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Role of $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$ Gene Modules in Catabolism of 3-Chlorobenzoate by *Ralstonia eutropha* JMP134(pJP4)

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The enzymes chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase allow *Ralstonia eutropha* JMP134(pJP4) to degrade chlorocatechols formed during growth in 2,4-dichlorophenoxyacetate or 3-chlorobenzoate (3-CB). There are two gene modules located in plasmid pJP4, $tfdC_ID_IE_IF_I$ (module I) and $tfdD_{II}C_{II}E_{II}F_{II}$ (module II), putatively encoding these enzymes. To assess the role of both *tfd* modules in the degradation of chloroaromatics, each module was cloned into the medium-copy-number plasmid vector pBBR1MCS-2 under the control of the *tfd*R regulatory gene. These constructs were introduced into *R. eutropha* JMP222 (a JMP134 derivative lacking pJP4) and *Pseudomonas putida* KT2442, two strains able to transform 3-CB into chlorocatechols. Specific activities in cell extracts of chlorocatechol-1,2-dioxygenase (*tfdC*), chloromuconate cycloisomerase (*tfdD*), and dienelactone hydrolase (*tfdE*) were 2 to 50 times higher for microorganisms containing module I compared to those containing module II. In contrast, a significantly (50-fold) higher activity of maleylacetate reductase (*tfdF*) was observed in cell extracts of microorganisms containing module II compared to module I. The *R. eutropha* JMP222 derivative containing $tfdR\text{-}tfdC_ID_IE_IF_I$ grew four times faster in liquid cultures with 3-CB as a sole carbon and energy source than in cultures containing $tfdR\text{-}tfdD_{II}C_{II}E_{II}F_{II}$. In the case of *P. putida* KT2442, only the derivative containing module I was able to grow in liquid cultures of 3-CB. These results indicate that efficient degradation of 3-CB by *R. eutropha* JMP134(pJP4) requires the two *tfd* modules such that TfdCDE is likely supplied primarily by module I, while TfdF is likely supplied by module II.

Ralstonia eutropha JMP134 is able to grow in media containing 2,4-dichlorophenoxyacetate (2,4-D) and 3-chlorobenzoate (3-CB), as well as other chloroaromatics (3, 5, 26). Most of its catabolic abilities are encoded in the plasmid pJP4 (5, 6). The enzymes for the catabolism of chloroaromatics in pJP4 have been intensively studied (19, 25–27, 32, 33, 35, 36). Catabolism of 2,4-D is started by the products of the 2,4-D/α-ketoglutarate dioxygenase (*tfdA*) (10, 11) and 2,4-dichlorophenol hydroxylase (*tfdB*) genes on pJP4, to form 3,5-dichlorocatechol (3,5-DCC). Metabolism of 3-CB is initiated by a chromosomally encoded, low-specificity benzoate dioxygenase and 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase to form 3-chlorocatechol (3-CC) and 4-chlorocatechol (4-CC), as has been reported for *Alcaligenes eutrophus* B9 (29). Chlorocatechol metabolism is supposed to be performed by the enzymes encoded in the $tfdC_ID_IE_IF_I$ gene module present in the *Eco*RI-B fragment of pJP4. Genes $tfdC_I$, $tfdD_I$, and $tfdE_I$ encode for chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase, respectively (7). Interruption of these genes by transposon mutagenesis resulted in mutants no longer able to grow in 2,4-D, supporting the notion that these gene products play a major role in the metabolism of this substrate. It has been proposed that the fourth gene of this module, $tfdF_I$, encodes a functional maleylacetate reductase (14). An additional chromosomally encoded maleylacetate reductase was reported to be recruited for chloroaromatic degradation in *R. eutropha* (20). The presence in pJP4 of a second module of genes ($tfdD_{II}C_{II}E_{II}F_{II}$) possibly coding for enzymes for chlorocatechol metabolism has only recently been reported (9, 22). Leveau and coworkers have observed that transcription of the genes of both modules I and II takes place during adaptation to 2,4-D in cells of *R. eutropha* JMP134 growing on fructose (23). It is not known if the tfd_{II} gene products are functional for chlorocatechol degradation.

The objective of this work was to investigate the function of the $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$ gene modules in *R. eutropha* growing on 3-CB as sole carbon and energy source. Each of the two gene modules was cloned into the medium-copy-number plasmid vector pBBR1MCS-2 (17), under the control of the LysR-type transcriptional activator *tfdR* (21) and its corresponding putative promoter sequences. The $tfdR/P_{tfd\text{-}I}tfdC_ID_IE_IF_I$ and $tfdR/P_{tfd\text{-}II}tfdD_{II}C_{II}E_{II}F_{II}$ gene modules were independently introduced into two strains able to transform 3-CB into chlorocatechols, i.e., *R. eutropha* JMP222, a derivative of strain JMP134 cured of plasmid pJP4, and *Pseudomonas putida* KT2442. In the derivatives obtained, the expression of Tfd enzymes and the ability to grow with 3-CB were assessed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* JMP222 and *P. putida* KT2442 were grown at 30°C in a chloride-free minimal medium (18) with 3 mM benzoate plus streptomycin (1,000 μg/ml) or rifampycin (50 μg/ml), respectively. *R. eutropha* JMP134 and 3-CB-mineralizing derivatives of JMP222 and KT2442 were grown in minimal medium with 3 mM 3-CB. Derivatives of strain KT2442 not capable of growing with 3-CB were grown in minimal medium with 3 mM benzoate plus kanamycin (50 μg/ml). *P. putida* KT2442(pJP4) was grown in minimal medium with 3 mM benzoate or in Luria-Bertani (LB) medium con-

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant phenotype or genotype[a]	Source or reference
Bacteria		
R. eutropha		
JMP134	2,4-D$^+$, 3-CB$^+$, Hg^r, pJP4	DSMZ[b]
JMP222	*R. eutropha* JMP134 derivative, 2,4-D$^-$, 3-CB$^-$, Sm^r	H. Knackmuss
P. putida		
KT2442	*P. putida* mt-2 derivative, *hsdR1 hsdM*$^+$, Rif^r	12
E. coli		
DH5α		1
HB101		1
Plasmids		
pRK600	Cm^r, IncPα, *tra*$^+$	4
pBluescript II KS	Ap^r	Stratagene
pUC18	Ap^r	GIBCO-BRL
pUC18Not (Sfi)	Ap^r	12
pUCP19	Ap^r	31
pBBR1MCS-2	Km^r, broad host range	17
pVJE22	Tc^r, *tfdC$_I$D$_I$E$_I$F$_I$*, *tfdB*	34
pJRC105	Ap^r, *tfdC$_I$D$_I$E$_I$F$_I$*, pUC18*Not* derivative	This work
pJRC67	Ap^r, *tfdC$_I$D$_I$E$_I$F$_I$*, pBSKSII derivative	This work
pJRC48	Ap^r, *tfdC$_I$D$_I$E$_I$F$_I$*, pUC18*Not* derivative	This work
pJRC42	Ap^r, *tfdC$_I$D$_I$E$_I$F$_I$*, pUC18*Not* derivative	This work
pUCLG	Ap^r, P$_{tfd\text{-}I}$*tfdC$_I$D$_I$′*, pUC18*Not* derivative	This work
pUCLG1	Ap^r, P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$*, pUC18*Not* derivative	This work
pUCLG2	Ap^r, *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$*, pUC18*Not* derivative	This work
pUCLG4	Ap^r, *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$′*, pUC18*Not* derivative	This work
pUCPM-I	Ap^r, *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$*, pUCP19 derivative	This work
pUCDP1	Ap^r, pJP4 *Eco*RI-E fragment, pUC18 derivative	This work
pUCDP2	Ap^r, pJP4 *Eco*RI-G fragment, pUC18 derivative	This work
pBSDP3	Ap^r, *tfdR*/P$_{tfd\text{-}II}$*tfdD$_{II}$C$_{II}$E$_{II}$′*, pBSKSII derivative	This work
pBSDP4	Ap^r, *tfdR*/P$_{tfd\text{-}II}$*tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$*, pBSKSII derivative	This work
pBBR1M-I	Km^r, *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$*, pBBR1MCS-2 derivative	This work
pBBR1M-II	Km^r, *tfdR*/P$_{tfd\text{-}II}$*tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$*, pBBR1MCS-2 derivative	This work

[a] 2,4-D and 3-CB, able to grow in 2,4-D and 3-CB, respectively; IncPα *tra*, IncP transference functions; *tfd*, catabolic genes from pJP4; *tfdR*, regulatory gene of pJP4; P$_{tfd\text{-}I}$, putative promoter region for the *tfdC$_I$D$_I$E$_I$F$_I$* cluster; P$_{tfd\text{-}II}$, putative promoter region for the *tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* cluster; Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Rf, rifampycin; Cf, cloramphenicol; Sm, streptomycin; pBSKSII, pBluescript II KS.

[b] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

taining 0.5 mM merbromin. *Escherichia coli* strains were maintained on LB agar plates containing the appropriate antibiotic: 50 μg of ampicillin, kanamycin, or rifampycin per ml or 20 μg of tetracycline or cloramphenicol per ml. Growth in 3-CB was determined as increase in optical density at 660 nm (OD_{660}). At least three replicate growth measurements were performed.

DNA manipulation. Restriction, ligation, and dephosphorylation reactions and purification and electroporation of DNA were performed by standard procedures (1). Derivatives of the broad-host-range plasmid vector pBBR1MCS-2 were mobilized from *E. coli* to *R. eutropha* JMP222 or *P. putida* KT2442 by using a triparental mating with *E. coli* HB101(pRK600) as the helper strain. A donor-to-helper-to-recipient ratio of 1:1:2 was used. After incubation, cells were resuspended, and the transconjugants were selected on agar plates containing minimal medium with 3 mM benzoate plus 50 μg of kanamycin per ml. Plasmid pJP4 was transferred to *P. putida* by biparental mating with *R. eutropha* JMP134 as the donor, and selection was performed on LB agar plates containing 0.5 mM merbromin plus 50 μg of rifampycin per ml. The presence of pJP4 in *P. putida* transconjugants was determined by plasmid extraction.

Construction of a *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$* gene module. The *tfdC$_I$D$_I$E$_I$F$_I$* genes were cloned (Fig. 1) from the *Eco*RI-B pJP4 fragment previously inserted in plasmid pVJE22 (34). Plasmid pJRC105 was generated by cloning the 10.5-kb *Eco*RI/*Bam*HI fragment of pVJE22 into pUC18*Not*. The 6.7-kb *Hpa*I/*Bam*HI fragment of pJRC105 was subcloned into pBluescript II KS digested with *Sma*I/*Bam*HI to make pJRC67. An *Eco*RI/*Dra*I digestion of pJRC67 produced a 4.8-kb fragment that was introduced into pUC18*Not* digested with *Eco*RI/*Hin*cII to form pJRC48. The insert in pJRC48 contains a 0.6-kb region upstream of the initiation codon of gene *tfd$_I$C*. Finally, digestion of pJRC48 DNA with *Pml*I and *Sph*I allowed cloning of *tfd$_I$CDEF* into pUC18*Not* digested with *Sma*I/*Sph*I, to form pJRC42.

The *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$* gene module was obtained as follows (Fig. 1). First, the PCR product with primer pairs PR-1 and VAL-2 (see below) from pJRC48 was digested with *Bam*HI and *Pst*I and was inserted into pUC18*Not* to give pUCLG. This 1.9-kb *Bam*HI/*Pst*I fragment includes a 219-base region upstream of the first nucleotide of *tfd$_I$C*, and, therefore, contains the putative *cis* regulatory region (P$_{tfd\text{-}I}$), plus the *tfdC$_I$D$_I$′* genes, but without the last 72 bases of *tfdD$_I$* (i.e., *tfdD$_I$′*). Then, a 2.2-kb *Pst*I fragment from pJRC42 containing the last 72 bases of *tfdD$_I$* plus *tfdE$_I$F$_I$* was cloned in pUCLG to give pUCLG1. The PCR product with primers PR-2 and PR-3 (see below) of the *tfdR*-containing *Eco*RI-E pJP4 fragment, previously cloned in pUC18 to give pUCDP1, was digested with *Sst*I and *Bcl*I and was introduced into pUCLG1 to give pUCLG2.

Plasmid pUCLG2 containing *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$* was digested with *Not*I, and 5′ overhangs of *Not*I fragment were filled in by Klenow DNA polymerase I (Gibco BRL) and were ligated to pUCP19 digested with *Sma*I to form pUCPM-I. An *Eco*RI/*Xba*I digestion of pUCPM-I produced a 5.1-kb fragment, containing the *tfdR*/P$_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$* gene module, that was introduced into pBBR1MCS-2 to generate pBBR1M-I.

To amplify P$_{tfd\text{-}I}$*tfdC$_I$D$_I$′*, primer pairs PR-1 (5′-CTGTCTTATTTCCAGGA TCCGTCCCG-3′ [bp 127 to 152 of GenBank accession no. M35097/XO7754, modified to introduce a *Bam*HI recognition sequence]) and VAL-2 (5′-GCCG TGGAATTCGCCAGTGGGAACCTGCAG-3′ [bp 2136 to 2165, modified to introduce an *Eco*RI recognition sequence]) were used. To amplify *tfdR*, primer pairs PR-2 (5′-CCACCAGGAGTGATCAATGGAGTTTCG-3′ [bp 40 to 66 of GenBank accession no. S80112, modified to create a *Bcl*I recognition sequence]) and PR-3 (5′-ACGTAGCCGAGCTCGCTATTTCTGTCCTTTCCCG-3′ [bp 984 to 1017, modified to create a *Sst*I recognition sequence]) were used. Conditions for PCR were 95°C for 2 min; 35 cycles of 95°C for 45 s, 55°C for 30 s, and 72°C for 3 min; and then 72°C for 10 min.

Construction of a *tfdR*/P$_{tfd\text{-}II}$*tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* gene module. The *tfdR* and *tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* genes with their intergenic region were cloned (Fig. 1) from the *Eco*RI-E and *Eco*RI-G pJP4 fragments previously inserted in pUC18 to give pUCDP1 and pUCDP2, respectively. pUCDP1 was digested with *Sac*I and *Eco*RI to yield a 4.2-kb fragment containing *tfdR*, an intergenic region, and *tfdD$_{II}$C$_{II}$E$_{II}$*. This fragment was subcloned into pBluescript II KS digested with *Sac*I/*Eco*RI, generating pBSDP3. The *Eco*RI-G fragment containing the *tfdF$_{II}$* gene was obtained from pUCDP2 and was inserted in the *Eco*RI site of pBSDP3 to give pBSDP4. The plasmid pBSDP4 was digested with *Sac*I/*Kpn*I, and the 5.9-kb fragment containing the *tfdR*/P$_{tfd\text{-}II}$*tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* gene module was introduced in pBBR1MCS-2 to give pBBR1M-II.

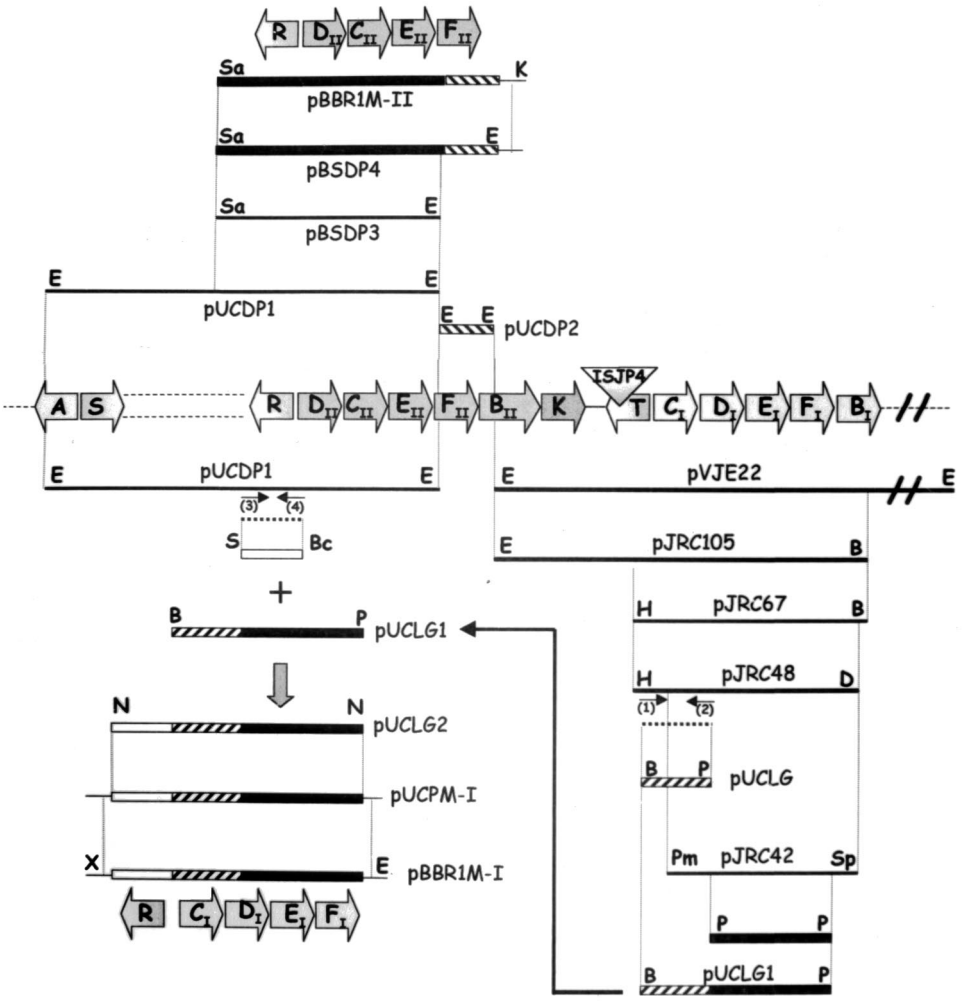

FIG. 1. Scheme of cloning for *tfdR*/$P_{tfd\text{-}I}$*tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ (bottom) and *tfdR*/$P_{tfd\text{-}II}$*tfdD*$_{II}$*C*$_{II}$*E*$_{II}$*F*$_{II}$ (top). The genetic map of the 22-kb region of pJP4 containing the *tfd* genes is depicted. DNA fragments used in cloning steps are indicated by restriction enzyme sites and by the names of plasmids that contain them (Table 1). The drawing is not to scale. B, *Bam*HI; Bc, *Bcl*I; D, *Dra*I; E, *Eco*RI; H, *Hpa*I; N, *Not*I; Pm, *Pml*I; P, *Pst*I; S, *Sst*I; Sa, *Sac*I; Sp, *Sph*I; X, *Xba*I. Dashed lines represent fragments obtained by PCR amplification. Small arrows indicate primer pairs: (1), PR-1; (2), VAL-2; (3), PR-2; (4), PR-3.

Enzyme assays. For enzyme assays, cells were grown in minimal medium containing 3 mM 3-CB. Additionally, strains not able to grow in 3-CB were grown in minimal medium containing 3 mM benzoate plus the corresponding antibiotic and were induced at late exponential growth phase with 1 mM 3-CB for 3 h. About 100 ml of each culture was harvested at the end of the exponential phase and was centrifuged, washed twice, and resuspended in 5 ml of a solution containing 50 mM Tris-acetate (pH 7.5) and 1 mM $MnSO_4$. Cells were disrupted by sonication (Vibracell; Sonics & Materials, Inc.). 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. Assays contained 50 μM substrate, 33 mM Tris-acetate buffer (pH 7.5), 1 mM $MnSO_4$, and a volume of crude extract corresponding to 1 to 100 μg of protein (0.002 to 0.02 enzyme units). One unit of enzyme activity was the amount of crude extract that forms or consumes 1 μmol of product or substrate, respectively, per min. Protein determinations were performed as previously described (2). Enzyme activities were determined in assays performed in a diode-array Hewlett Packard HP 8452-A UV/Vis spectrophotometer.

(i) Chlorocatechol-1,2-dioxygenase. Chlorocatechol-1,2-dioxygenase activity was measured with 3,5-dichlorocatechol (3,5-DCC), 4-chlorocatechol (4-CC), or 3-chlorocatechol (3-CC) (Helix Biotechnology, Inc., Vancouver, British Columbia, Canada) as substrate by following product formation as indicated by OD_{260}. The molar absorption coefficients were 2,4-dichloromuconate (2,4-DCM), ε_{260} = 12,000 $M^{-1}cm^{-1}$; 3-chloromuconate (3-CM), ε_{260} = 12,400 $M^{-1}cm^{-1}$; and 2-chloromuconate (2-CM), ε_{260} = 17,100 $M^{-1}cm^{-1}$, respectively (8).

(ii) Chloromuconate cycloisomerase. Chloromuconate cycloisomerase activity was measured by substrate consumption (as indicated by OD_{260}) with 2,4-DCM, 3-CM, or 2-CM. With 2,4-DCM, a reaction coefficient of ε_{260} = 5,800 $M^{-1}cm^{-1}$ was used (for explanation, see reference 19). These muconates were prepared by incubation of the corresponding chlorocatechols with a cell suspension of *E. coli* DH5α harboring plasmid pUCLG4. This pUC18*Not* derivative contains the *tfdR*/$P_{tfd\text{-}I}$*tfdC*$_I$*D*$_I$′ genes but lacks the last 72 bases of the gene coding for the chloromuconate cycloisomerase and, therefore, expresses an active chlorocatechol-1,2-dioxygenase but an inactive chloromuconate cycloisomerase. *E. coli* DH5α(pUCLG4) was grown in 50 ml of LB broth containing 100 μg of ampicillin per ml and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After overnight growth, 1 mM IPTG was added to the culture, and cells were incubated for an additional 2 h. Cells were pelleted, washed twice, and resuspended in 5 ml of 10 mM Tris-HCl, pH 8.5. The suspension was incubated in a shaker at 30°C for 120 min with three additions of a 0.5 mM solution of the respective chlorocatechol, performed at 0, 15, and 60 min. After 120 min, the cell suspension was centrifuged, and the supernatant was stored at −20°C. Quantitative transformation of chlorocatechols and no formation of dienelactones were verified by high-pressure liquid chromatography analysis. This was carried out with an LC-10AD Shimadzu liquid chromatograph equipped with an SC 125- by 4.6-cm Lichrospher 100 RP8 5.0-μm-particle-size column (Bischoff, Leonberg, Germany) and by using as elution solvent an aqueous system containing 36% methanol plus 0.1% phosphoric acid.

(iii) Dienelactone hydrolase. Dienelactone hydrolase activity was measured by consumption of *cis*-dienelactone as indicated by OD_{280} (ε_{280} = 17,000 $M^{-1}cm^{-1}$) (30). The assay contained 10 mM histidine-HCl buffer, pH 6.5, and 0.08 μM *cis*-dienelactone, in addition to crude extract. *cis*-Dienelactone was a gift of W. Reineke and S. Kaschabek (15).

(iv) Maleylacetate reductase. Maleylacetate reductase was measured by consumption of NADH as indicated by OD_{340} (ε_{340} = 6,300 $M^{-1}cm^{-1}$). The sub-

TABLE 2. Growth in 3-CB of *R. eutropha* JMP134 and JMP222 and *P. putida* KT2442 derivatives containing *tfdR*/$P_{tfd\text{-}I}$*tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ and/or *tfdR*/$P_{tfd\text{-}II}$*tfdD*$_{II}$*C*$_{II}$*E*$_{II}$*F*$_{II}$

Strain/derivative	Growth in 3-CB[a]	Generation time (h)
R. eutropha JMP134(pJP4)	+	5.0
R. eutropha JMP222(pBBR1M-I)	+	3.4
R. eutropha JMP222(pBBR1M-II)	+	13.4
P. putida KT2442(pJP4)	−	Nonapplicable
P. putida KT2442(pBBR1M-I)	+	6.0
P. putida KT2442(pBBR1M-II)	−	Nonapplicable

[a] Growth was tested in liquid cultures containing 3 mM 3-CB incubated for up to 15 days. Growth was observed after 1 to 2 days of incubation for the wild-type strain and derivatives with pBBR1M-I and after 4 to 5 days of incubation for the derivative with pBBR1M-II.

strates were generated in situ with a crude extract of *R. eutropha* JMP134 pregrown in 2 mM 2,4-D. The diluted crude extract was incubated at room temperature with 100 μM of 4-CC or 3,5-DCC until complete conversion (approximately 120 min) to maleylacetate or chloromaleylacetate, respectively, had occurred. During the incubation, UV spectral changes corresponding to the complete removal of the chlorocatechol, along with formation of the maleylacetate (maximum OD at 245 to 253 nm) were observed. No signals for aromatic compounds were detected by gas chromatography-mass spectrometry analysis at the end of incubation. Only one peak, corresponding to 2-chloromaleylacetate or maleylacetate was observed by high-pressure liquid chromatography analysis (L. Padilla, V. Matus, P. Zenteno, and B. González, unpublished data). The formation of maleylacetates was also supported by the fact that no product was detected in incubations without chlorocatechol or in incubations with chlorocatechol plus NADH (because the maleylacetate was converted to β-ketoadipate). The final concentration of maleylacetate was 0.1 mM. These compounds were used immediately. After the incubation of the crude extract of *R. eutropha* JMP134, the maleylacetate reductase lost its activity. That was confirmed by the addition of NADH at the end of incubation. These control assays did not show spectral changes at 340 nm. Contribution of the NADH oxidation due to components other than maleylacetate was determined by adding fresh crude extract plus NADH to incubations performed without chlorocatechol, and this contribution was subtracted from reported measurements. The assay was started after the addition of 200 μM NADH and fresh crude extract.

RESULTS

Growth in 3-CB of derivatives of *R. eutropha* JMP222 and *P. putida* KT2442 containing pBBR1M-I or pBBR1M-II. *R. eutropha* JMP222 derivatives containing pBBR1M-I were able to grow in liquid cultures with 3-CB as the sole carbon and energy source after 1 to 2 days of incubation. Surprisingly, *R. eutropha* JMP222 derivatives containing pBBR1M-II were also able to grow in liquid cultures with 3-CB after 4 to 5 days of incubation. The generation time for these derivatives growing in 3 mM 3-CB are shown in Table 2. *R. eutropha* JMP222 (pBBR1M-I) grew about four times faster than strain JMP222 (pBBR1M-II) and even faster than wild-type strain JMP134 (pJP4). Of all the *P. putida* KT2442 derivatives, only the derivative containing pBBR1M-I was able to grow in liquid cultures of 3-CB, with a generation time about twice that of the corresponding *R. eutropha* strain (Table 2). *P. putida* derivatives containing pJP4 or pBBR1M-II were unable to grow in liquid cultures with 3-CB, even after 15 days of incubation. However, *P. putida* KT2442(pJP4) and strain KT2442 (pBBR1M-II) formed small colonies on 3-CB agar plates. *P. putida* KT2442(pJP4) was also able to form small colonies on 2,4-D agar plates. These small colonies were clearly different from those occasionally seen in plates without a carbon source or those inoculated with strains without plasmids.

The effect of substrate concentration on 3-CB growth was studied with the four strains that grew in liquid cultures. *R. eutropha* JMP222 containing module I exhibited a better growth performance than the wild type at high 3-CB concentrations (Fig. 2). In contrast, the slow-growing derivative containing module II reached lower growth yields at concentrations up to 6 mM, then declined in yield at 8 and 10 mM 3-CB. *P. putida* (pBBR1M-I) exhibited a behavior similar to *R. eutropha* JMP222(pBBR1M-I), but with lower yields (Fig. 2).

Expression of Tfd gene products in derivatives of *R. eutropha* JMP222 containing pBBR1M-I or pBBR1M-II. To further study the role of *tfd* modules I and II in the degradation of 3-CB, the activity of Tfd enzymes was determined in derivatives of strain JMP222 containing only one of these modules. The activities of chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase in crude extracts of strains JMP222(pBBR1M-I), JMP222(pBBR1M-II), and JMP134(pJP4) grown in 3 mM 3-CB are shown in Table 3. The activity for chlorocatechol-1,2-dioxygenase in strain JMP222(pBBR1M-I) was two times

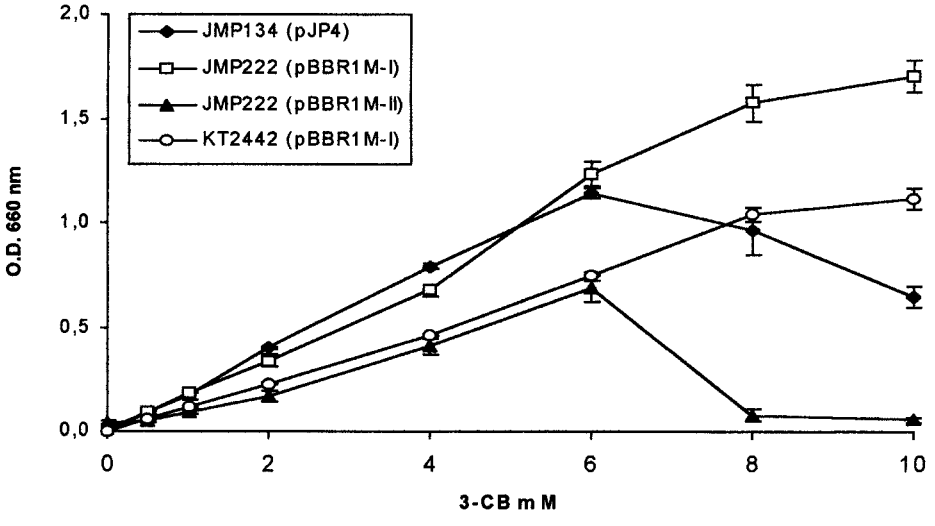

FIG. 2. Effect of substrate concentration in growth of *R. eutropha* and *P. putida* strains in 3-CB. OD was measured at stationary phase, i.e., 1 to 2 days of incubation for the wild type and derivatives with pBBR1M-I and 4 to 5 days of incubation for the derivative with pBBR1M-II. Values correspond to means ± deviation of triplicates. ◆, *R. eutropha* JMP134(pJP4); □, *R. eutropha* JMP222(pBBR1M-I); ▲, *R. eutropha*(pBBR1M-II); and ○, *P. putida* KT2442(pBBR1M-I).

TABLE 3. Specific activities of enzymes encoded in modules *tfdR*/$P_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$* and *tfdR*/$P_{tfd\text{-}II}$*tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* in crude extracts of *R. eutropha* JMP134 and *R. eutropha* JMP222 derivatives grown in 3 mM 3-CB[a]

Enzyme	Substrate	Sp act of enzymes in *R. eutropha* strain:			
		JMP134(pJP4)[a]	JMP222[b]	JMP222(pBBR1M-I)[a]	JMP222(pBBR1M-II)[a]
CC-1,2-DO	3,5-DCC	0.46 ± 0.02	<0.02	0.95 ± 0.23	0.32 ± 0.02
	4-CC	0.26 ± 0.03	<0.02	0.47 ± 0.22	0.19 ± 0.02
	3-CC	0.27 ± 0.04	<0.01	0.52 ± 0.21	0.28 ± 0.02
CMCI	2,4-DCM	0.53 ± 0.03	No activity	1.08 ± 0.13	0.26 ± 0.008
	3-CM	0.13 ± 0.01	No activity	0.39 ± 0.02	0.05 ± 0.002
	2-CM	0.01 ± 0.001	No activity	0.03 ± 0.002	<0.005
DLH	DL	0.61 ± 0.11	No activity	1.96 ± 0.25	0.04 ± 0.008
MAR	MA	0.43 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	1.57 ± 0.03
	2-CMA	0.32 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	1.12 ± 0.02

[a] Cells grown in 3 mM 3-CB.

[b] Cells grown in 3 mM benzoate and induced with 3-CB. Values for *R. eutropha* JMP134 grown on 3 mM benzoate and induced with 3-CB were approximately 20 to 30% (T. Ledger, C. Varela, R. Céspedes, D. Pérez-Pantoja, L. Guzmán, D. Pieper, and B. González, unpublished data) of those found in cell extracts grown on 3-CB CC-1,2-DO, chlorocatechol-1,2-dioxygenase; CMCI, chloromuconate cycloisomerase; DLH, dienelactone hydrolase; MAR, maleylacetate reductase: DL, *cis*-dienelactone; MA, maleylacetate; and 2-CMA, 2-chloromaleylacetate. Values are expressed as units per milligram and correspond to averages of two or three independent experiments.

higher than the activity of the wild-type strain JMP134(pJP4) and was two to three times higher than the activity encoded in module II. A very low activity against chlorocatechols was found in the crude extract of the recipient strain JMP222 (Table 3), supporting the notion that both modules encode an active chlorocatechol-1,2-dioxygenase. The highest activity for chloromuconate cycloisomerase (Table 3) was also found in the crude extract of strain JMP222(pBBR1M-I). This activity was two to three times higher than that observed in the wild type and was more than four times higher than that observed in derivatives containing module II. No chloromuconate cycloisomerase activity was found in the recipient strain JMP222. Therefore, the differences in amount and specificity (see below) of chloromuconate cycloisomerase in both crude extracts are completely due to the *tfd* genes. Significant differences in substrate specificity of chloromuconate cycloisomerase were observed in cell extracts of *R. eutropha* derivatives containing module I or II. The chloromuconate cycloisomerase of module II showed higher ratios of 2,4-DCM to 3-CM and 2,4-DCM to 2-CM utilization (5.2 and 52, respectively [Table 3]) than the corresponding enzyme in module I (2.8 and 36, respectively [Table 3]). Another important difference was found for the dienelactone hydrolase activity (Table 3). The dienelactone hydrolase activity of module I was 50 times higher than that of module II. In contrast, the highest activity of maleylacetate reductase was detected in strain JMP222(pBBR1M-II). It was approximately 3 times higher than in the wild-type JMP134(pJP4). Maleylacetate reductase activity was observed at low rates in both JMP222 grown in 3 mM benzoate and in strain JMP222(pBBR1M-I) grown in 3-CB (Table 3). *tfdF$_I$* was cloned under the control of the P_{tac} promoter in pVLT35, a medium-copy-number plasmid that replicates in *R. eutropha* (4), and was introduced in *R. eutropha* JMP222. No significant differences in maleylacetate reductase activity were found in IPTG-induced cells with respect to noninduced cells or cells of strain JMP222. It can therefore be concluded that a functional maleylacetate reductase was not produced from *tfdF$_I$* at all, or it has a very low activity. The activity of maleylacetate reductase observed in JMP222 cells grown on benzoate and induced with 3-CB (Table 3) may correspond to the chromosomal activity involved in the ability of *R. eutropha* JMP222 to grow in 2,4,6-trichlorophenol (3; L. Padilla, V. Matus, P. Zenteno, and B. González, unpublished data).

P. putida derivatives were studied for expression of chlorocatechol dioxygenase. Crude extracts of cells grown in 3-CB or in benzoate and induced with 3-CB showed specific activities that were 25 to 50% lower than those of the corresponding *R. eutropha* derivatives.

DISCUSSION

This work showed that both *tfd* modules found in *R. eutropha* JMP134(pJP4) encode functional enzymes for chlorocatechol metabolism. High levels of activity were found for enzymes encoded by genes *tfdC$_I$*, *tfdC$_{II}$*, *tfdD$_I$*, *tfdD$_{II}$*, *tfdE$_I$*, and *tfdF$_{II}$*. Low activity levels were found for the enzyme encoded by *tfdE$_{II}$*. No, or very low, activity was observed for the enzyme encoded by *tfdF$_I$*. This work also showed that cloning of each module in the medium-copy-number plasmid vector pBBR1MCS-2 and introduction of each module into strains that accumulate chlorocatechols from 3-CB allow such derivatives to grow in it. In order to study the role in chlorocatechol degradation of the two *tfd* modules harbored in pJP4, we chose to clone each of these modules into a medium-copy-number plasmid vector that replicates in *R. eutropha* and *P. putida*. Two reasons explain that choice. First, preliminary work performed with *tfdR*/$P_{tfd\text{-}I}$*tfdC$_I$D$_I$E$_I$F$_I$* and *tfdR*/$P_{tfd\text{-}II}$*tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* in *E. coli* showed very little expression of Tfd enzymes. Second, introduction of a single copy of these *tfd* gene modules into the *R. eutropha* chromosome, by using miniTn*5*-derived vectors, also gave very low expression of the Tfd enzymes (T. Ledger, C. Varela, R. Céspedes, D. Pérez-Pantoja, L. Guzmán, D. Pieper, and B. González, unpublished data). These *R. eutropha* derivatives have 2 to 10% of the enzyme activity levels found in the wild-type strain or in derivatives containing module II or I in pBBRMCS-2 (this work) and were not able to grow in 3-CB (T. Ledger, C. Varela, R. Céspedes, D. Pérez-Pantoja, L. Guzmán, D. Pieper, and B. González, unpublished data). Both chlorocatechol metabolism gene modules were cloned under the control of *tfdR*. The role of *tfdR*, located upstream of *tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$*, as a regulator of the *tfdA* gene and *tfdC$_I$D$_I$E$_I$F$_I$* and *tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* gene modules has been proposed (13, 21, 24). Leveau and van der Meer (21) showed that expression of *tfdC$_I$* is activated in *R. eutropha* by *tfdR*, which was previously thought to encode a repressor protein, whereas *tfdT*, located upstream of *tfdC$_I$D$_I$E$_I$F$_I$*, does not encode a functional regulatory protein, as it is inactivated by insertion of the ISJP4 element. The presence of *tfdR* ensures proper recognition of $P_{tfd\text{-}I}$,

and $P_{tfd\text{-}II}$, the putative promoter sequences for modules I and II, respectively.

The introduction of each *tfdR*-regulated module into bacterial strains that accumulate chlorocatechols from 3-CB allowed us to assess the role of these modules for the degradation of chloroaromatics. Two observations arose from this part of the work. First, the introduction of module I resulted in a more-efficient 3-CB-degrading phenotype than introduction of module II. Second, the growth properties related to *tfd* genes were better expressed in the *R. eutropha* strain than in the *P. putida* strain. The last observation can be explained by the expected differences in gene expression and gene background between a homologous and a heterologous system. The first observation, however, may be explained in several ways. One of them is that transcriptional activation of module I is higher than that of module II. However, steady-state mRNA levels of transcripts from modules I and II show no obvious difference (23). Another possibility is that the very low activity of $TfdD_{II}$ with 2-CM or $TfdE_{II}$ becomes the rate-limiting step in catabolism of 3-CB by strains containing module II.

The expression of chlorocatechol metabolism enzymes was studied in *R. eutropha* JMP222 derivatives containing module II or I that were able to grow in 3-CB. Both modules expressed a chlorocatechol-1,2-dioxygenase activity whose substrate specificity resembled that of the wild-type strain. It has been recently reported that both genes are transcribed during growth of strain JMP134 in a chemostat fed with 2,4-D (23). Therefore, it is highly probable that both genes play a role in catabolism of 3-CB. Both modules express the second enzyme of the pathway, chloromuconate cycloisomerase. Although sequence comparisons indicate that the $tfdD_{II}$ gene is clustered apart from the other chloromuconate cycloisomerase genes reported in gram-negative bacteria (9), both chloromuconate cycloisomerase activities possess the higher dichlorinated-to-monochlorinated substrate activity ratio of all known chloromuconate cycloisomerases (36). However, the enzyme encoded in module II has a more pronounced preference for 2,4-DCM with very little activity toward 2-CM. In this context, it is worth mentioning that 2-CM is the main muconate formed during catabolism of 3-CB (28). This may be the main reason for the poor growth on 3-CB observed with module II. The activities of dienelactone hydrolases were also tested in this work. There was a significant (50-fold) difference of this activity in both modules. However, as accumulation of dienelactones was never observed during transformation of 2,4-DCM, 3-CM, or 2-CM, it can be assumed that the activity of each dienelactone hydrolase is not rate limiting. Another important difference between enzyme expression from both *tfd* modules was observed in the activity of maleylacetate reductase. Maleylacetate reductase encoded by $tfdF_{II}$ was, by far, more active than $tfdF_I$. Although similar steady-state levels of mRNAs corresponding to both $tfdF_I$ and $tfdF_{II}$ are found in *R. eutropha* JMP134 growing in 2,4-D (23), it should be noted that the NH_2-terminal sequence of the maleylacetate reductase purified from a culture of *R. eutropha* JMP134 grown in 2,4-D (32) perfectly matched the amino acid sequence deduced for $tfdF_{II}$ (GenBank accession no. U16782). Therefore, the expression and substrate specificity of the enzyme encoded by $tfdF_{II}$ makes it highly probable that this maleylacetate reductase plays the main role in 3-CB, as well as 2,4-D, metabolism. Our observations may explain early reports of no phenotype associated with the $tfdF_I$ gene (7). It should be noted that derivatives containing module I, and therefore not having a maleylacetate reductase encoded in the plasmid, grow similarly to the wild type. The expression, at low levels, of a chromosomally encoded maleylacetate reductase (Table 3) (20, 32; L. Padilla, V. Matus, P. Zenteno, and B. González, unpublished data) should be enough to support growth of such derivatives in 3-CB. The higher level of activity of the maleylacetate reductase encoded in module II may be needed for growth in 2,4-D (but not for 3-CB), since this carbon source produces 2-chloromaleylacetate which is not as good a substrate as maleylacetate and requires two steps catalyzed by this enzyme (16).

The expression profile of Tfd enzymes reported here may explain the presence of both *tfd* modules in pJP4 (a very unusual feature in genes encoding chloroaromatic metabolism) with both modules complementing each other (e.g., $tfdC_ID_IE_I$ plus $tfdF_{II}$).

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