCP hydroxylation steps (42). The enzyme responsible for both hydroxylation reactions on 2,4,5-TCP, chlorophenol-4-monooxygenase, was subsequently purified and shown to be composed of a 58-kDa polypeptide and a 22-kDa polypeptide (48).

Natural horizontal transfer of genes responsible for biodegradation has been described for several bacterial systems (43, 44) and seems to play a major role in the acclimation of bacterial communities to environmental pollutants. *B. cepacia* AC1100 acquired its catabolic ability to metabolize 2,4,5-T over a period of several months after being subjected for a prolonged period to strong selective pressure in a continuous culture (3). The evolution of this catabolic capacity occurred most likely through recruitment of genes present in the chemostat consortium and their integration into its genome. The two 2,4,5-T catabolic gene clusters *tftAB* and *tftEFGH* have previously been identified and shown to be specific for the upper and the lower portion of the pathway. The presence of copies of the three known insertion sequence (IS) elements in AC1100, namely, IS931, IS932, and IS1490, adjacent to both gene clusters supports their possible role in stimulating rapid evolution through gene acquisition and expression (6, 9, 15, 16, 19, 41).

In this paper, the identification and characterization of *tftCD* provide the missing link in the characterization of the upper

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TABLE 1. Bacterial strains and plasmid vectors

Strain or plasmid	Genotype, phenotype, or description ^a	Source or reference
Strains		
<i>E. coli</i> K-12		
TG1	K-12 $\Delta(lac-pro)$ <i>supE thi hsdD5 F' traD36 proA⁺B⁺ lacI^q lacZΔM15</i>	Amersham Corp.
AC80	<i>thr leu met hsdR hsdM</i>	2
<i>B. cepacia</i>		
AC1100	2,4,5-T ⁺ wild type	21
PT88	2,4,5-T ⁻	41
AH88	2,4,5-T ⁻	This work
Plasmids		
pCP13	IncP1, Tc ^r Km ^r <i>cos⁺</i> , <i>tra</i> mutant, <i>mob⁺</i>	7
pBBR1MCS	Cm ^r , <i>tra</i> mutant, <i>mob⁺</i>	23
pRHC21	Tc ^r , contains a 27.5-kb insert encoding the <i>tftA</i> and <i>tftB</i> genes (pCP13)	16
pSW200	IncP1, Amp ^r	13, 29
PRS1100	pUC119 containing a 2.5-kb <i>Hind</i> III- <i>Eco</i> RI fragment encoding IS931	This work
pAH12	pUC19 containing a 1.3-kb <i>Hind</i> III- <i>Eco</i> RI fragment encoding IS1413	19
pAH154	pUC19 containing a random <i>Hind</i> III genomic fragment	This work
pTFT1	Tc ^r , contains a 20-kb <i>Bam</i> HI insert with <i>tftC</i> and part of <i>tftD</i> (pCP13)	This work
pTFT2	Ap ^r , contains a 7.5-kb <i>Bam</i> HI insert subcloned from pTFT1 (pSW200)	This work
pTFT3	Tc ^r , contains a 24-kb <i>Bam</i> HI insert with <i>tftCD</i> (pCP13)	This work
pTFT4	Cm ^r , contains a 5-kb <i>Sst</i> II- <i>Eco</i> RV insert with <i>tftCD</i> (pBBR1MCS)	This work

^a The plasmids in parentheses represent cloning vectors.

portion of the 2,4,5-T pathway. Through the use of pulsed-field gel electrophoresis (PFGE) we demonstrate that the three 2,4,5-T gene clusters are localized on different replicons, which supports the hypothesis that the 2,4,5-T pathway was assembled by a series of independent gene transfers. Comparison of AC1100 with different 2,4,5-T-negative mutants shows that the *tft* genes are deleted and rearranged at high frequencies.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The relevant strains and constructions used in this work are summarized in Table 1. Luria broth medium (Difco Laboratories) was used for normal culturing of these strains at 37°C. When antibiotic selection was necessary, ampicillin was used at a concentration of 50 µg/ml and chloramphenicol was used at a concentration of 30 µg/ml for *E. coli* strains. *B. cepacia* AC1100 was grown in basal salts mineral medium (BSM) at 30°C as previously described (20). For the preparation of high-molecular-weight DNA in agarose plugs as described below, cells were grown overnight in BSMGYT (BSM, 0.5% glucose, 0.01% yeast extract, 1% 2,4,5-T [Sigma and Aldrich]). Spontaneous 2,4,5-T-negative mutants of AC1100 were obtained by growing AC1100 for more than 20 generations in the absence of 2,4,5-T (BSMGY) with subsequent selection for the 2,4,5-T-negative phenotype.

Routine DNA manipulations. Large-scale purification of cosmids was performed with plasmid-preparative columns as described in the instructions of the manufacturer of the columns (Qiagen Inc.). Minipreparations of plasmid DNA from *E. coli* were obtained by a modification of the protocol by Majumdar and Williams (27). Restriction enzyme digestion, transformation, and cloning techniques were performed as described by Sambrook et al. (36). DNA fragments used in Southern hybridization experiments were internally labeled with [α -³²P] dCTP by the random-priming labeling technique (NEBlot kit; New England Biolabs Inc.).

Inverse PCR. To obtain the complete open reading frame (ORF) of *tftD*, we designed outwardly oriented primers complementary to the known *tftC* sequence of pTFT2 (Fig. 1) in order to perform inverse PCR (28, 30). To generate a suitable PCR template, chromosomal DNA of AC1100 was first digested with *Sac*II and intramolecular ligation was then achieved at a low DNA concentration as described previously (36). The PCR was performed on the ligated circular template under standard conditions.

Nucleotide sequencing. DNA sequences of both strands were generated from plasmid constructs with a Sequitherm dideoxy chain termination cycle sequencing kit supplied by Epicentre Technologies and by primer walking. Additional sequencing was performed at the Genetic Engineering Facility of the University of Illinois at Champaign-Urbana.

RNA extraction, slot blotting, and transcript mapping. Wild-type AC1100 was grown to mid-log phase (optical density at 600 nm, 1.0) in BSM containing 1% glucose at 30°C. The cells were then resuspended in BSM containing either 1% glucose (uninduced) or 4 mM 2,4,5-T (induced) after being washed with BSM. The cultures were grown with shaking at 30°C for 6.5 h. The cells were then pelleted, and RNA was extracted with Trizol reagent (Gibco BRL) according to the manufacturer's instructions. Thirty micrograms of RNA from uninduced or induced culture was used for slot blot experiments with a Hybri-Slot filtration manifold (Gibco BRL) and blotted onto N⁺ nylon filters (Amersham Corp.). The RNA was hybridized to 10 pmol of a [γ -³²P]ATP-labeled 22-mer oligonucleotide (dech7), which is complementary to a region of the *tftC* coding strand, 85 bp downstream of the ATG codon. The sequence of this oligonucleotide is 3'-GGG CAGTGGCATCACCGTCTGCT-5'. Control hybridizations were performed with an 800-bp PCR-generated and [α -³²P]CTP-labeled fragment representing an internal region of the *tftA* gene (6). For determination of the transcriptional start site, 50 µg of RNA from induced or uninduced culture was employed. Hybridization with the end-labeled dech7 primer and the reverse transcriptase (Promega Corp.) reaction were performed by a modification of the procedure described by Hendrickson and Misra (18). The sequencing ladder was generated with the same primer. The template for the sequencing reaction was pTFT4, which contains a 5-kb *Sst*II-*Eco*RV fragment that carries *tftCD*. The primer extension and sequencing reactions were run on a 6% polyacrylamide-7 M urea gel. Quantitation of radioactivity for the slot blotting and primer extension and sequencing reactions were done and images were made with a PhosphorImager and ImageQuant software (Molecular Dynamics).

Preparation of genomic DNA for PFGE, PFGE, and isolation of chromosomes. Agarose plugs containing intact chromosomal DNA were obtained from overnight cultures essentially as described by Cheng and Lessie (4). Additionally, cells were washed once in phosphate-buffered saline (PBS). After the cells were resuspended in PBS such that the optical density at 600 nm per milliliter was 15 to 20, an equal volume of 1.5% InCert agarose (FMC) was added. Approximately 6.4×10^8 cells were imbedded in 80-µl agarose plugs with commercially available plug molds (Bio-Rad). Plugs used to separate intact replicons were soaked for 30 min in 40% glycerol, shock-frozen in a mixture of dry ice and ethanol, and subsequently thawed at 37°C. Prior to electrophoresis, the plugs were washed two times for 20 min in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) to remove the glycerol.

PFGE was performed in a contour-clamped homogeneous electric field apparatus (model DRII or DRIII; Bio-Rad). Routinely, samples were electrophoretically separated at 12°C in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.3]). For resolution in the megabase size range, electrophoresis was performed with a 0.6% gel matrix (FastLane; FMC) at 50 V with pulsed times increasing from 450 to 3,400 s over a period of 170 h. For resolution in the size range of 150 kb to 1.5 Mbp, electrophoresis was conducted with a 1% agarose gel (ultraPURE; Gibco BRL) at 200 V for 23 h in one ramp, with pulse times linearly increasing from 50 to 90 s. If isolation of replicons was to be carried out, low-melting-point agarose (type VII; Sigma) was chosen. The bands corresponding to replicons III and IV were excised and submitted to GELase (Epicentre Technologies) digestion according to the manufacturer's instructions.

Southern blot DNA transfer and hybridization. After being stained with ethidium bromide (0.5 µg/ml), pulsed-field gels were exposed to UV radiation at 600 mJ/cm² in a UV cross-linker (Fisher Scientific). Prior to the DNA transfer, gels were denatured for 45 min in 1.5 M NaCl and 0.5 M NaOH and neutralized for 45 min in 1.0 M Tris-1.5 M NaCl (pH 7.5). The transfer onto nylon membranes (MSI) was performed overnight with a TurboBlotter (Schleicher & Schuell). The DNA was immobilized in a UV cross-linker at 2,500 mJ/cm². To determine the chromosomal localization of the known *tft* gene clusters (*tftAB*,

FIG. 1. Restriction maps of the constructs used to clone the genes encoding the chlorophenol-4-monooxygenase (*tftC* and *tftD*). The dashed line represents contiguous unmapped DNA regions in constructs pTFT1 and pTFT3. B, *Bam*HI; SI, *Sst*II; SII, *Sst*II; H, *Hind*III; X, *Xma*I; E, *Eco*RV.

A

1	HpaB	MKPEDFRASQRPFT	GEEYLKSLQDGREIY	IYGERVKDVTTHPAF	RNAAASVAQLYDALH	KPEMQSLCWNTDTG	SGGYTHKFFRVAKSA	90
2	HpaA	MKPENFRADTKRPLT	GEEYLKSLQDGREIY	IYGERVKDVTTHPAF	RNAAASVAQLYDALH	NPELQNTLCWGTDTG	SGGYTHKFFRVAKSA	90
3	TftD	-----MR--T	GKQYLESINDGRVVM	GGNEKIDNVATHPLT	RDYAERVAQFYDLHH	RPDLQDVLT-VDAD	-GVRRSRQWDPKDA	76
4	HadA	-----MIR--T	GTQYLESINDGRVVM	VGNEKIDNVATHPKT	RDYAQRHADFYDLHH	RPDLQDVMTY-IDEG	-GQRAMQWFGHRDK	77
	consensus		R T G YL SL DGR	E	THP	R A A YD H P Q	G	
1	HpaB	DDLRRHERDAIAEWSR	-LSYQWGMGRTPDYKA	AFGCALGCTP----	---GFYQQ-FEQNAR	NWYTRIQTETGLYFNH	AIYNPPIDRHLPTDK	170
2	HpaA	DDLRRQRDATAEWSR	-LSYQWGMGRTPDYKA	AFGGGLGANP----	---GFYQQ-FEQNAR	DWYTRIQTETGLYFNH	AIYNPPIDRHKPADE	170
3	TftD	AGLRVKKRYHETILR	EIAAGSYGRLPDAHN	YTFTTYADDPEVWEK	QSIGAEGRNLTQNIH	NFLKLLREKDLNCP	NFADPQTRSSDAQ	166
4	HadA	EQLRRKKRYHETVMR	EMAGASFPRTPDVNN	VVLTTYIDDPAPWET	QSIGDDGHKAGKIV	DFIRYAREHDLNCP	QFVDPQDRSNPDAQ	167
	consensus		L R R	R PD	G G	L	P DR	
1	HpaB	VKDVIYKLEKETDAG	IIVSGAKVVATNSAL	THYNMIGFSAQVMG	ENPDFALMFVA-PMD	ADGVKLISRASYEMV	AGATGSPYDPLSSR	259
2	HpaA	VKDVIYKLEKETDAG	IIVSGAKVVATNSAL	THYNMIGFSAQVMG	ENPDFALMFVA-PMD	AEGDKLISRASYELV	AGATGSPYDPLSSR	259
3	TftD	ARSPNLRIEGETDDG	IIVNGVKAVGTGIAF	GEYMHIGCLYRP--G	-IPGEQVIFAALPTN	TPGVTVCRES--TV	K---NDPAEHPLASQ	248
4	HadA	ERSPLRVVKEKDEG	IIVNGVKAIGTCVAF	ADWIHIGVFFRP--G	-IPGDQVIFAATPVN	TPGVTVCRES--LV	K---DDKVEHPLAAQ	249
	consensus		G I V G K	IG	G	A P	R S V	PL
1	HpaB	FDENDAILVMDNVLI	PWENVLIYRDFDRCR	RWT-MEGGFARMYPL	QACVRLAVKLDIFITA	LLKKSLECTGTLEFR	GVQADLGEVVAWRNT	348
2	HpaA	FDENDAILVMDNVLI	PWENVLIYRDFDRCR	RWT-MEGGFARMYPL	QACVRLAVKLDIFITA	LLKKSLECTGTLEFR	GVQADLGEVVAWRNT	348
3	TftD	GDELDTSTVFDNVFI	PWEQVFIHIGNPEHAK	LYPLYPQIRIFDWMHY	HILIRQVLAELIVG	LAILITEHIGTSKLP	TVSARVAKLVAFHAK	348
4	HadA	GDELDMGTVEFNVFI	PWSHVFHIGNPNHAK	LY---PQVDFDMLHY	HALIRQVLAELIVG	LAILITEHIGTSKLP	AVQTRVAKLVGFHQA	336
	consensus		DE D V NV I	PW V		L	T	
1	HpaB	FWALSDSMCSAEATPW	VNGAYLPDHAALQTY	RVLAPMAYAKIKNII	ERNVTSGLIYLPSSA	RDNNPQIDQYLAKY	VRGSGMDHVRQRIKI	438
2	HpaA	FWALSDSMCSAEATPW	VNGAYLPDHAALQTY	RVMAMPYAKIKNII	ERSVTSGLIYLPSSA	RDNNPQINDTLAKY	VRGSGMDHVRQRIKI	438
3	TftD	MQAHLIAESETGFHT	KGGRYKPNPLIYDFG	RAHFLQNMVSMYEL	LDLAGRSSIMTPSRG	Q-WDDSQSGQWFKVL	NNGPGRNPR-BRVQI	426
4	HadA	MLAHLIAESELGFHT	PGGHYKPNPLIYDFG	RALYLENFSQMIYEL	VDLSCRSALIPASE	Q-WNDDKLNQWFERM	NNGPVGRPH-DRVQI	424
				R		S	I	

B

1	HpaC	-----MQLDE---	--QRLRFDMASLS	AAVNIITTEGDADNA	GLRQRPSCSVTDTPP	SLMVCINANSAMNPV	FQNGKGLCVNVLNHE	78
2	HpaH	-----MQLDE---	--QRLRFDMASLS	PAVNVITTEABAGAA	VSPHRPSCSVTDTPP	SVMVCINANSAMNPV	FQNGKGLCVNVLNHE	78
3	TftC	MHAGEAVQQLKKAPE	TVASFDFRDLASKAS	TPVTVTVAINGPFGLA	GLTCSAVCSVCDDRP	TVLLCINRKSAYAAGT	IKSNGVLSVNWLAAG	90
	consensus		QL	FRDA S	T A	CSV D PP	CIN S	NG L L
1	HpaC	QEVMARHFAAGMTGMA	MEERFSLSCWQKGLP	AQPVKGLSLASLEGE	IRDVQAIGTHLVVYL	EIKNIILSARGHGLI	YFKRRFHPVMELEMA	168
2	HpaH	QEEMARHFAAGMTGMA	MDRFRGLSGWQKGL	QOPVLKGLASLEGE	ISQVQTIGSHLVVYL	EIRNITLSQGHGLI	YFKRRFHPVMEHMDV	168
3	TftC	QAVISQTFAGVGSVP	MEERFADKGWQTIAT	GAPYRMDAAVSFDCT	IANIIVDVGSHSVIFA	EVVARNHABECTPLI	YHRRQYATTRSLAE-	179
	consensus		Q	FAG	M RF	WQ	P	S I
						G H V	E	LI Y R

FIG. 2. Amino acid alignments of TftD with the hydroxylase components (A) and of TftC with the smaller components (B) of the aromatic-ring monooxygenases. The HpaB sequence (A) and the HpaC sequence (B) are from Prieto et al. (33, 34), the HpaA sequence (A) and the HpaH sequence (B) are from Gibello et al. (14), and the HadA sequence (A) is from Takizawa et al. (39). The residues which are identical in all sequences are shown in the line labeled consensus. Gaps (-) are introduced into the sequences to allow optimal alignment. The number to the right of each sequence represents the last amino acid residue position at each line.

tftCD, and *tftEFGH*), fragments representing an internal region of each gene cluster were radiolabeled with α - 32 P (as described above) and used as probes. Prehybridization was carried out at 68°C in 10% polyethylene glycol-7% sodium dodecyl sulfate-1.5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7])—100 µg of salmon sperm DNA per ml. The hybridization was done in the same solution overnight at 65°C. The membranes were washed as described by Sambrook et al. (36). Blots were visualized with a Phosphor-Imager apparatus as described above.

Nucleotide sequence analysis. Searches of homologous proteins were performed with the FASTA program (32) at the National Center for Biotechnology Information (NCBI). Multiple amino acid sequence alignments were done with the CLUSTAL W program (40).

Nucleotide sequence accession number. The nucleotide sequence of *tftCD* has been reported to the NCBI and assigned accession no. U83405.

RESULTS

Cloning and sequence analysis of the genes encoding the chlorophenol-4-monooxygenase from AC1100. The N-terminal sequence of the 58-kDa polypeptide component of the chlorophenol-4-monooxygenase was determined to be Met-Arg-Thr-Gly-Lys-Gln-Tyr-Leu-Glu-Ser-Leu-Asn-Asp-Gly (48). The N-terminal amino acid sequence was compared to sequences in the protein database at the NCBI and found to show strong homology to the chlorophenol hydroxylase from *Burkholderia pickettii* DTP0602 (39). A degenerate oligonucleotide, TCPCA, was designed from the first nine amino acid residues with the following 5'-to-3' sequence: ATGCGIACI GGIAAGCAGTA(C/T)(G/T)TIGAG, where I is inosine. TCPCA was end labeled and hybridized to a *Bam*HI-generated genomic library of wild-type AC1100 (37). Clones (9,600) were screened by colony hybridization, and one positive clone, pTFT1, was selected for further study. A 7.5-kb *Bam*HI fragment demonstrating positive hybridization was subcloned to create plasmid pTFT2.

To determine if the construct contained the relevant genes, pTFT2 was sequenced, and the translated sequence was compared to sequences in the FASTA protein sequence database (NCBI). An ORF at one end showed very high homology to the N-terminal part of the *hadA* gene product, which is a 58-kDa polypeptide, one of the components of the 2,4,6-TCP hydroxylase from *B. pickettii* DTP0602 (39). It appeared that this clone contained part of the 5' end of the gene encoding the 58-kDa polypeptide from AC1100 (Fig. 1; 5' part of *tftD*), since the first nine amino acids on translation matched those identified for the 58-kDa polypeptide of chlorophenol-4-monooxygenase (48). Further sequencing in pTFT2 revealed a complete 537-bp ORF, which we have designated *tftC*. The first 10 amino acids of the translated *tftC* sequence (Fig. 2) match the first 10 amino acids of the purified small component of chlorophenol-4-monooxygenase. The partial ORF encoding a portion of the 58-kDa polypeptide ended with a *Bam*HI restriction site, after which was vector-encoded sequence. Since the genomic library did not appear to contain inserts overlapping the *Bam*HI site, inverse PCR (28, 30) was performed with primers designed from the known sequence to obtain the rest of this partial ORF. Sequence analysis of the resulting 1-kb PCR product confirmed that the ORF encoded the *tftD* gene. This DNA fragment was then used as a probe to obtain from the genomic library a cosmid, pTFT3, containing a 24-kb insert. pTFT4 was constructed by ligating a 1-kb *Ssi*II-*Bam*HI fragment of pTFT2 carrying *tftC* and part of *tftD* to a 4.5-kb *Bam*HI-*Eco*RV fragment of pTFT3 containing the rest of *tftD* (Fig. 1).

The calculated molecular masses of the *tftC* and *tftD* gene products are 20 and 59 kDa, respectively, which are the approximate molecular masses of both components of the chlo-

rophenol-4-monooxygenase (48). The translational start site of *tftD* is a GTG codon, as has been observed previously for other *tft* genes of this strain (9). A comparison of the deduced amino acid sequences of the *tftC* and *tftD* ORFs with the sequences of other bacterial aromatic monooxygenases revealed a high degree of similarity. The translated ORF encoding the 59-kDa polypeptide (*tftD*) showed highest homology to HadaA (chlorophenol-4-hydroxylase) from *B. pickettii* DTP0602 (39) (64% identity and 76% homology over a 281-amino-acid overlap), followed by HpaB (4-hydroxyphenyl 3-monooxygenase) (48% identity and 68% homology over a 54-amino-acid overlap) from *E. coli* W (33, 34) and HpaA (4-hydroxyphenyl 3-monooxygenase) from *Klebsiella pneumoniae* (48% identity and 68% homology over a 54-amino-acid overlap) (14) (Fig. 2A). The translated ORF encoding the 22-kDa polypeptide (*tftC*) showed strong homology to the smaller components of these monooxygenase enzymes, starting with HpaC (34% identity and 52% homology over a 150-amino-acid overlap) from *E. coli* (33, 34) and HpaH (35% identity and 52% homology over a 150-amino-acid overlap) from *K. pneumoniae* (14) (Fig. 2B).

Transcriptional regulation of the *tftCD* operon. When *B. pickettii* DTP0602 is grown with succinate as a sole source of carbon and energy, its cells lose their ability to dechlorinate 2,4,6-TCP (22). This same phenomenon was observed with resting-cell suspensions of AC1100. Karns et al. (20) demonstrated that when AC1100 was grown with glucose, succinate, or lactate, the cells were unable to dechlorinate 2,4,5-T. To determine the nature of this regulation, AC1100 was grown with 1% glucose as a sole carbon source and then resuspended in 4 mM 2,4,5-T or 1% glucose. After an incubation period of 6.5 h, the cells were harvested and RNA was extracted. RNA from induced and uninduced cells was employed to map the transcriptional start site of the *tftC* gene by primer extension. The same primer was used for both the reverse transcription and sequencing reactions. A broad band encompassing several adjacent residues was obtained at the sequence CATT (Fig. 3A). The intensity of the band in the induced sample was much greater than the intensity of the band of the uninduced sample, suggesting transcriptional regulation of these genes. Quantitative hybridization of total cellular RNA with a *tftC* probe confirmed that, under inducing conditions, the amount of *tftC* expression was increased by approximately eightfold (Fig. 3B). Analysis of the promoter region revealed a motif in the -10 region which resembles a σ^{70} -like *E. coli* -10 consensus sequence (TAGTAT) (Fig. 3C). A typical -35 consensus sequence was not present, perhaps suggesting regulation by a protein that binds in the upstream region. These results are consistent with previous observations that the ability to dechlorinate 2,4,5-T is greatly reduced when AC1100 is grown with alternate carbon sources (20).

The cloning and characterization of *tftCD* allow the definitive assignment of functions to the upper portion of the 2,4,5-T degradative pathway (6, 8, 9). The two components encoded by *tftAB* are responsible for the conversion of 2,4,5-T to 2,4,5-TCP. *TftCD* then converts 2,4,5-TCP to 5-CHQ (48). The lower part of the pathway, specified by *tftEFGH*, proceeds through *ortho*-cleavage of CHQ to generate β -keto adipate. Further dissimilation of β -keto adipate is mediated by chromosomal genes leading to tricarboxylic acid cycle intermediates (10).

Genome structure of AC1100 and chromosomal locations of the three *tft* gene clusters. The previously cloned *tftAB* and *tftEFGH* gene clusters were found on separate cosmids. Considering the short-term evolution of the catabolic pathway for 2,4,5-T degradation in AC1100 over a period of several months (3), and the apparent transfer of this capacity from a consortium to a single isolate, it is possible that the pathway evolved

FIG. 3. (A) Determination of the *tftC* transcriptional start site. RNA (50 μ g) from uninduced (U) and induced (I) cultures of AC1100 was used for primer extension analysis. The same primer was used for the reverse transcription reaction and generation of the sequencing ladder. The asterisks next to the residues on the sense strand represent the transcriptional start sites. (B) Slot blots of AC1100 RNA extracted from induced and uninduced cultures. The top position contains 30 μ g of RNA from uninduced cultures of AC1100, and the bottom position contains an equal amount of RNA from induced cultures. The left blot was probed for *tftCD*, and the right blot was probed for *tftAB* as described in Materials and Methods. (C) Sequence of the promoter region of the *tftCD* gene cluster. The arrow indicates the initiating residues. The -10 region is indicated. TATAAT represents the *E. coli* σ^{70} consensus sequence. The asterisks above the consensus sequences indicate residues on the sense strand of *tftCD* which match those of the consensus sequence. No apparent -35 consensus sequence was identified. n, any nucleotide.

by a series of independent gene transfers. If so, one would expect the genes to be in separate genomic locations.

Before identifying genomic locations of the *tft* genes, we needed a better understanding of the AC1100 genome organization. *B. cepacia* has an unusual genomic structure consisting of multiple replicons. Three large replicons have been reported for *B. cepacia* ATCC 17616 (4) and the type strain, ATCC 25416 (35). This observed chromosome multiplicity prompted us to examine the number of replicons present in AC1100 by PFGE. Prior to electrophoresis, agarose plugs were submitted to a freeze-thaw procedure that results in reproducible linearization of replicons, a prerequisite for migration of whole, undigested replicons into the gel matrix (see Materials and Methods). We have previously demonstrated that the genome of AC1100 is comprised of five replicons (designated replicons I through V) with a total genome size of about 7.6 Mbp, determined by adding up the sizes of linearized replicons (17). AC1100 contains two large chromosomes with estimated

FIG. 4. PFGE (contour-clamped homogeneous electric field) analysis of the AC1100 genome. (A) Ethidium bromide-stained PFGE gel with resolution in the megabase size range. Lane 1, *Schizosaccharomyces pombe* chromosomes; lane 2, *Hansenula wingei* chromosomes; lane 3, undigested DNA of *B. cepacia* AC1100. (B) Replicon locations of *tft* genes. Lane 1, *B. cepacia* AC1100 DNA stained with ethidium bromide; lanes 2 to 4, autoradiograms of the Southern hybridizations with internal fragments of the three *tft* gene clusters, *tftEFGH* (lane 2), *tftAB* (lane 3), and *tftCD* (lane 4). Separation conditions are described in Materials and Methods.

sizes of 4 (replicon I) and 2.7 (replicon II) Mbp (Fig. 4A) and three smaller replicons with estimated sizes of 530, 340, and 150 kb (replicons III to V) (Fig. 4B and 5). DNAs of the cloned *tft* gene clusters were used to probe linearized AC1100 replicons. As shown in Fig. 4B, *tftEFGH* hybridizes to the 0.53-Mbp replicon (III) (lane 2), whereas *tftAB* hybridizes to the 0.34-Mbp replicon (IV) (lane 3). *tftCD* is present in two copies, one on the 0.53-Mbp replicon and the other on the 0.34 Mbp replicon (lane 4). These Southern blot hybridizations were done with labeled DNA fragments as probes, which in all cases comprised almost the complete gene cluster. Consequently, it could not be distinguished whether the hybridization pattern observed for *tftCD* resulted from a duplication of the entire cluster or from that of one of the individual genes, *tftC* or *tftD*. We repeated the hybridization using as probes γ -³²P-end-labeled oligonucleotides complementary to a region of either the *tftC* or *tftD* coding strand. Both probes hybridized to replicons III and IV (data not shown). To discriminate between gene duplication and cross-hybridization of highly related genes, we isolated DNA from each replicon and then amplified an internal fragment of the *tftCD* gene cluster from each replicon by high-fidelity PCR. Sequences of the PCR products obtained from both replicons were identical, suggesting that the gene duplication occurred after gene recruitment.

We have previously shown the genes encoding the 2,4,5-T oxygenase (*tftAB*) to be located on a 27.5-kb DNA cosmid clone, pRHC21 (6, 16). Since the enzymatic reactions specified by the *tftAB* and *tftCD* operons are successive in the biodegradation of 2,4,5-T and since both the *tftAB* and the *tftCD* gene clusters are present in the 0.34-Mbp replicon (IV), it was of interest to know if the *tftCD* genes were also present on this 27.5-kb DNA fragment. To answer this question, we performed a Southern hybridization experiment using a 0.7-kb *Bam*HI-*Hind*III DNA fragment harboring *tftC* and part of *tftD* as the probe. No hybridization was seen with pRHC21 or the vector pCP13 digests (data not shown), indicating that the *tftCD* copy on the 0.34-Mbp replicon is not present within 12 kb upstream or 8 kb downstream of *tftAB*.

Genomic instability and rearrangements of *tft* genes. In earlier studies, mutants in the pathway, such as PT88 (41), were

obtained by Tn5 mutagenesis. PT88, when grown on glucose in the presence of 2,4,5-T, accumulates in the medium a bright-red compound which is an autooxidation product of the intermediate 5-CHQ. This product is the result of a metabolic block in the lower part of the pathway specified by the *tftEFGH* operon (9, 37). AH88, a spontaneous mutant of PT88, was isolated based on its failure to excrete the colored compound. This failure indicated a defect upstream of the 5-CHQ formation step, either in *tftAB* or *tftCD*. To answer the question of whether the observed phenotypes of PT88 and AH88 were the result of point mutations, insertional inactivation, deletions, or genetic rearrangements, we decided to compare these

FIG. 5. Locations of *tft* genes in AC1100 and two *tft*-negative mutants of AC1100, PT88, and AH88. Several identical PFGE agarose gels were run in parallel and subjected to Southern blot analysis. (A) Ethidium bromide-stained gel containing undigested chromosomal DNAs of *B. cepacia* AC1100 (lane 1), PT88 (lane 2), and AH88 (lane 3). (B to D) Autoradiograms of Southern hybridizations showing localization of *tftAB* (B), *tftCD* (C), and a random clone, pAH154 (D).

TABLE 2. Chromosomal analysis of *tft* mutants

Strain	Phenotype ^a	Presence ^b of:		
		<i>tftAB</i>	<i>tftCD</i>	<i>tftEFGH</i>
AC1100	Pink	+	++	+
PT88	Bright red	+	+	Δ
AH88	No color	Δ	+	Δ
WHP4	Bright red	+	+	Δ
WHP5	Bright red	+	+	Δ
WH1	No color	Δ	++	+
WH51	No color	Δ	++	+
SM3C	No color	Δ	Δ	Δ
SM7C	No color	Δ	+	Δ

^a Color produced by accumulation in liquid medium of intermediates in the 2,4,5-T pathway. No color indicates a block at the first step, and bright red indicates a block in the lower portion of the pathway.

^b + represents one copy of the indicated *tft* gene cluster. ++ represents two copies of the indicated *tft* gene cluster on separate replicons. Δ represents a deletion of the indicated *tft* gene cluster.

mutant strains with AC1100 to determine the chromosomal locations of the individual *tft* gene clusters. The smaller replicons were resolved in a pulsed-field gel (Fig. 5A) and then subjected to Southern hybridization analysis (Fig. 5B and C) as described above.

We found that PT88 had undergone extensive rearrangements of the small chromosomes, including deletion of the *tftEFGH* gene cluster (data not shown) and one copy of *tftCD* (Fig. 5C, lane 2). The *tftAB* gene cluster was rearranged in conjunction with a 60-kb fragment from the 0.34-Mbp replicon (IV) to the 0.53-Mbp replicon (III) (Fig. 5B, lane 2). In strain AH88 we observed a more dramatic deletion of about 340 kb that included *tftAB* (Fig. 5A and B, lanes 3). To decide whether the 0.28-Mbp replicon of PT88 (Fig. 5A, lane 2) was indeed the remainder of the 0.34-Mbp replicon (IV) of AC1100 after transposition of about 60 kb to the 0.53-Mbp replicon (III), and to exclude more complicated rearrangements, we isolated random clones from the 0.28-Mbp replicon and used them as probes on whole replicons in Southern hybridization experiments. The hybridization pattern of pAH154, which is presented in Fig. 5D, establishes a direct relationship between the 0.28-Mbp replicons of PT88 and AH88 and the 0.34-Mbp replicon (IV) of AC1100. Our results clearly indicate that the main mechanism of the loss of *tft* function in PT88 and AH88 is deletion.

Using the phenotypic selection procedure described above for PT88 and AH88, we isolated six more spontaneous *tft*-negative mutants of AC1100 in order to examine them for the presence and replicon locations of the *tft* genes (Table 2). We found that all three gene clusters were deleted in an independent manner. However, the simultaneous loss of one copy of *tftCD* along with *tftEFGH*, as was observed for all six *tftEFGH*-negative mutants, suggests that the distance between these gene clusters may be relatively small. All but one of the *tft*-negative mutants contained at least one copy of *tftCD*. Total loss of *tftCD*, as observed for the mutant SM3C, coincided with the loss of all three *tft* functions. No viable mutant that had retained *tftAB* but lost both copies of *tftCD* could be isolated (see Discussion). Overall, we observe that AC1100 is prone to spontaneous chromosomal deletions and rearrangements of the *tft* genes at high frequencies. Our data, which are summarized in Table 2, explain the gradual loss of the 2,4,5-T-positive phenotype upon growth under nonselective conditions, as was documented for AC1100 (3).

DISCUSSION

In this paper we report the cloning, sequencing, and characterization of a locus encoding the chlorophenol-4-monooxygenase from *B. cepacia* AC1100. The predicted amino acid sequences of the *tftC* and *tftD* gene products reveal strong homology to other bacterial aromatic monooxygenases. The *tftD* gene product shows a high degree of similarity to the larger subunits of aromatic hydrocarbon monooxygenases, such as HadA from *B. pickettii* DTP0602 (39), HpaB from *E. coli* W (33, 34), and HpaA from *K. pneumoniae* (14) (Fig. 2A). TftC has homology with the smaller subunits of this class of monooxygenases, such as HpaC from *E. coli* W (34) and HpaH from *K. pneumoniae* (14) (Fig. 2B). The larger components of these enzymes appear to be hydroxylases which often contain a ferrous ion (46). Surprisingly, HadB, the smaller-molecular-weight component of the 2,4,6-trichlorophenol-4-dechlorinase (39), displayed no significant homology to either TftC or any other of the smaller-molecular-weight components of the other aromatic monooxygenases. Less is known about the function of these components; however, Xun (48) has shown that TftC is capable of NADH-dependent reduction of cytochrome *c* in the presence of flavin adenine dinucleotide. Thus, TftC may function as an electron transfer protein. This may also be true of HpaC and HpaH. Although HadB has no homology to TftC, HpaC, or HpaH, it is necessary for the efficient conversion of 2,4,6-trichlorophenol to 2,6-dichloro-*p*-hydroquinone in *B. pickettii* DTP0602. Furthermore, HadB shows similarity to the *nox* gene product, an NADH oxidase from *Thermus thermophilus* (31). Therefore, HadB may also serve as an electron transfer protein. More recently, a 2,4,6-trichlorophenol-4-monooxygenase was isolated from *Azotobacter* sp. strain GP1 (45). This enzyme was found to be inducibly expressed; that is, the enzyme was isolated only from 2,4,6-trichlorophenol-grown cells. The N-terminal amino acid sequence of this single component enzyme has high degrees of homology to HadA (39) and HpaB (33, 34). Similarly to the 2,4,6-TCP monooxygenase from *B. pickettii*, the 2,4,6-TCP monooxygenase from *Azotobacter* does not require an additional protein for in vitro activity.

The majority of aromatic biodegradative pathways involve steps which are transcriptionally regulated. When strain DTP0602 is grown with succinate or glucose as sole carbon sources, its ability to dechlorinate 2,4,6-TCP is suppressed (22). Sequence analysis of the region upstream of *hpaB* revealed a partial ORF (*hpaA*) which showed homology with MelR, a member of the XylS or AraC family of transcriptional regulators (12). When AC1100 was grown with glucose, succinate, or lactate as the sole carbon source, these cells were unable to dechlorinate 2,4,5-TCP or pentachlorophenol over a 5-h period (20). As shown in the slot blot and primer extension experiments (Fig. 3A and B), the amount of *tftCD* transcript being synthesized by AC1100 was significantly increased under inducing conditions. In each case, there appeared to be a small amount of transcript being synthesized even under uninduced conditions; however, dechlorinase activity, if present, was below the level detectable in our whole-cell assays. The induction of the *tftCD* transcript may have been due to the presence of regulators that interact with inducers such as 2,4,5-TCP to activate the *tftC* and *tftD* genes. The nature of the regulatory elements as well as the mode of their regulation are unknown at present.

Genes encoding enzymes which degrade aromatic compounds, especially those for degradation of the central intermediate chlorocatechol, are often organized as coherent gene clusters and regulated as individual operons (43, 44). The genes of the entire 4-hydroxyphenylacetic acid pathway in *E. coli* W are

positioned next to each other within a 14,855-bp DNA region (33). It has been hypothesized that AC1100 acquired the ability to degrade 2,4,5-T in the chemostat over 8 to 10 months by recruiting the necessary genes from various genetic sources present in the consortium (8). Using PFGE and Southern blot analysis, we showed that the genome of AC1100 consists of five replicons of different sizes (4.0, 2.7, 0.53, 0.34, and 0.15 Mbp, referred to as replicons I to V, respectively) and that the three sets of *tft* genes are dispersed between the 0.53- and 0.34-Mbp replicons, III and IV (8, 17). We found that the genes encoding the 2,4,5-T oxygenase, *tftAB*, are located on replicon IV. The *tftCD* operon, which codes for the chlorophenol-4-monooxygenase, is present in two identical copies on replicons III and IV, and *tftEFGH*, which specifies the lower portion of the degradative pathway, was found exclusively on replicon III. Duplication of the *tftCD* cluster after its recruitment into the cell might be of selective advantage, since it allows a more efficient conversion of 2,4,5-TCP, the toxic intermediate generated from 2,4,5-T by the gene products of *tftAB*. In this paper, we described for the first time a catabolic pathway which is split between two different replicons. The recruitment of foreign degradative genes as part of two individual replicons, as described here for strain AC1100, suggests that independent genetic events might have led to the evolution of an organism able to use 2,4,5-T as its sole energy and carbon source. The isolation and genomic analysis of various mutants that are unable to catabolize 2,4,5-T allow further insight into the evolution of the 2,4,5-T pathway as well as the limitations of AC1100 in field experiments, as the result of its genomic instability. Mapping of the three *tft* gene clusters to whole replicons of these mutants revealed that the *tft* gene clusters are deleted and rearranged at high frequencies. Clearly, no close physical linkage could be detected between the individual *tft* gene clusters; their deletions occurred in an independent manner. However, our failure to isolate viable mutants that had lost both copies of *tftCD*, but still contained *tftAB*, confirms a physiological linkage between *tftAB* and *tftCD*. Retention of at least one copy of *tftCD* might be necessary to avoid an intracellular buildup of toxic 2,4,5-TCP.

IS elements have been implicated in assisting evolution of biodegradative pathways through recruitment of foreign genes (25) and insertional activation of the expression of adjacent genes (1, 24, 38). Numerous copies of transposon-like IS elements are present in the genome of AC1100 (37, 41). Haugland et al. (15) have shown that these IS elements are capable of translocating themselves from the AC1100 genome to plasmids introduced into the cells. These IS elements, IS931 and IS932, are associated with the *tftAB* operon (15) and the *tftEFGH* operon (9, 37), and their involvement in the evolution of the 2,4,5-T-degradative pathway has been discussed previously (16, 41). A new family of IS elements that are distinct from previously identified elements were recently described for AC1100. One member, IS1490, was recently linked to 2,4,5-T metabolism. Creation of a fusion promoter about 110 bp upstream of the *tftA* initiation codon is responsible for the constitutive expression of *tftAB* (5, 19). Southern hybridization experiments indicated the presence of sequences similar to that of IS1490 upstream of *tftCD* (data not shown). A 558-bp DNA region starting 757 bp upstream of *tftC* was sequenced and was found to be 86% identical to IS1490. This evidence suggests that the 2,4,5-T-degradative pathway may have evolved in AC1100 through IS element-mediated events and that the final organization of the various genes encoding different steps of the pathway is therefore somewhat random. In strain AC1100 we find IS elements disproportionately concentrated on the two smaller replicons, III and IV, which also harbor the *tft*

genes (data not shown). Such clustering of IS elements makes these replicons a prime target for homologous recombination that results in deletions and rearrangements of adjacent genes. The extraordinary plasticity of these replicons causes *B. cepacia* AC1100 without selective pressure to quickly lose its ability to degrade 2,4,5-T.

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