

# Chlorophenol Hydroxylases Encoded by Plasmid pJP4 Differentially Contribute to Chlorophenoxyacetic Acid Degradation

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Phenoxyalkanoic compounds are used worldwide as herbicides. *Cupriavidus necator* JMP134(pJP4) catabolizes 2,4-dichlorophenoxyacetate (2,4-D) and 4-chloro-2-methylphenoxyacetate (MCPA), using *tfd* functions carried on plasmid pJP4. TfdA cleaves the ether bonds of these herbicides to produce 2,4-dichlorophenol (2,4-DCP) and 4-chloro-2-methylphenol (MCP), respectively. These intermediates can be degraded by two chlorophenol hydroxylases encoded by the *tfdB<sub>I</sub>* and *tfdB<sub>II</sub>* genes to produce the respective chlorocatechols. We studied the specific contribution of each of the TfdB enzymes to the 2,4-D/MCPA degradation pathway. To accomplish this, the *tfdB<sub>I</sub>* and *tfdB<sub>II</sub>* genes were independently inactivated, and growth on each chlorophenoxyacetate and total chlorophenol hydroxylase activity were measured for the mutant strains. The phenotype of these mutants shows that both TfdB enzymes are used for growth on 2,4-D or MCPA but that TfdB<sub>I</sub> contributes to a significantly higher extent than TfdB<sub>II</sub>. Both enzymes showed similar specificity profiles, with 2,4-DCP, MCP, and 4-chlorophenol being the best substrates. An accumulation of chlorophenol was found to inhibit chlorophenoxyacetate degradation, and inactivation of the *tfdB* genes enhanced the toxic effect of 2,4-DCP on *C. necator* cells. Furthermore, increased chlorophenol production by overexpression of TfdA also had a negative effect on 2,4-D degradation by *C. necator* JMP134 and by a different host, *Burkholderia xenovorans* LB400, harboring plasmid pJP4. The results of this work indicate that codification and expression of the two *tfdB* genes in pJP4 are important to avoid toxic accumulations of chlorophenols during phenoxyacetic acid degradation and that a balance between chlorophenol-producing and chlorophenol-consuming reactions is necessary for growth on these compounds.

Bacteria play a fundamental role in the degradation of many chlorinated aromatic pollutants. *Cupriavidus necator* (formerly *Ralstonia eutropha*) JMP134(pJP4) was isolated for its ability to use 2,4-dichlorophenoxyacetate (2,4-D) as a sole carbon and energy source (5). This strain has been the focus of intensive studies because of its ability to degrade a wide spectrum of aromatic compounds, including 4-chloro-2-methylphenoxyacetate (MCPA) (29), 2,4,6-trichlorophenol (3), 4-fluorobenzoate (32), and 3-chlorobenzoate (5), among many others (D. Pérez-Pantoja, J. E. González-Pastor, D. H. Pieper, V. de Lorenzo, and B. González, unpublished data). The genes responsible for metabolism of 2,4-D/MCPA are localized in a 22-kb DNA region of plasmid pJP4 (37). The first step in 2,4-D/MCPA degradation involves cleavage of the ether bond by a 2,4-D/ $\alpha$ -ketoglutarate dioxygenase encoded by the *tfdA* gene (8) to yield 2,4-dichlorophenol (2,4-DCP) and 4-chloro-2-methylphenol (MCP), respectively (Fig. 1A). These compounds are then transformed by a 2,4-DCP hydroxylase to produce 3,5-dichlorocatechol (3,5-DCC) or 5-chloro-3-methylcatechol (MCC) (6). A four-step modified *ortho* ring cleavage pathway consisting of a chlorocatechol 1,2-dioxygenase (TfdC), a chloromuconate cycloisomerase (TfdD), a dienelactone hydrolyase (TfdE), and a maleylacetate reductase (TfdF) transforms

3,5-DCC into  $\beta$ -keto adipate (Fig. 1B), which can be channeled to the central metabolism by two additional, chromosomally encoded enzymes (10). The pJP4 plasmid has two homologous, but nonidentical, gene clusters, *tfdC<sub>I</sub>D<sub>I</sub>E<sub>I</sub>F<sub>I</sub>* and *tfdC<sub>II</sub>D<sub>II</sub>E<sub>II</sub>F<sub>II</sub>*, encoding chlorocatechol degradation enzymes. Two different 2,4-dichlorophenol hydroxylases are encoded by the *tfdB* genes located immediately downstream of each of these clusters (Fig. 1C) (18, 37).

Studies with *C. necator* JMP134 have demonstrated that each *tfd* gene module on pJP4 encodes functional enzymes, but the contribution of the enzymes encoded by each cluster to chlorocatechol degradation is not fully understood (18, 27, 30). Except for chloromuconate cycloisomerases, no great differences in substrate specificity between the enzymes encoded by each *tfd* module have been found, although it has been shown that the maintenance of both modules in pJP4 results in a higher expression of key catabolic activities that prevents the accumulation of toxic chlorocatechols (28, 30). An inactivation analysis of *tfd* functions was recently reported (19), suggesting that the *tfdC<sub>II</sub>D<sub>II</sub>E<sub>II</sub>F<sub>II</sub>B<sub>II</sub>* genes are not essential for growth on 2,4-D but are relevant for growth on MCPA. Since no further information was given on intermediate accumulation during the degradation of chlorophenoxyacetates by these mutants, and since gene complementation was not reported, it is unclear if some of these phenotypes could be derived from polar effects on the chlorophenol hydroxylase genes. This is particularly relevant since chlorophenol substantially accumulates during 2,4-D degradation (22). Furthermore, no information on the contributions to total chlorophenol hydroxylase

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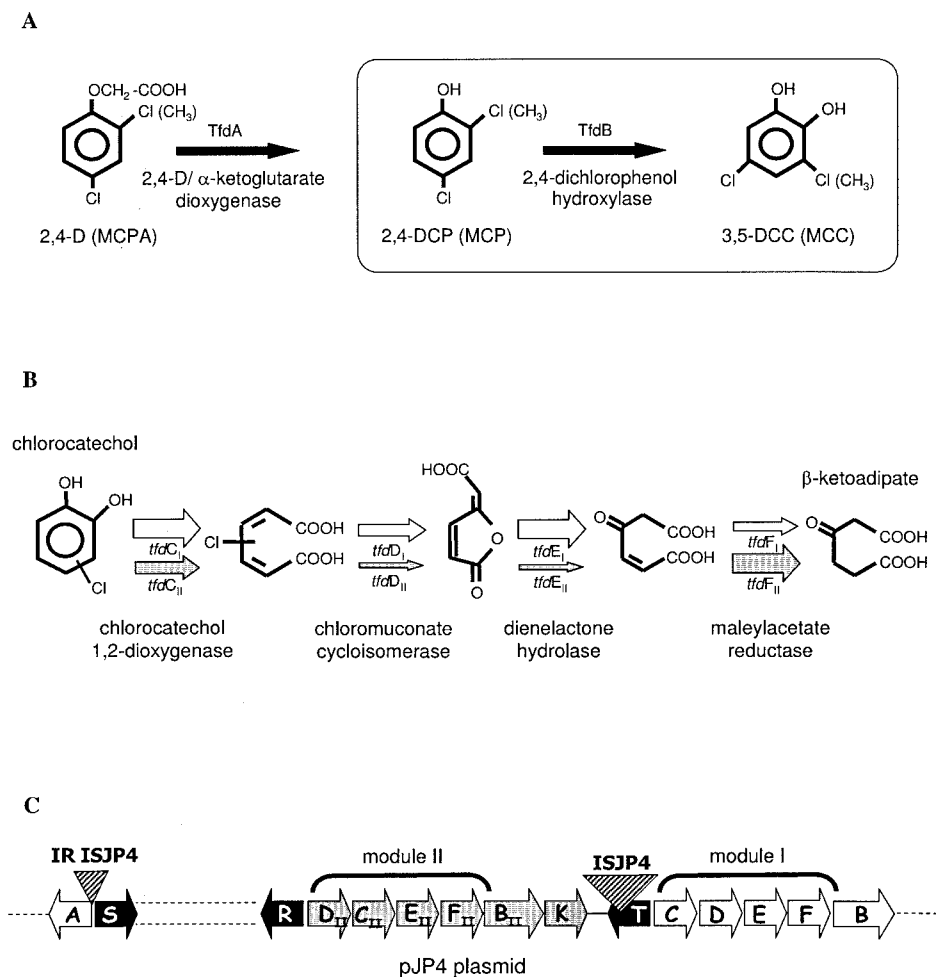


FIG. 1. Genes involved in 2,4-D and MCPA degradation. (A) Chlorocatechol-producing peripheral reactions for 2,4-D and MCPA are carried out by enzymes encoded by the *tfdA* and *tfdB* genes in plasmid pJP4. (B) Chlorocatechol 1,2-dioxygenase (TfdC), chloromuconate cycloisomerase (TfdD), dienelactone hydrolase (TfdE), and maleylacetate reductase (TfdF) catalyze the conversion of chlorocatechols to chloromuconate, *cis*-dienelactone, maleylacetate, and  $\beta$ -ketoadipate, respectively. The arrow thicknesses indicate the relative specific activities of the enzymes encoded by each module (28, 30). (C) Organization of *tfd* genes in pJP4, including the *tfdC<sub>I</sub>D<sub>I</sub>E<sub>I</sub>F<sub>I</sub>B<sub>I</sub>* and *tfdD<sub>II</sub>C<sub>II</sub>E<sub>II</sub>F<sub>II</sub>B<sub>II</sub>* gene clusters. The diagram is not drawn to scale.

activity or differences in substrate specificity between TfdB<sub>I</sub> and TfdB<sub>II</sub> have been reported so far. In this work, we assess the contribution of each *tfdB*-encoded chlorophenol hydroxylase in this archetypal bacterial chlorophenoxyalkanoic acid degradation pathway.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *C. necator* JMP134(pJP4), *Burkholderia xenovorans* LB400(pJP4), and 2,4-D/MCPA-mineralizing derivatives of these strains were grown at 30°C in liquid minimal medium (17), with 0.5 to 10 mM 2,4-D or MCPA or with 5 mM fructose as the sole carbon source. The plasmid pJP4-free derivative strain *C. necator* JMP222 was grown with 2 mM 2-methylphenol or with 5 mM fructose. Growth on each chlorophenoxyacetate was determined by measuring the increase in optical density at 600 nm (OD<sub>600</sub>). At least three replicates were used for each growth measurement. *C. necator* derivatives not able to proliferate on 2,4-D or MCPA were grown on 5 mM benzoate plus the appropriate antibiotic (Table 1) or on 5 mM fructose for induction experiments. *Escherichia coli* strains DH5 $\alpha$  and BW25113 were maintained on Luria-Bertani (LB) agar plates. Antibiotics were used at the following

concentrations: ampicillin, 100  $\mu$ g/ml; kanamycin, 50  $\mu$ g/ml; chloramphenicol, 20  $\mu$ g/ml; and trimethoprim, 100  $\mu$ g/ml.

**DNA manipulation.** Restriction, ligation, and dephosphorylation reactions and the purification and electroporation of DNA were performed by standard procedures (1). Derivatives of the pJP4 plasmid were transferred from *E. coli* BW25113 to *C. necator* JMP222 by mating. Derivatives of the broad-host-range plasmid vector pMLS7 (pMLB1 and pMLB3) (20) were mobilized from *E. coli* DH5 $\alpha$  to *C. necator* JMP222 by triparental mating with the helper strain *E. coli* HB101(pRK600), as previously described (27). Transconjugants were selected on minimal medium agar plates supplemented with 2 mM benzoate plus kanamycin or trimethoprim.

**Inactivation of *tfdB<sub>I</sub>* and *tfdB<sub>II</sub>* genes.** The *tfdB<sub>I</sub>* and *tfdB<sub>II</sub>* genes were independently inactivated in *E. coli* BW25113 cells harboring the pJP4 plasmid by the method of Datsenko and Wanner (4). PCR primers FORTB1 (5'-**AACAATTGACGGAGGAAGACATGGCATTGACGATCGAAACTGTAGGCTGGAGCTGCTTCG**-3') and REVTB1 (5'-**ATAGATCGCGACGCCACTCCTTATATAGCTCGAGACGTAACAATGATATCCTCCTTAG**-3') (for *tfdB<sub>I</sub>*) and FORTB2 (5'-**CC TATCGATAAGGAGACTTCATGACGAAAAACCAACTGTAGGCTGGAGCTGGAGCTGCTTCG**-3') and REVTB2 (5'-**CCGATCGGGTTTTTCTGGATCAGTGGCGCAGAGCGCCCATATGAATATCCTCCTTAG**-3') (for *tfdB<sub>II</sub>*), which contain 40-bp homology extensions of the *tfdB<sub>I</sub>* or *tfdB<sub>II</sub>* gene sequence (in bold) and 20-bp priming sequences for pKD4 (4), were synthesized. These primer pairs

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype and/or genotype <sup>a</sup>	Source or reference
<i>C. necator</i> strains		
JMP134(pJP4)	2,4-D <sup>+</sup> MCPA <sup>+</sup> , pJP4	DSMZ <sup>b</sup>
JMP222	pJP4-free derivative, 2,4-D <sup>-</sup> MCPA <sup>-</sup> Sm <sup>r</sup>	H.-J. Knackmuss
JMP222(pJP4)	2,4-D <sup>+</sup> MCPA <sup>+</sup> , pJP4	This study
<i>B. xenovorans</i> strains		
LB400	2,4-D <sup>-</sup> MCPA <sup>-</sup>	DSMZ <sup>b</sup>
LB400(pJP4)	2,4-D <sup>+</sup> MCPA <sup>+</sup>	This study
<i>E. coli</i> strains		
BW25113	pKD46, red recombinase	4
BL21(DE3)	λDE3 lysogen, T7 RNA Pol	
Plasmids		
pRK600	Cm <sup>r</sup>	11
pGEM-T Easy	Ap <sup>r</sup>	Promega Corp., Madison, WI
pGEMTFDB1	Ap <sup>r</sup> , <i>tfdB</i> <sub>I</sub>	This study
pGEMTFDB2	Ap <sup>r</sup> , <i>tfdB</i> <sub>II</sub>	This study
pGEMB3	Ap <sup>r</sup> , <i>pheA</i>	This study
pJP4- <i>tfdB</i> <sub>II</sub> mutant	<i>tfdB</i> <sub>I</sub>	This study
pJP4- <i>tfdB</i> <sub>I</sub> mutant	<i>tfdB</i> <sub>II</sub>	This study
pMLS7	<i>P</i> <sub>S7</sub> promoter, Tp <sup>r</sup>	20
pMLB1	<i>tfdB</i> <sub>I</sub> , pMLS7 derivative	This study
pMLB3	ORF4010, pMLS7 derivative	This study
pBBRMCS-2	Km <sup>r</sup>	16
pBTFDA	<i>tfdA</i> , pBBRMCS-2 derivative	Lab stock

<sup>a</sup> 2,4-D<sup>+</sup> and MCPA<sup>+</sup>, able to grow in 2,4-D and MCPA, respectively; *tfd*, catabolic genes from pJP4; Ap<sup>r</sup>, ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Sm<sup>r</sup>, streptomycin resistance; Tp<sup>r</sup>, trimethoprim resistance; Km<sup>r</sup>, kanamycin resistance.

<sup>b</sup> DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

were used with pKD4 as the template to amplify the kanamycin resistance gene flanked by 40 bp of the *tfdB*<sub>I</sub> or *tfdB*<sub>II</sub> gene sequence. The following PCR program was used: 95°C for 5 min, 28 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s, and then 72°C for 10 min. PCR products were used to inactivate the *tfdB*<sub>I</sub> or *tfdB*<sub>II</sub> gene in an *E. coli* BW25113(pJP4) strain harboring RecBCD recombinase by a previously described procedure (28). pJP4 derivatives containing an inactivated *tfdB*<sub>I</sub> or *tfdB*<sub>II</sub> gene were then transferred to strain JMP222 by biparental conjugation as previously described (27). Primer pair FORWB1 (5'-GGAGGAATTCATGGCATTGAC-3'; includes an EcoRI restriction site [in italics]) and REVWB1 (5'-GTTACGTCCGGTCCAGAAAA-3') and primer pair FORWB2 (5'-TTCATATGTCTCCTTCGGGC-3') and REVWB2 (5'-GCATTGCTGCGATCACTT-3') were used to verify correct recombinational insertion of the kanamycin resistance cassette in place of each *tfdB* gene. This was then confirmed by direct sequencing of the insertion region using these primers.

**Constitutive and IPTG-induced expression of *tfdB* genes.** In order to clone the *tfdB*<sub>I</sub> gene into the pMLS7 constitutive expression vector, the FORWB1/REVWB1 primer pair was used to amplify a 1.9-kb DNA sequence corresponding to the complete *tfdB*<sub>I</sub> open reading frame (ORF) (the translation start site is included in the primer and is located immediately downstream of the EcoRI site). This fragment was cloned into pGEM-T Easy (Promega Corp., Madison, WI), and the resulting plasmid was digested with EcoRI to obtain a 2.0-kb fragment, which was inserted, in turn, into the pMLS7 plasmid (20) to produce pMLB1. The ORF4010 chromosomal gene was amplified from *C. necator* JMP134 genomic DNA using the primer pair FORWB3 (5'-GCGAATCTGGA GGAGAAATTCATGAAGACG-3'; includes an EcoRI restriction site [in italics]) and REVWB3 (5'-ATTTTACAGACCCAGACG-3') to obtain a 1.8-kb DNA sequence. This PCR product was cloned into pGEM-T Easy, and the

resulting plasmid, digested with EcoRI to release a 1.9-kb fragment, was then cloned into pMLS7 to produce pMLB3. The identities of the sequences cloned in pMLS7 with either the *tfdB*<sub>I</sub> or ORF4010 gene were confirmed by sequencing of the cloned regions in pMLB1 and pMLB3. For IPTG (isopropyl-β-D-thiogalactopyranoside)-induced expression of the *tfdB* genes in *E. coli* BL21(DE3), the primer pairs FORWB1L (5'-CTAGTCTGGTACCGCAGATGAACAAT CCCTGT-3')/REVWB1 and FORWB2/REVWB2 were used for amplification of the *tfdB*<sub>I</sub> and *tfdB*<sub>II</sub> gene sequences, respectively. Each product was then cloned into pGEM-T Easy to give pGEMTFDB1 and pGEMTFDB2, respectively. The proper orientation of the insert was verified in each case by separate digestion with EcoRI, KpnI, and SacI. Selected plasmid clones were transferred to strain BL21(DE3) by electroporation. Expression of TfdB<sub>I</sub> and TfdB<sub>II</sub> was achieved by growing the resulting strains in LB broth, with 100 μg ml<sup>-1</sup> of IPTG added at the exponential growth phase. Cells were harvested after 4 h of induction, and enzyme extracts were prepared as described below.

**Toxicity tests for chlorophenols.** The MICs of 2,4-DCP and MCP were determined for strain JMP222(pJP4), the *tfdB*<sub>I</sub> mutant, the *tfdB*<sub>II</sub> mutant, and strain JMP222. Each strain was grown overnight in 5 mM fructose, and 10-μl aliquots of these cultures were transferred to fresh medium supplemented with fructose in the presence of different concentrations of 2,4-DCP or MCP (50 to 500 μM at increments of 25 μM). Growth was determined by the increase in OD<sub>600</sub> after 24 h. Degradation of the added 2,4-DCP was also measured for each inoculum. At least three independent determinations were carried out for each reported MIC. The viability of cells growing on fructose and exposed to 2,4-DCP was studied by measuring the number of CFU on solid medium. Cells were grown on fructose, washed, resuspended in fresh medium without a carbon source to an OD<sub>600</sub> of 0.5, and treated or not with 2 mM 2,4-DCP (time zero), and aliquots were taken every hour for up to 8 h. Appropriate dilutions were plated on agar supplemented with phenol as the sole carbon source. The reported CFU values are averages of three independent experiments.

**Enzyme assays.** For enzyme assays, cells were grown in minimal medium containing 2 mM 2,4-D. Strains not able to proliferate on 2,4-D were grown in minimal medium containing 5 mM fructose plus the corresponding antibiotic and were induced at late exponential growth phase with 0.5 mM 2,4-D for 3 h. One hundred milliliters of each culture was harvested at the end of the exponential growth phase (OD<sub>600</sub> = 0.8 on fructose and 0.5 on 2,4-D), centrifuged, washed twice, and resuspended in 5 ml of a solution containing 50 mM Tris-HCl (pH 7.5) for chlorocatechol 1,2-dioxygenase activity measurements and 75 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.6), 1 mM dithiothreitol, 0.1 mM EDTA, 0.01 mM flavin adenine dinucleotide (FAD), and 0.1 mM 2,4-DCP for chlorophenol hydroxylase activity determinations. Cells were disrupted by sonication (Vibracell; Sonics & Materials, Inc., Newton, CT). The soluble protein fraction was obtained after 1 h of centrifugation at 130,000 × g in a Beckman L-80 ultracentrifuge. Cell extracts (0.1 to 5.0 mg of protein per ml) were used without further purification. Chlorocatechol 1,2-dioxygenase assays were performed as previously described (27). For chlorophenol hydroxylase activity, assay mixtures contained 50 mM Na<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.6) and a volume of crude extract corresponding to 1 to 100 μg of protein (0.002 to 0.2 enzyme units). One unit of enzyme activity is the amount of crude extract that forms or consumes 1 μmol of product or substrate, respectively, per min. Protein determinations were performed using the Bradford reagent as previously described (27). Enzyme activities were determined in assays performed with a diode array Hewlett Packard HP 8452-A UV/Vis spectrophotometer. Chlorophenol hydroxylase activity was measured by the consumption of NADH, as indicated by the decrease in absorbance at 340 nm (ε<sub>340</sub> = 6,300 M<sup>-1</sup> cm<sup>-1</sup>). All assays were performed in the presence of 10 μM of FAD. Phenol-independent NADH oxidation was determined for each extract, and this contribution was subtracted from reported measurements. Each assay was started with the addition of 200 μM NADH to the reaction mixture containing fresh crude extract. Reported values are averages of at least three independent experiments. Transformation into the corresponding catechol was confirmed by high-performance liquid chromatography (HPLC) (see below) for every substituted phenol producing an NADH turnover rate higher than 5% of the rate produced by 2,4-DCP (see Table 2). Most chemicals were purchased from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; Baker Chemikalien, Griesheim, Germany; and Merck AG, Darmstadt, Germany. Chlorocatechols were obtained from Promochem, Wesel, Germany.

**Detection of catabolic intermediates.** The accumulation of catabolic intermediates during the degradation of chloroaromatic compounds was determined by HPLC analysis. Cells were grown on 5 mM fructose and induced for at least 2 h with 0.5 mM 2,4-D. Experiments were designed to be performed under these conditions due to the slow growth of the *tfdB*<sub>I</sub> mutant on 2,4-D. Cells were pelleted, washed twice with minimal medium, and resuspended in this medium to an OD<sub>600</sub> of 1.0. 2,4-D or MCPA (1 mM) was added, and the cells were

TABLE 2. Chlorophenol hydroxylase activities against different phenolic substrates

Substrate	Activity of <i>C. necator</i> strain against indicated substrate <sup>a</sup>	
	JMP222(pJP4- <i>tfdB</i> <sub>II</sub> ) (TfdB <sub>I</sub> )	JMP222(pJP4- <i>tfdB</i> <sub>I</sub> ) (TfdB <sub>II</sub> )
2,4-Dichloro	100 (0.31)	100 (0.13)
2-Chloro	11	31
3-Chloro	3	<2
4-Chloro	29	68
2,3-/2,5-Dichloro <sup>b</sup>	15	<5
3,4-Dichloro	<2	<2
2,3,5-/2,4,5-/2,4,6-Trichloro	<5	<2
4-Chloro-2-methyl	46	75
2-Methyl	12	20
3-Methyl	<5	<5
4-Methyl	10	15
2,4-Dimethyl	11	<2
2-Fluoro/4-fluoro	<2	<2
2-Nitro/4-nitro	<2	<2
2,4-Dinitro	4	<2
Phenol	3	<2

<sup>a</sup> Relative values with respect to activity with 2,4-DCP. Absolute values (units of protein<sup>-1</sup>) are given in parenthesis.

<sup>b</sup> Not transformed to chlorocatechols at detectable levels.

incubated at 30°C in an orbital shaker. Two-hundred-microliter aliquots were taken at different times and cleared by centrifugation at 20,817 × g for 10 min, and supernatants were collected for HPLC. To detect catechols produced by chlorophenol hydroxylase, the enzyme reaction was stopped by acidification to pH 2 with HCl. Precipitated proteins were eliminated by centrifugation, and supernatants were collected. Samples (20 μl) from cell-free supernatants or enzyme reactions were injected directly into a Shimadzu LC-10AD liquid chromatograph system equipped with an SC125 Lichrospher 5-μm column (Bischoff, Leonberg, Germany). A methanol-H<sub>2</sub>O (60:40) mixture containing 0.1% (vol/vol) *ortho*-phosphoric acid was used as the solvent, at a flow rate of 1 ml min<sup>-1</sup>. The column effluent was monitored simultaneously at 210, 260, and 270 nm with an SPD-M10A diode array detector. The reported values are representative of at least two independent experiments. Retention volumes for relevant compounds were as follows: 3,5-dichlorocatechol, 1.7 ml; 3-methyl-5-chlorocatechol, 1.5 ml; 4-chlorocatechol, 1.3 ml; 3-chlorocatechol, 1.2 ml; 3-methylcatechol, 1 ml; 4-methylcatechol, 1.1 ml; 2,4-dichloromuconate, 0.7 ml; 2-methyl-4-chloromuconate, 0.6 ml; 2,4-D, 5.6 ml; MCPA, 6.0 ml; 2,4-DCP, 7.8 ml; and MCP, 7.1 ml.

## RESULTS AND DISCUSSION

***C. necator* JMP222 derivatives containing *tfdB*<sub>I</sub> or *tfdB*<sub>II</sub> mutant pJP4 plasmid grow at different rates and extents on chlorophenoxyacetates.** The specific contribution of each *tfdB*-encoded chlorophenol hydroxylase to growth on 2,4-D and MCPA was assessed by independently inactivating each *tfdB* gene in plasmid pJP4. Mutants of the pJP4 plasmid were constructed in *E. coli* BW25113 by allelic replacement of each of the chlorophenol hydroxylase-encoding genes (*tfdB*<sub>I</sub> or *tfdB*<sub>II</sub>) with a kanamycin resistance cassette. Recombination of the antibiotic resistance genes and disruption of each *tfdB* gene were verified by PCR amplification and direct sequencing of the target region (data not shown). The resulting pJP4 derivatives were then transferred by mating to the plasmid-free derivative strain JMP222. Growth of the *tfdB*<sub>I</sub> mutant and the *tfdB*<sub>II</sub> mutant on 2 mM 2,4-D or MCPA as the sole carbon and energy source was compared to the growth of *C. necator* JMP222 harboring the wild-type plasmid. The phenotype of the last strain is essentially equivalent to that of wild-type JMP134 (data not shown). Both *tfdB* mutant strains displayed

slower growth on 2,4-D than strain JMP222(pJP4) (Fig. 2A). A much more pronounced effect was observed for the *tfdB*<sub>I</sub> mutant, which exhibited a growth rate of only 10% that of strain JMP222(pJP4), with a doubling time (*Dt*) of 40 h versus 4.5 h, and reached only 50% of the growth yield in stationary phase. In contrast, the growth rate of the *tfdB*<sub>II</sub> mutant was only slightly retarded (*Dt* = 7.7 h), and no reduction of the final growth yield was observed.

Similar patterns of growth were found when the mutant strains were incubated with MCPA as the carbon source (Fig. 2A). In this case, however, the change in phenotype was more severe, since growth was completely abolished in the *tfdB*<sub>I</sub> mutant, while the *tfdB*<sub>II</sub> mutant grew four times slower (*Dt* = 30.8 h) than strain JMP222(pJP4) (*Dt* = 8 h) and reached only 60% of the final growth yield. Differences between the growth of the *tfdB* mutants and that of strain JMP222(pJP4) could also be appreciated at higher substrate concentrations. As shown in Fig. 2B, the highest growth yield of strain JMP222(pJP4) was obtained at 6 mM 2,4-D (OD<sub>600</sub> = 0.8), while both *tfdB* mutants reached lower yields at the same concentration. Accordingly, higher concentrations of MCPA as a substrate resulted in a strong impact on the growth of the *tfdB*<sub>II</sub> mutant strain, while the *tfdB*<sub>I</sub> mutant did not grow on this compound at any of the concentrations tested (Fig. 2B).

Growth measurements showed a differential contribution of each pJP4-encoded chlorophenol hydroxylase to the degradation of 2,4-D and MCPA. This observation correlates with total chlorophenol hydroxylase activity in cell extracts of the strain grown on fructose and induced with 0.5 mM 2,4-D. While total activity with 2,4-DCP was reduced from 0.38 ± 0.03 units mg of protein<sup>-1</sup> in strain JMP222(pJP4) to 0.13 ± 0.05 units mg of protein<sup>-1</sup> in the *tfdB*<sub>I</sub> mutant, the *tfdB*<sub>II</sub> mutant exhibited only a minor reduction in activity (0.31 ± 0.02 units mg of protein<sup>-1</sup>). Total hydroxylase activity with MCP was reduced to less than half the activity of strain JMP222(pJP4) in the *tfdB*<sub>I</sub> mutant (0.21 ± 0.04 units mg of protein<sup>-1</sup> versus 0.10 ± 0.03 units mg of protein<sup>-1</sup>) and by about one-third in the *tfdB*<sub>II</sub> mutant (0.14 ± 0.02 units mg of protein<sup>-1</sup>). Chlorocatechol dioxygenase activities were compared as a reference and were found to be very similar in these three crude extracts (data not shown).

To ensure that the reduced growth on chlorophenoxyacetates by mutant strains was a result of *tfdB* gene inactivation, a plasmid for constitutive expression of the *tfdB*<sub>I</sub> gene was constructed to complement the *tfdB*<sub>I</sub> mutant strain, which shows the most severe phenotype. The pMLB1 plasmid, containing the *tfdB*<sub>I</sub> gene cloned downstream of the *rps-7* promoter of *Burkholderia cepacia* (20), was introduced into the *tfdB*<sub>I</sub> mutant by mating. The resulting strain, JMP222(pJP4-*tfdB*<sub>I</sub>)(pMLB1), was able to grow on 2,4-D and MCPA, at concentrations up to 4 mM (Fig. 2B), but did not reach the same performance as strain JMP222(pJP4) at higher chlorophenoxyacetate concentrations. The chlorophenol hydroxylase activity in these cell extracts was 0.33 ± 0.03 units mg of protein<sup>-1</sup> with 2,4-DCP as a substrate, which was higher than the activity of the *tfdB*<sub>I</sub> mutant but still lower than the activity of strain JMP222(pJP4). This may be due to a low level of expression of the *tfdB*<sub>I</sub> gene obtained with the exogenous *P*<sub>S7</sub> promoter. Polar effects in the mutants were disregarded, since no catabolic function is encoded downstream of the *tfdB*<sub>I</sub> gene



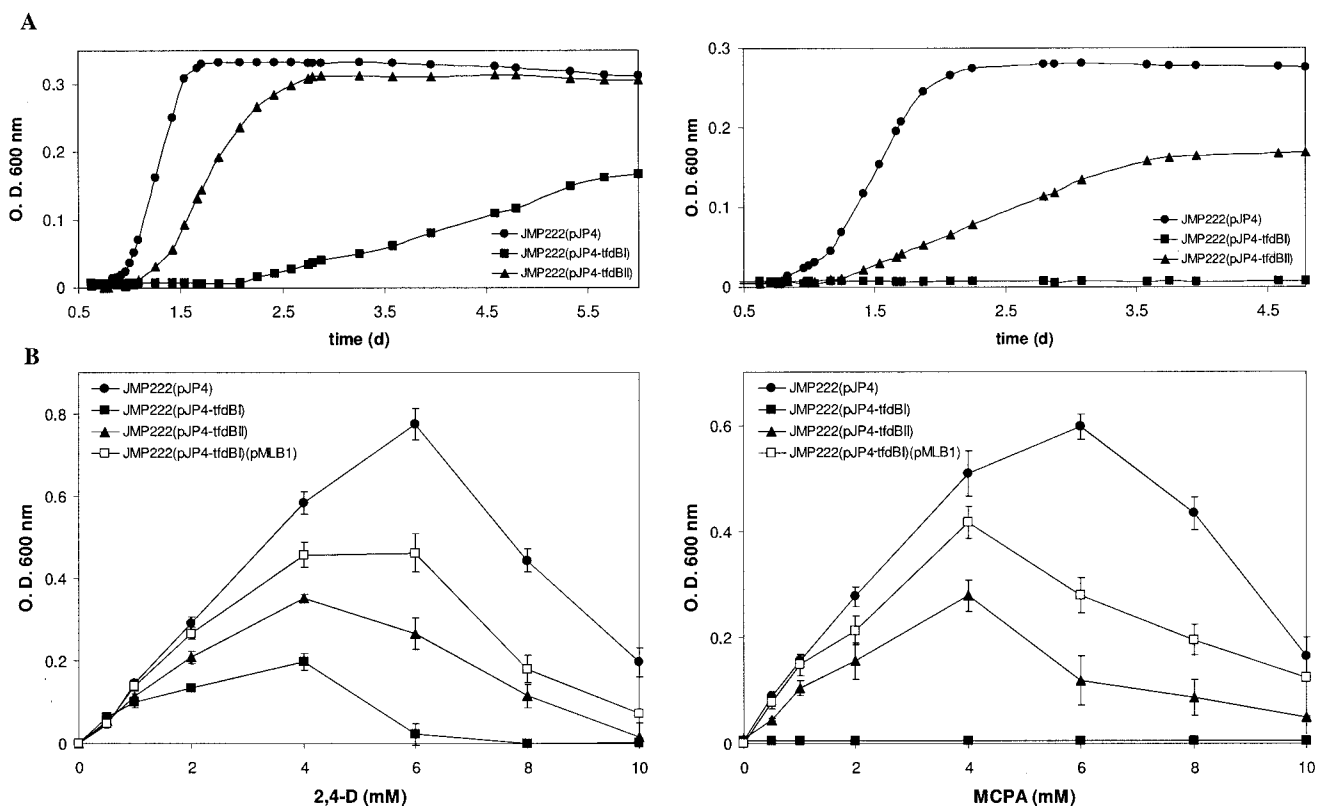


FIG. 2. Growth of *C. necator* *tfdB*<sub>I</sub> and *tfdB*<sub>II</sub> mutants on chlorophenoxyacetates is differentially reduced. (A) Growth curves of strain JMP222(pJP4) (circles), the *tfdB*<sub>I</sub> mutant (squares), and the *tfdB*<sub>II</sub> mutant (triangles) on 2 mM 2,4-D (left) or MCPA (right). Representative OD<sub>600</sub> values are shown for at least three independent experiments. (B) Growth of *C. necator* derivatives with different 2,4-D or MCPA concentrations. Data for the *tfdB*<sub>I</sub> mutant (pMLB1) constitutively expressing *tfdB*<sub>I</sub> are included. OD<sub>600</sub> values were measured at stationary phase for all strains. Values shown are means of three independent experiments.

that could be affected by its inactivation, while the insertion in the *tfdB*<sub>II</sub> gene could only affect the expression of the *tfdK* gene, whose function as a 2,4-D uptake protein gene is only relevant for growth at very low concentrations of this compound (21).

The results showing that TfdB<sub>I</sub> is of major importance for 2,4-DCP or MCP transformation are in agreement with the inactivation analyses performed by Laemmler et al. (19) that indicated that the *tfdB*<sub>II</sub> gene is nonessential. However, we have shown that the *tfdB*<sub>II</sub>-encoded chlorophenol hydroxylase does make a contribution to the degradation of these compounds and that its function seems more relevant for the degradation of MCPA than for that of 2,4-D. In fact, *C. necator* JMP222(pJP4-*tfdB*<sub>I</sub>) is able to grow on 2,4-D, relying solely on the *tfdB*<sub>II</sub>-encoded chlorophenol hydroxylase.

**The chlorophenol hydroxylases encoded by the pJP4 plasmid exhibit narrow substrate specificity profiles.** The differences in activity stated above for the TfdB enzymes with 2,4-DCP and MCP suggest that they have slightly different substrate specificities. Inactivation of each of the chlorophenol hydroxylase-encoding genes in pJP4 permitted the differentiation of each enzyme activity from its counterpart in a crude extract and allowed further investigation of the substrate preference of each of the TfdB enzymes. The results of the assays with crude extracts are summarized in Table 2. Both pJP4-encoded chlorophenol hydroxylases exhibited a very narrow

specificity profile, being able to transform almost exclusively chlorinated phenols as long as they were substituted in the 2-, 4-, or 2,4- positions and allowing slow transformation of 2- and 4-methylphenol. The detection of catechols produced in the enzymatic assays by HPLC (not shown) allowed discrimination of true substrates, such as 2- and/or 4-chlorosubstituted phenols, from compounds such as 2,3- and 2,5-dichlorophenol, which elicited NADH oxidation but were not transformed to detectable levels. This has also been observed for other chlorophenol hydroxylases (2, 23) and described in detail for salicylate hydroxylase (36).

Apparent kinetic constants for 2,4-DCP calculated from double-reciprocal plots of activity versus substrate concentration were determined for the TfdB<sub>I</sub> and TfdB<sub>II</sub> hydroxylases by cloning and expression of the *tfdB*<sub>I</sub> and *tfdB*<sub>II</sub> genes in *E. coli*, using the pGEM-T Easy T7 promoter. Unfortunately, expression of the *tfdB* genes in *E. coli* was not high enough to allow quantification of each chlorophenol hydroxylase and a direct comparison of their  $V_{max}$  values. The apparent  $V_{max}$  of TfdB<sub>I</sub> under these conditions was 0.09 units mg of protein<sup>-1</sup> (calculated with total protein), which was five times higher than the value obtained for TfdB<sub>II</sub> (0.02 units mg of protein<sup>-1</sup>). Assuming that both *tfdB* genes are similarly expressed from the IPTG-inducible T7 promoter in pGEM-T Easy, this difference would suggest that TfdB<sub>I</sub> is indeed more active than TfdB<sub>II</sub>. The apparent  $K_m$  value determined for TfdB<sub>I</sub> was 11  $\mu$ M,

which is higher than the  $K_m$  values reported for the TfdB proteins from *B. cepacia* 2A and *Deftuvibacter lusatiensis* S1 (2, 24), which range from 3 to 4  $\mu\text{M}$ , but is similar to the  $K_m$  value of 14  $\mu\text{M}$  reported for a hydroxylase purified from *B. cepacia* CSV90, another 2,4-D-degrading strain (31). The apparent  $K_m$  for the TfdB<sub>II</sub> enzyme with 2,4-DCP was 2.5  $\mu\text{M}$ , which is similar to the  $K_m$  values of the reported hydroxylases and also similar to the  $K_m$  previously described by Liu and Chapman for a strain JMP134 hydroxylase (23). The apparent kinetic constants described here suggest that the differences between the TfdB<sub>I</sub> and TfdB<sub>II</sub> hydroxylases are not merely a consequence of their differential expression but that the TfdB<sub>I</sub> enzyme is indeed more active than its counterpart, although TfdB<sub>II</sub> can be active at lower substrate concentrations.

A protein with chlorophenol hydroxylase activity from *C. necator* JMP134 was purified for an early report by Liu and Chapman (23) that suggested that dichlorophenol hydroxylase is a heterotetramer. However, using a different purification scheme, it was later shown that dichlorophenol hydroxylase is a homotetramer (6), and it was suggested that its identification as a heterotetramer was due to the presence of a copurified protein. No protein sequence data were provided by Liu and Chapman, and the amino acid composition of the copurified protein did not fit with either TfdB<sub>I</sub> or TfdB<sub>II</sub> sequence information or a mixture of both. Two other dichlorophenol hydroxylases have thus far been purified and characterized concerning substrate specificity, namely, the enzyme of *B. cepacia* 2a (formerly *Acinetobacter*) (2) and the enzyme from strain S1, a member of the  $\alpha$ -2 subgroup of the *Proteobacteria*, recently identified as *Deftuvibacter lusatiensis* (7, 24). Both enzymes have also been found to exhibit very narrow substrate preferences, being highly active with 2,4-dichloro- and 4-chloro-2-methylphenol but not able to transform phenol, and activity with 3-chlorophenol was either low or absent, in contrast to what is observed for multicomponent alkylphenol hydroxylases (13). Because TfdA, performing the first step in phenoxyalkanoic herbicide degradation, is an enzyme with relaxed substrate specificity (29), the strict substrate preference of TfdB enzymes may be the limiting factor in the range of chlorophenoxyacetates that can be degraded by *C. necator* JMP134. For the degradation of nonchlorinated substrates such as 2- and 4-methylphenoxyacetate, strain JMP134 can combine the use of TfdA with the recruitment of chromosomally encoded multicomponent phenol hydroxylases that degrade the corresponding phenols, probably via a *meta* cleavage pathway (29).

Although it is certain that the transformation of chlorophenols in *C. necator* JMP134 is achieved by the action of pJP4-encoded enzymes, a role of a chromosomally encoded chlorophenol hydroxylase activity in 2,4-DCP degradation has been suggested (15). This activity was only evident when the strain was grown on 2-methylphenol as a carbon source. *C. necator* carries a putative protein (ORF4010) with significant amino acid identity to either of the two plasmid-encoded chlorophenol hydroxylases (43% amino acid identity to TfdB<sub>I</sub> and 46% identity to TfdB<sub>II</sub>) and other phenol hydroxylases, which could indicate an enzyme capable of hydroxylating 2,4-DCP/MCP. In order to determine if ORF4010 codes for a functional chlorophenol hydroxylase, the corresponding DNA segment was cloned immediately downstream of the S7 promoter of pMLS7 to produce pMLB3. This plasmid was introduced into strain

JMP222, and chlorophenol hydroxylase activity was measured with 2,4-DCP, MCP, and other substituted phenols in crude extracts of the strain growing on fructose. No activity against 2,4-DCP was found in strain JMP222(pMLB3), but some activity was detected with 2-methylphenol ( $0.05 \pm 0.01$  units mg of protein<sup>-1</sup>). The protein encoded by ORF4010 was more active with phenol ( $0.25 \pm 0.02$  units mg of protein<sup>-1</sup>) and 3-methylphenol ( $0.23 \pm 0.02$  units mg of protein<sup>-1</sup>), which contrasted with the negligible activities of the TfdB<sub>I</sub> and TfdB<sub>II</sub> enzymes for these substrates (Table 2). No phenol hydroxylase activity was found in crude extracts of strain JMP222 grown on fructose. These results indicate that the contribution of a chromosomally encoded putative chlorophenol hydroxylase to the degradation of phenoxyalkanoates seems to be negligible. Thus, despite the amino acid identity of the chromosomally encoded hydroxylase with the TfdB proteins, it exhibits a completely different substrate specificity which places it near other monocomponent flavin phenol hydroxylases, such as the PheA protein from *Pseudomonas* sp. strain 1001 (14), that can only transform phenol and 3-methylphenol (25).

**Inactivation of the *tfdB<sub>I</sub>* or *tfdB<sub>II</sub>* gene results in the accumulation of toxic levels of chlorophenol during chlorophenoxyacetate degradation.** A discrete reduction in chlorophenol hydroxylase activity produced notorious changes in the ability of *C. necator* JMP134 derivatives to grow on chlorophenoxyacetates. The extent of reduction in growth of the *tfdB* mutants suggests that it was not merely due to kinetic pathway limitations but was probably raised from the toxic effects of both 2,4-DCP and MCP intermediates. Toxic effects of chlorophenols are mainly due to an increase in membrane fluidity due to their hydrophobicities and to their possible role as uncouplers of the electron transport chain (33, 35). Thus, relatively small changes in the levels of enzymes such as chlorophenol hydroxylase which are capable of transforming toxic intermediates can be responsible for substantial effects on growth rates and yields. In order to test if a higher level of 2,4-DCP accumulation during 2,4-D degradation could be detected in the mutant strains than in strain JMP222(pJP4), the presence of catabolic intermediates was analyzed in resting cells. As shown in Fig. 3A and D, strain JMP222(pJP4) accumulated low levels of the corresponding phenols and muconates when exposed to 2,4-D or MCPA. In contrast, 2,4-DCP was rapidly accumulated to a concentration of about 0.4 mM in the *tfdB<sub>I</sub>* mutant when 1 mM 2,4-D was given as the initial substrate, while 2,4-D degradation was almost completely stopped (to 7% of the initial rate) (Fig. 3B). A smaller chlorophenol accumulation (about 0.1 mM) was observed when the *tfdB<sub>II</sub>* mutant strain was incubated under the same conditions (Fig. 3C). This was associated with a reduction in the 2,4-D degradation rate to 41% of the initial value, while strain JMP222(pJP4) maintained a constant degradation rate throughout the same period (Fig. 3A).

A similar scenario was observed for MCPA degradation (Fig. 3E and F), where the mutants also accumulated MCP to higher concentrations than did strain JMP222(pJP4), with 0.35 and 0.2 mM MCP in the *tfdB<sub>I</sub>* and *tfdB<sub>II</sub>* mutants, respectively, compared with 0.05 mM in the reference strain. When 0.4 mM 2,4-DCP was added directly to strain JMP222(pJP4) cells during 2,4-D degradation, a reduction in the rate of 2,4-D degradation was observed (to 14% of the initial rate), mimicking the phenotype of the *tfdB<sub>I</sub>* mutant. As the 2,4-DCP concentration

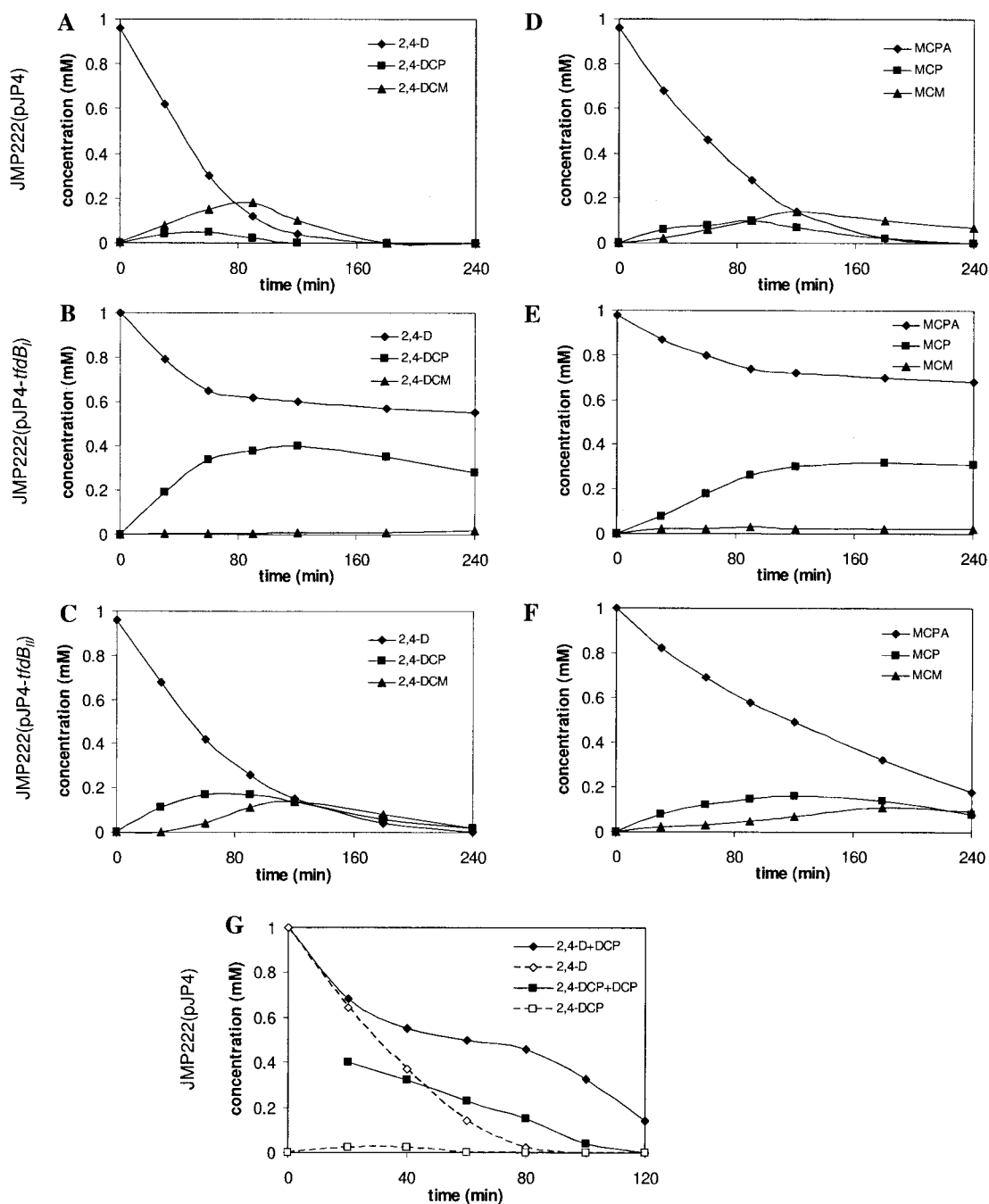


FIG. 3. Intermediate accumulation during chlorophenoxyacetate degradation by *C. necator* JMP134 derivatives. Chlorinated intermediates were detected by HPLC, using samples of supernatants after incubation of preinduced cell suspensions ( $OD_{600}$ , 1.6) of strain JMP222(pJP4), the *tfdB<sub>1</sub>* mutant, and the *tfdB<sub>11</sub>* mutant with 1 mM 2,4-D (A to C) or MCPA (D to F). (G) Degradation of 2,4-D by strain JMP222(pJP4), as in panel A. 2,4-DCP (0.5 mM) was added to the cells after 20 min of incubation (+DCP). The discontinuous lines represent 2,4-D/2,4-DCP degradation in the absence of externally added 2,4-DCP. Symbols: diamonds, 2,4-D/MCPA; squares, 2,4-DCP/MCP; triangles, 2,4-dichloromuconate (2,4-DCM)/2-methyl-4-chloromuconate (MCM).

decreased, the rate of 2,4-D degradation increased again, to 82% of the initial value (Fig. 3G). These observations confirmed that reductions in chlorophenol hydroxylase activity result in large increases in the concentration of chlorophenol, which can severely affect the growth of *C. necator* mutant strains on 2,4-D or MCPA.

Since chlorophenols are accumulated during the degradation of chlorophenoxyacetates, the effect of direct exposure to 2,4-DCP on cell viability was compared for the strain JMP222(pJP4) and the mutant strains. Cells grown on fructose were washed and resuspended in fresh medium to an  $OD_{600}$  of 0.25 for treatment with different 2,4-DCP concentrations.

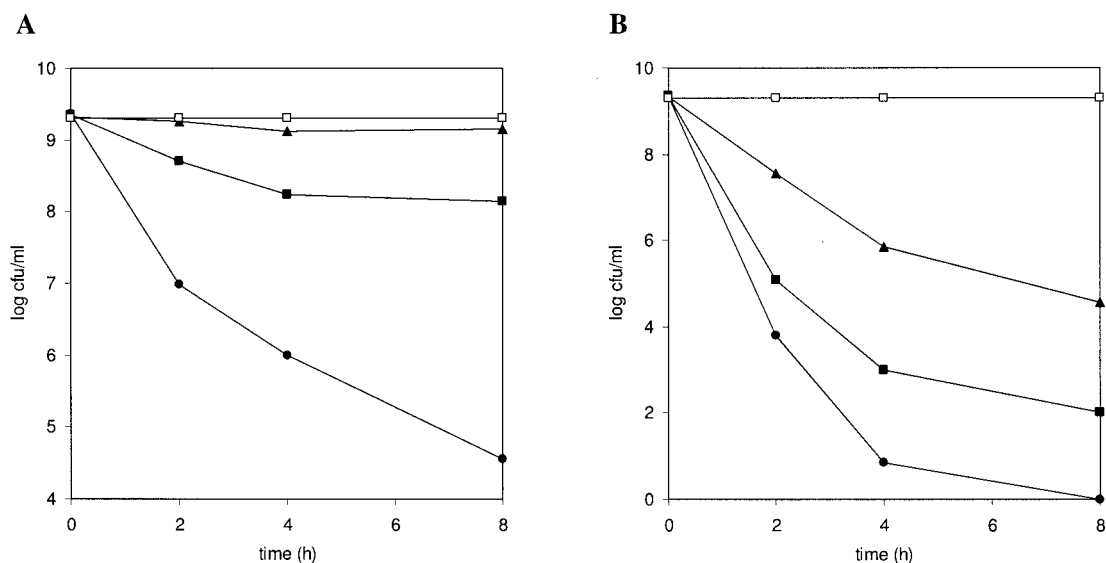


FIG. 4. Effect of 2,4-DCP on cell viability of *C. necator* JMP134 derivatives. Cell suspensions of strain JMP222(pJP4) (triangles), the *tfdB*<sub>1</sub> mutant (circles), or the *tfdB*<sub>II</sub> mutant (squares) previously grown in 5 mM fructose were exposed to 1 mM (left) or 2 mM (right) 2,4-DCP, and samples were analyzed to determine the CFU at different times. Data for nonexposed *tfdB*<sub>II</sub> mutant cells (open squares) are included in each panel as a control of viability during the time of the assay. The values are averages based on two replicates.

Compared to that of strain JMP222(pJP4), survival of the *tfdB*<sub>1</sub> mutant was reduced by 2 and 3 orders of magnitude after 4 h and 6 h of 1 mM 2,4-DCP treatment, respectively (Fig. 4A). Survival of the *tfdB*<sub>II</sub> mutant was 1 order of magnitude lower than that of strain JMP222(pJP4) in the 8-h experiment. Treatment with 2 mM 2,4-DCP resulted in complete killing of the *tfdB*<sub>1</sub> mutant in 8 h, while the viability of the *tfdB*<sub>II</sub> mutant was decreased by 6 orders of magnitude (Fig. 4B). The viability of strain JMP222(pJP4) was reduced by 4 orders of magnitude at the end of the incubation period. By this time, strain JMP222(pJP4) degraded 15% of the initial 2,4-DCP, while the *tfdB*<sub>1</sub> mutant did not degrade any detectable amount of the compound. Therefore, chlorophenol disappearance alone does not explain the increased survival of strain JMP222(pJP4), but the metabolism of chlorophenol during this interval may allow energy-consuming adaptation mechanisms such as efflux pumps to function more efficiently (35). Despite these differences in survival, it is important that only a slight reduction in the viability of all strains tested was found when 0.5 mM 2,4-DCP was added to the cell suspensions (data not shown). The level of 2,4-DCP accumulated during 2,4-D degradation by the *tfdB*<sub>1</sub> mutant (Fig. 3B) is therefore unlikely to produce immediate cell death but is toxic enough to prevent 2,4-D degradation.

In order to test if chlorophenol hydroxylase activity is relevant for the protection of *C. necator* directly exposed to chlorophenols, the MICs of 2,4-DCP and MCP for strain JMP222(pJP4) and *tfdB* mutant strains were compared. Strain JMP222(pJP4) was able to grow at concentrations of <0.4 or <0.3 mM of 2,4-DCP or MCP. The *tfdB*<sub>1</sub> mutant strain could only grow up to a concentration of 0.2 or 0.125 mM of 2,4-DCP or MCP, which was closer to the MICs for strain JMP222, at 0.075 mM and 0.05 mM, respectively. The elimination of *tfdB*<sub>II</sub> from pJP4 also reduced the MICs of chlorophenols, permitting growth of the mutant strain only at 2,4-DCP concentrations

lower than 0.325 mM and resulting in a stronger effect on growth in MCP (MIC of 0.2 mM). These differences in the MICs between strain JMP222(pJP4) and the mutant strains can be explained because growth inhibition by 2,4-DCP is more effective in a strain that cannot degrade the compound. A strain JMP222(pJP4) inoculum completely degrades 2,4-DCP after 24 h of incubation in fructose plus 0.4 mM 2,4-DCP. In contrast, the *tfdB*<sub>1</sub> mutant can only degrade 16% of the total initial 2,4-DCP under the same incubation conditions. The ability to degrade 2,4-DCP thus seems intimately related to resistance to chlorophenol toxicity, explaining the differences in the above reported MICs.

**Imbalance between production and degradation of chlorophenol affects growth on chlorophenoxyacetate by *C. necator* and *B. xenovorans*.** The results described so far show that chlorophenol turnover is a critical factor for chlorophenoxyacetate degradation by *C. necator* JMP134 due to the toxicity of this intermediate. If this is true, then a perturbation of this balance by an increase in the chlorophenol-producing enzyme TfdA should also have a negative effect on growth with these substrates. In order to test this possibility, the plasmid pBTFDA, harboring a constitutively expressed version of the *tfdA* gene, was introduced into strain JMP222(pJP4). Strain JMP222(pJP4)(pBTFDA) displayed reduced growth on 2,4-D compared with strain JMP222(pJP4), and this reduction became increasingly pronounced at higher 2,4-D concentrations (data not shown). Conversely, the introduction of plasmid pMLB1, harboring *tfdB*<sub>1</sub>, into strain JMP222(pJP4) resulted in a strain showing improved growth on the same substrate with respect to strain JMP222(pJP4) (data not shown). The total chlorophenol hydroxylase activity against 2,4-DCP measured in strain JMP222(pJP4)(pMLB1) reached up to  $0.49 \pm 0.04$  units mg of protein<sup>-1</sup>, compared to  $0.38 \pm 0.03$  units mg of protein<sup>-1</sup> in strain JMP222(pJP4). These results imply that a moderate increase in chlorophenol hydroxylase activity can improve the



efficiency of 2,4-D degradation in strain JMP222(pJP4). However, increased chlorophenol degradation can also result in intoxication of the strain because of chlorocatechol accumulation (28). This intermediate is highly toxic for bacteria, possibly due to oxidative damage of membranes and proteins (34). Nevertheless, the accumulation of chlorocatechols during 2,4-D degradation was negligible, even in strains harboring a constitutively expressed version of TfdB<sub>I</sub> (data not shown). This was probably due to the high activities of TfdC chlorocatechol dioxygenases towards 3,5-DCC, which is the best substrate for these enzymes (27).

To determine if chlorophenol turnover has a similar effect on a different catabolic strain, the pJP4 plasmid derivatives were transferred to *B. xenovorans* LB400. The resulting strain, *B. xenovorans* LB400(pJP4), had a growth yield on 2,4-D of about 50% that of strain JMP222(pJP4). The total chlorophenol hydroxylase activity for 2,4-DCP was  $0.29 \pm 0.02$  units mg of protein<sup>-1</sup>, indicating that expression of the *tfdB* genes was not as high as that in *C. necator*, although TfdC activity reached similar levels (data not shown). When the pMLB1 plasmid harboring the constitutively expressed version of the *tfdB<sub>I</sub>* gene was introduced into strain LB400(pJP4), an increase in 2,4-DCP chlorophenol hydroxylase activity to twice (230%) the activity of strain LB400(pJP4) was produced. This resulted in a significant improvement (ranging from 1.5 to 2 times) in growth yields of strain LB400(pJP4)(pMLB1) in a wide range of 2,4-D concentrations (data not shown). Constitutive expression of the *tfdB<sub>I</sub>* gene in *B. xenovorans* LB400(pJP4) enabled the strain to grow faster on 2,4-D than strain LB400(pJP4), with a *D<sub>t</sub>* of 3.2 h versus 5.9 h. On the other hand, the introduction of the *tfdB<sub>I</sub>* or *tfdB<sub>II</sub>* mutant pJP4 plasmid into strain LB400 resulted in a strain unable to grow on MCPA at all concentrations tested (data not shown), whereas only strain LB400(pJP4-*tfdB<sub>II</sub>*) could grow on 2,4-D, at concentrations below 4 mM. Increased expression of TfdA in strain LB400(pJP4)(pBTFDA) led to a reduction of growth on 2,4-D, which was more pronounced at higher 2,4-D concentrations. The results clearly indicate that avoiding chlorophenol accumulation is an important advantage for productive growth and for the degradation of chlorophenoxyacetates in  $\beta$ -proteobacteria. Intoxication due to unbalanced turnover of chlorophenols produces an immediate effect on chlorophenoxyacetate degradation and should be considered an important factor determining the extent and rate of growth of chlorophenoxyacetate-degrading strains on these xenobiotic compounds. Interestingly, toxicity by an imbalance between the production and degradation of initial intermediates in aromatic metabolism has also been reported for 3-chlorobenzoate (28), hydroxycinnamate (26), and protocatechuate (9).

**TfdB enzyme sequences in pJP4 belong to different FAD monocomponent hydroxylase groups.** The results reported here have significant implications concerning the evolution of chloroaromatic compound metabolism pathways and also on the construction of chloroaromatic compound-degrading bacterial strains. Avoiding chlorophenol toxicity can be a selective pressure for an increased dosage of chlorophenol hydroxylase genes, produced either by gene duplication or by recruitment of different genes. A comparison of the two pJP4-encoded enzymes with other reported FAD monocomponent hydroxylases indicated that they may have been recruited from differ-

ent pathways. The TfdB<sub>II</sub> protein sequence clusters together with homologues usually observed in isolates for which 2,4-D catabolic gene clusters have been analyzed (dendrogram not shown). Thus, TfdB<sub>II</sub> of strain JMP134 is highly related to TfdB enzymes from *B. cepacia* 2a, *Delftia acidovorans* P4a, *Achromobacter xylooxidans* subsp. *denitrificans* EST4002, and *Variovorax paradoxus* TV1, sharing >86% amino acid sequence identity with them, while the TfdB<sub>I</sub> enzyme shares only approximately 60% amino acid sequence identity with members of this group. This supports the notion that the *tfdB<sub>II</sub>* gene is derived from a specialized 2,4-D degradation cluster. However, the TfdB<sub>II</sub> enzyme in *C. necator* JMP134 exhibits a low activity that is barely enough to allow growth of the strain on 2,4-D. This could indicate that *tfdB<sub>II</sub>* codes for a chlorophenol hydroxylase with intrinsically low activity or merely that this gene reaches a low level of expression in strain JMP134. This last possibility seems unlikely, since expression analyses have reported similar levels of transcription for the *tfdB<sub>I</sub>* and *tfdB<sub>II</sub>* genes when *C. necator* JMP134 was grown in continuous culture on fructose and 2,4-D was added as an inducer (22). In addition, transcriptomic preliminary data indicate that *tfdB<sub>II</sub>* transcripts are three to four times more abundant than *tfdB<sub>I</sub>* transcripts in cultures of *C. necator* JMP134 exposed to 2,4-D (D. Pérez-Pantoja, J. E. González-Pastor, D. H. Pieper, V. de Lorenzo, and B. González, unpublished data).

The TfdB<sub>I</sub> sequence belongs to a different group of FAD-dependent hydroxylases (data not shown). The absence of close relatives of *tfdB<sub>I</sub>* in all sequenced 2,4-D-degrading clusters suggests that this gene does not necessarily belong to a cluster specialized for 2,4-D degradation. However, partial amino acid sequences (covering approximately 12% of the protein) indicated that enzymes highly similar to TfdB<sub>I</sub> are present in 2,4-D-degrading *Ralstonia* and *Burkholderia mallei* strains (38), and a similar enzyme (DcpA) with 96% amino acid sequence identity with TfdB<sub>I</sub> has been reported for a *Pseudomonas* isolate (AY496436). The difference in chlorophenol hydroxylase activity of the TfdB<sub>I</sub> enzyme with respect to its TfdB<sub>II</sub> counterpart suggests that the function of TfdB<sub>I</sub> is not restricted to chlorophenoxyacetate degradation but that the enzyme also takes part in direct degradation of chlorophenols. This idea is supported by the significant identity of the TfdB<sub>I</sub> protein to ClpB (73% amino acid identity), a chlorophenol hydroxylase involved in 2,4-DCP degradation by *D. lusatiensis*, a strain that does not grow on 2,4-D (7). In fact, the *tfdTC<sub>I</sub>D<sub>I</sub>E<sub>I</sub>F<sub>I</sub>B<sub>I</sub>* gene cluster organization in pJP4 is different from the *tfdRCEBKA* organization of the 2,4-D-degrading gene clusters reported for strains where the *tfd* gene organization is known, i.e., *V. paradoxus* TV1(pTV1), *B. cepacia* 2a, *A. xylooxidans* subsp. *denitrificans* EST4002, and *D. acidovorans* P4a (12).

The *tfdA* gene in strain JMP134 is separated by about 4 and 15 kb from *tfd<sub>II</sub>* and *tfd<sub>I</sub>* gene clusters, respectively, encoding enzymes for chlorophenol and chlorocatechol degradation, and it apparently forms a separate transcriptional unit (Fig. 1C). This may imply a different regulation for the *tfdA* gene and the rest of the *tfd* gene modules, making coordination between 2,4-DCP production and degradation difficult. Expression of these activities can be more balanced in *tfdRCEBKA* gene clusters, eliminating the need for an extra-high-performance chlorophenol hydroxylase like TfdB<sub>I</sub>. Accord-

ingly, proteomic evidence obtained by our group shows that a larger amount of TfdA is produced with respect to both pJP4-encoded TfdB proteins when *C. necator* JMP134 is grown on 2,4-D (F. Flores-Aceituno, D. H. Pieper, and B. González, unpublished data).

Chlorophenols are highly toxic pollutants that can also be catabolic intermediates, as with phenoxyalkanoic herbicides. Due to this toxicity, the performance of catabolic bacteria is not dependent only on the presence of the characteristic enzymatic steps. This work clearly indicates that additional studies are required concerning the role in detoxification of reported chlorophenol and, possibly, phenol hydroxylases, focusing on their substrate specificities, activities, and regulation of gene expression.

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#### REFERENCES

- Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl (ed.). 1992. Short protocols in molecular biology, 2nd ed. Greene Publishing Associates, New York, N.Y.
- Beadle, C. A., and A. R. W. Smith. 1982. The purification and properties of 2,4-dichlorophenol hydroxylase from a strain of *Acinetobacter* species. *Eur. J. Biochem.* **123**:323–332.
- Clément, P., V. Matus, L. Cárdenas, and B. González. 1995. Degradation of trichlorophenols by *Alcaligenes eutrophus* JMP134. *FEMS Microbiol. Lett.* **127**:51–55.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Don, R. H., and J. M. Pemberton. 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *J. Bacteriol.* **145**:681–686.
- Farhana, L., and P. B. New. 1997. The 2,4-dichlorophenol hydroxylase of *Alcaligenes eutrophus* JMP134 is a homotetramer. *Can. J. Microbiol.* **43**:202–205.
- Fritsche, K., G. Auling, J. R. Andreessen, and U. Lechner. 1999. *Deftuvibacter lusatiae* gen. nov., sp. nov., a new chlorophenol-degrading member of the alpha-2 subgroup of proteobacteria. *Syst. Appl. Microbiol.* **22**:197–204.
- Fukumori, F., and R. P. Hausinger. 1993. *Alcaligenes eutrophus* JMP134 “2,4-dichlorophenoxyacetate monooxygenase” is an  $\alpha$ -ketoglutarate-dependent dioxygenase. *J. Bacteriol.* **175**:2083–2086.
- Gerischer, U., and L. N. Ornston. 1995. Spontaneous mutations in *pcaH* and *-G*, structural genes for protocatechuate 3,4-dioxygenase in *Acinetobacter calcoaceticus*. *J. Bacteriol.* **177**:1336–1347.
- Gobel, M., K. Kassel-Cati, E. Schmidt, and W. Reineke. 2002. Degradation of aromatics and chloroaromatics by *Pseudomonas* sp. strain B13: cloning, characterization, and analysis of sequences encoding 3-oxoadipate:succinyl-coenzyme A (CoA) transferase and 3-oxoadipyl-CoA thiolase. *J. Bacteriol.* **184**:216–223.
- Herrero, M., V. de Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic selection markers for cloning and stable chromosomal insertion of foreign DNA in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
- Hoffmann, D., S. Kleinstuber, R. H. Muller, and W. Babel. 2003. A transposon encoding the complete 2,4-dichlorophenoxyacetic acid degradation pathway in the alkalitolerant strain *Delftia acidovorans* P4a. *Microbiology* **149**:2545–2556.
- Jeong, J. J., J. H. Kim, C. K. Kim, I. Hwang, and K. Lee. 2003. 3- and 4-alkylphenol degradation pathway in *Pseudomonas* sp. strain KL28: genetic organization of the *lap* gene cluster and substrate specificities of phenol hydroxylase and catechol 2,3-dioxygenase. *Microbiology* **149**:3265–3277.
- Kivisaar, M., R. Horak, L. Kasak, A. Heinaru, and J. Habicht. 1990. Selection of independent plasmids determining phenol degradation in *Pseudomonas putida* and the cloning and expression of genes encoding phenol monooxygenase and catechol 1,2-dioxygenase. *Plasmid* **24**:25–36.
- Koh, S. C., M. V. McCullar, and D. D. Focht. 1997. Biodegradation of 2,4-dichlorophenol through a distal meta-fission pathway. *Appl. Environ. Microbiol.* **58**:1276–1283.
- Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson. 1994. pBBR1MCS: a broad-host-range cloning vector. *BioTechniques* **16**:800–802.
- Kröckel, L., and D. D. Focht. 1987. Construction of chlorobenzene-utilizing recombinants by progenitive manifestation of a rare event. *Appl. Environ. Microbiol.* **53**:2470–2475.
- Laemml, C. M., J. H. Leveau, A. J. B. Zehnder, and J. R. van der Meer. 2000. Characterization of a second *tfd* gene cluster for chlorophenol and chlorocatechol metabolism on plasmid pJP4 in *Ralstonia eutropha* JMP134 (pJP4). *J. Bacteriol.* **182**:4165–4172.
- Laemml, C. M., C. Werlen, and J. R. van der Meer. 2004. Mutation analysis of the different *tfd* genes for degradation of chloroaromatic compounds in *Ralstonia eutropha* JMP134. *Arch. Microbiol.* **181**:112–121.
- Lefebvre, M. D., and M. Valvano. 2002. Construction and evaluation of plasmid vectors optimized for constitutive and regulated gene expression in *Burkholderia cepacia* complex isolates. *Appl. Environ. Microbiol.* **68**:5956–5964.
- Leveau, J. H., A. J. B. Zehnder, and J. R. van der Meer. 1998. The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP134 (pJP4). *J. Bacteriol.* **180**:2237–2243.
- Leveau, J. H., F. König, H. Fuchslin, C. Werlen, and J. R. van der Meer. 1999. Dynamics of multigene expression during catabolic adaptation of *Ralstonia eutropha* JMP134 (pJP4) to the herbicide 2,4-dichlorophenoxyacetate. *Mol. Microbiol.* **33**:396–406.
- Liu, T., and P. J. Chapman. 1984. Purification and properties of a plasmid-encoded 2,4-dichlorophenol hydroxylase. *FEBS Lett.* **173**:314–318.
- Makdessi, K., and U. Lechner. 1997. Purification and characterization of 2,4-dichlorophenol hydroxylase isolated from a bacterium of the alpha-2 subgroup of the Proteobacteria. *FEMS Microbiol. Lett.* **157**:95–101.
- Nurk, A., L. Kasak, and M. Kivisaar. 1991. Sequence of the gene (*pheA*) encoding phenol monooxygenase from *Pseudomonas* sp. EST1001: expression in *Escherichia coli* and *Pseudomonas putida*. *Gene* **102**:13–18.
- Parke, D., and L. N. Ornston. 2004. Toxicity caused by hydroxycinnamoyl-coenzyme A thioester accumulation in mutants of *Acinetobacter* sp. strain ADP1. *Appl. Environ. Microbiol.* **70**:2974–2983.
- Pérez-Pantoja, D., L. Guzmán, M. Manzano, D. H. Pieper, and B. González. 2000. Role of *tfdC<sub>D</sub>E<sub>1</sub>F<sub>1</sub>* and *tfdD<sub>1</sub>C<sub>11</sub>E<sub>11</sub>F<sub>11</sub>* gene modules in catabolism of 3-chlorobenzoate by *Ralstonia eutropha* JMP134 (pJP4). *Appl. Environ. Microbiol.* **66**:1602–1608.
- Pérez-Pantoja, D., T. Ledger, D. H. Pieper, and B. González. 2003. Efficient turnover of chlorocatechols is essential for growth of *Ralstonia eutropha* JMP134 (pJP4) in 3-chlorobenzoic acid. *J. Bacteriol.* **185**:1534–1542.
- Pieper, D. H., W. Reineke, K. H. Engesser, and H.-J. Knackmuss. 1988. Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. *Arch. Microbiol.* **150**:95–102.
- Plumeier, I., D. Pérez-Pantoja, S. Heim, B. González, and D. H. Pieper. 2002. The importance of different *tfd* genes for the degradation of chloroaromatics by *Ralstonia eutropha* JMP134. *J. Bacteriol.* **184**:4054–4064.
- Radjendirane, V., M. A. Bhat, and C. S. Vaidyanathan. 1991. Affinity purification and characterization of 2,4-dichlorophenol hydroxylase from *Pseudomonas cepacia*. *Arch. Biochem. Biophys.* **288**:169–176.
- Schlömann, M., E. Schmidt, and H.-J. Knackmuss. 1990. Different types of diene lactone hydrolase in 4-fluorobenzoate-utilizing bacteria. *J. Bacteriol.* **172**:5112–5118.
- Schüürmann, G., H. Segner, and K. Jung. 1997. Multivariate mode of action analysis of acute toxicity of phenols. *Aquat. Toxicol.* **38**:277–296.
- Schweigert, N., A. J. B. Zehnder, and R. I. L. Eggen. 2001. Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environ. Microbiol.* **3**:81–91.
- Sikkema, J., J. A. M. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**:201–222.
- Takemori, S., M. Kakamura, K. Suzuki, and M. Katagiri. 1970. The kinetics of salicylate hydroxylase reaction. *FEBS Lett.* **6**:305–308.
- Trefault, N., R. De la Iglesia, A. M. Molina, M. Manzano, T. Ledger, D. Pérez-Pantoja, M. A. Sánchez, M. Stuardo, and B. González. 2004. Genetic organization of the catabolic plasmid pJP4 from *Ralstonia eutropha* JMP134 (pJP4) reveals mechanisms of adaptation to chloroaromatic pollutants and evolution of specialized chloroaromatic degradation pathways. *Environ. Microbiol.* **6**:655–668.
- Vallaes, T., L. Courde, C. McGowan, A. D. Wright, and R. R. Fulthorpe. 1999. Phylogenetic analyses indicate independent recruitment of diverse gene cassettes during assemblage of the 2,4-D catabolic pathway. *FEMS Microbiol. Ecol.* **28**:373–382.