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Efficient Turnover of Chlorocatechols Is Essential for Growth of *Ralstonia eutropha* JMP134(pJP4) in 3-Chlorobenzoic Acid

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Ralstonia eutropha **JMP134(pJP4) degrades 3-chlorobenzoate (3-CB) by using two not completely isofunctional, pJP4-encoded chlorocatechol degradation gene clusters, *tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ and *tfdD*$_{II}$*C*$_{II}$*E*$_{II}$*F*$_{II}$. Introduction of several copies of each gene cluster into *R. eutropha* JMP222, which lacks pJP4 and thus accumulates chlorocatechols from 3-CB, allows the derivatives to grow in this substrate. However, JMP222 derivatives containing one chromosomal copy of each cluster did not grow in 3-CB. The failure to grow in 3-CB was the result of accumulation of chlorocatechols due to the limiting activity of chlorocatechol 1,2-dioxygenase (TfdC), the first enzyme in the chlorocatechol degradation pathway. Micromolar concentrations of 3- and 4-chlorocatechol inhibited the growth of strains JMP134 and JMP222 in benzoate, and cells of strain JMP222 exposed to 3 mM 3-CB exhibited a 2-order-of-magnitude decrease in viability. This toxicity effect was not observed with strain JMP222 harboring multiple copies of the *tfdC*$_I$ gene, and the derivative of strain JMP222 containing *tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ plus multiple copies of the *tfdC*$_I$ gene could efficiently grow in 3-CB. In addition, *tfdC*$_I$ and *tfdC*$_{II}$ gene mutants of strain JMP134 exhibited no growth and impaired growth in 3-CB, respectively. The introduction into strain JMP134 of the *xylS-xylXYZL* genes, encoding a broad-substrate-range benzoate 1,2-dioxygenase system and thus increasing the transformation of 3-CB into chlorocatechols, resulted in derivatives that exhibited a sharp decrease in the ability to grow in 3-CB. These observations indicate that the dosage of chlorocatechol-transforming genes is critical for growth in 3-CB. This effect depends on a delicate balance between chlorocatechol-producing and chlorocatechol-consuming reactions.**

Ralstonia eutropha JMP134(pJP4) grows in pollutant compounds like 3-chlorobenzoate (3-CB) and 2,4-dichlorophenoxyacetic acid. Metabolism of 3-CB is initiated by a chromosomally encoded, low-specificity benzoate dioxygenase and a 1,2-dihydro-1,2-dihydroxybenzoate dehydrogenase and forms 3-chlorocatechol (3-CC) and 4-CC (Fig. 1) (28, 33). The chlorocatechols are metabolized by the enzymes chlorocatechol 1,2-dioxygenase (TfdC), chloromuconate cycloisomerase (TfdD), dienelactone hydrolase (TfdE), and maleylacetate reductase (TfdF) encoded in the *tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ and *tfdD*$_{II}$*C*$_{II}$*E*$_{II}$*F*$_{II}$ gene clusters from pJP4 (Fig. 1) (9, 20–22, 27, 29, 30). The simultaneous presence of these two apparently isofunctional chlorocatechol-degrading gene clusters (Fig. 1) is very uncommon in bacterial chloroaromatic catabolism (38, 40), which raises a question concerning the specific roles, if any, of each of these operons in chlorocatechol catabolism. Both gene clusters are induced during adaptation to 2,4-dichlorophenoxyacetic acid in cells of *R. eutropha* JMP134(pJP4) growing in fructose (24) and expressing active enzymes for catabolism of this compound and 2,4-dichlorophenol (21). Introduction of multiple copies of the *tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ or *tfdD*$_{II}$*C*$_{II}$*E*$_{II}$*F*$_{II}$ genes into strain JMP222, a derivative of strain JMP134 lacking pJP4 and therefore unable to grow in 3-CB, allows the derivatives to grow in this compound (27). However, we observed that derivatives of strain JMP222 harboring single chromosomal copies of each *tfd* gene module or one copy of the *tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ module and one copy of the *tfdD*$_{II}$*C*$_{II}$*E*$_{II}$*F*$_{II}$ module did not grow in 3-CB (23). A *tfd* gene dosage effect has been shown to be important for efficient growth in 3-CB of strain JMP134 derivatives harboring a rearranged form of the pJP4 plasmid (5). In addition, recent evidence from our laboratory indicates that pJP4 occurs naturally at a level of several copies per genome in strain JMP134 (37). These observations led us to hypothesize that expression of single copies of the *tfd* genes is limiting for growth in 3-CB because toxic intermediates are accumulated. In this study, we obtained evidence that accumulation of chlorocatechols impairs the growth of derivatives of strain JMP134 in 3-CB. This effect is observed when chlorocatechol-producing reactions overtake chlorocatechol-consuming reactions, and it is prevented if several copies of the *tfdC* genes, encoding chlorocatechol 1,2-dioxygenase, are present.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* JMP134(pJP4) and 3-CB-mineralizing derivatives of this strain were grown at 30°C in minimal medium (19) with 0.5 to 10 mM 3-CB as the sole carbon source. Growth in 3-CB was determined by measuring the increase in the optical density at 600 nm (OD_{600}). At least three replicates were used for each growth measurement. *R. eutropha* derivatives not able to proliferate in 3-CB were grown in 3 to 5 mM benzoate plus the appropriate antibiotic (Table 1). *Escherichia coli* strains were maintained on Luria-Bertani (LB) agar plates containing 50 μg of ampicillin ml^{-1}, 50 μg of kanamycin ml^{-1}, 20 μg of gentamicin ml^{-1}, 20 μg of chloramphenicol ml^{-1}, or 40 μg of tellurite ml^{-1}.

DNA manipulation. Restriction, ligation, and dephosphorylation reactions, purification, and electroporation of DNA were performed by standard proce-

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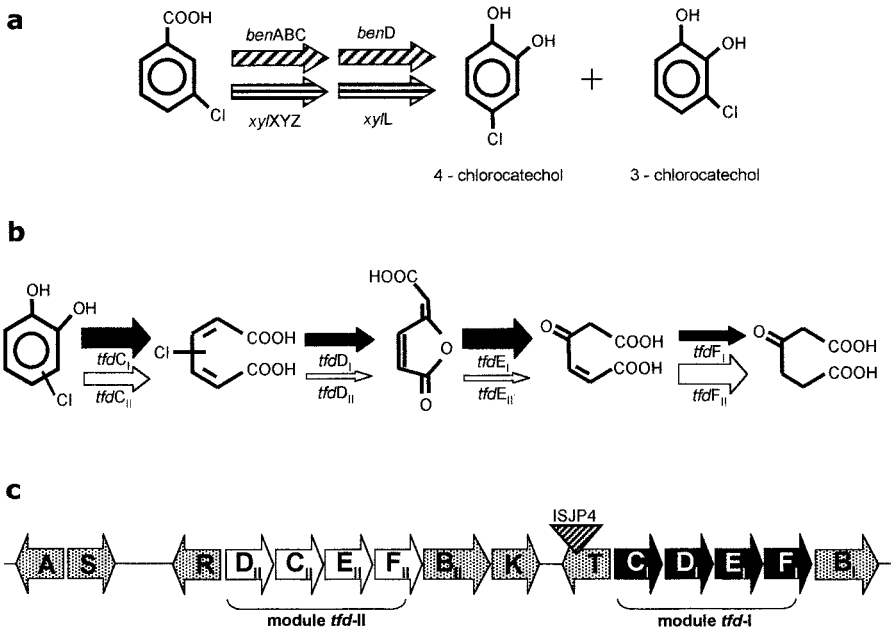

FIG. 1. Genes involved in 3-CB degradation. (a) Chlorocatechol-producing peripheral reactions for 3-CB encoded in the chromosome of *R. eutropha* (*ben* genes), and the 1,2-toluate dioxygenase system (*xyl* genes) from pWW0. (b) Chlorocatechol 1,2-dioxygenase (TfdC), chloromuconate cycloisomerase (TfdD), dienelactone hydrolase (TfdE), and maleylacetate reductase (TfdF) catalyze the conversion of chlorocatechols to chloromuconate, *cis*-dienelactone, maleylacetate, and β-ketoadipate, respectively. The arrow thickness indicates the relative specific activity of the enzymes encoded by each module for intermediates of 3-CB metabolism (27, 30). (c) Organization of *tfd* genes in pJP4, including the $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$ gene clusters. The diagram is not to scale.

dures (3). Derivatives of the broad-host-range plasmid vectors pBBR1MCS-2 (pBBRCI, pBBRPI) and pUT (pSPM100) were mobilized from *E. coli* to *R. eutropha* 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 3 mM benzoate plus kanamycin or tellurite.

Construction of *tfd* gene modules. The two pJP4-encoded chlorocatechol-degrading *tfd* modules were cloned into the medium-copy-number plasmid vector pBBR1MCS-2 to obtain pBBR1M-I and pBBR1M-II, as described elsewhere (27). To allow insertion into the chromosome, the *tfdR*-$P_{tfd\text{-}I}$ $tfdC_ID_IE_IF_I$ gene module, the *tfdR*-$P_{tfd\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$ gene module, and both modules were cloned into the mini Tn*5*-derived plasmid vector pBSL202 to obtain pR1TFD, pR2TFD, and pR12TFD, respectively, as reported elsewhere (23).

Cloning of *tfdR*-$P_{tfd\text{-}I}$ $tfdC_I$ and *tfdR*-$P_{tfd\text{-}I}$ in multiple copies. To clone the *tfdR*-regulated $tfdC_I$ gene into pBBR1MCS-2, pBBR1M-I (27) was digested with *Kpn*I to obtain a 2.2-kb fragment containing *tfdR*-$P_{tfd\text{-}I}$ $tfdC_ID_I^{\nabla}$. This DNA

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype and/or genotype[a]	Source or reference
R. eutropha strains		
JMP134	2, 4-D^+ 3-CB^+, pJP4	DSMZ[b]
JMP134-F3	3-CB^+, pJP4-F3	5
JMP222	pJP4-free derivative, 2,4-D^- 3-CB^- Sm^r	H.-J. Knackmuss
Plasmids		
pRK600	Cf^r IncPα *tra*$^+$	15
pBBR1MCS-2	Km^r, broad host range	18
pUT	Km^r Ap^r	15
pBSL202	Gm^r Ap^r	2
pBBRCI	Km^r *tfdR*-$P_{tfd\text{-}I}$ $tfdC_ID_I^{\nabla}$, pBBR1MCS-2 derivative	This study
pBBRPI	Km^r *tfdR*-$P_{tfd\text{-}I}$, pBBR1MCS-2 derivative	This study
pSPM100	Ap^r Te^r *xyIS-xyIXYZL*, pUT derivative	V. de Lorenzo
pR1TFD	Gm^r Ap^r *tfdR*-$P_{tfd\text{-}I}$ $tfdC_ID_IE_IF_I$, pBSL202 derivative	23
pR2TFD	Gm^r Ap^r *tfdR*-$P_{tfd\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$, pBSL202 derivative	23
pR12TFD	Gm^r Ap^r *tfdR*-$P_{tfd\text{-}I}$ $tfdC_ID_IE_IF_I$-*tfdR*-$P_{tfd\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$, pBSL202 derivative	23
pBBR1M-I	Km^r *tfdR*-$P_{tfd\text{-}I}$ $tfdC_ID_IE_IF_I$, pBBR1MCS-2 derivative	27
pBBR1M-II	Km^r *tfdR*-$P_{tfd\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$, pBBR1MCS-2 derivative	27
pJP4-Δ$tfdC_I$	$tfdC_I$	This study
pJP4-Δ$tfdC_{II}$	$tfdC_{II}$	This study

[a] 2,4-D^+ and 3-CB^+, able to grow in 2,4-dichlorophenoxyacetate and 3-CB respectively; IncPα *tra*,$^+$ IncP transference functions; *tfd*, catabolic genes from pJP4; *tfdR*, regulatory gene of pJP4; $P_{tfd\text{-}I}$, promoter region for $tfdC_ID_IE_IF_I$ cluster; $P_{tfd\text{-}II}$, promoter region for $tfdD_{II}C_{II}E_{II}F_{II}$ cluster; Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Cf^r, cloramphenicol resistance; Sm^r, streptomycin resistance.

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

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fragment was then introduced into pBBR1MCS-2 to obtain pBBRCI, which harbors a complete $tfdC_I$ gene and a truncated $tfdD_I$ gene ($tfdD_I^{\nabla}$). For control purposes, plasmid pBBR1M-I was digested with *Pml*I and *Xba*I to delete a 3.9-kb fragment containing the $tfdC_ID_IE_IF_I$ genes. The *Xba*I end of the plasmid backbone was blunt ended by using Mung bean nuclease. The plasmid was religated to obtain pBBRPI, which encodes only the $tfdR$-$P_{tdf\text{-}I}$ region. The pBBR1MCS-2 derivatives containing *tfd* genes were shown to be present at levels of about 10 copies per cell (37).

Inactivation of the $tfdC_I$ and $tfdC_{II}$ genes in pJP4. The $tfdC_I$ and $tfdC_{II}$ genes were independently inactivated in *E. coli* cells harboring the pJP4 plasmid by using the method of Datsenko and Wanner (7). PCR primers MutC1FW (5′-TCATGACGGAGGCAAAGTGAACAAAAGAGTCAAGGATGTTGTGTAGGCTGGAGCTGCTTC-3′) and MutC1RE (5′-GGGTTTGCCCCCGCCTGCGCACGCGCGGGCTCGATAACGAATTCCGGGGATCCGTCGACC-3′) and primers MutC2FW (5′-TTCATCTTTTTTGAAGAGAAAGCACCATGACAAATCCCCGGTGTAGGCTGGAGCTGCTTC-3′) and MutC2RE (5′-GCCTTCGCCGTCTCAGGCGCGGGACTTCTCGATGACGAAGATTCCGGGGATCCGTCGACC-3′), which contain 40-bp homology extensions on $tfdC_I$ or $tfdC_{II}$ sequences and 20-bp priming sequences for pKD13 (7), were synthesized. These primer pairs were used with pKD13 as the template to amplify the kanamycin-resistant gene flanked by 40 bp of the $tfdC_I$ or $tfdC_{II}$ gene sequences. 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. The PCR products were used to inactivate $tfdC_I$ or $tfdC_{II}$ genes in an *E. coli*(pJP4) strain harboring RecBCD recombinase by a previously described procedure (7). pJP4 derivatives containing inactivated $tfdC_I$ or $tfdC_{II}$ genes were transferred to strain JMP222 by biparental conjugation (6).

Toxicity tests for chlorocatechols. The MICs of 3-CC and 4-CC were determined for strains JMP134 and JMP222 growing in 5 mM benzoate or in LB broth. Growth was determined by determining the increase in OD_{600} after 24 h. To avoid interference by colored metabolic products, net OD_{600} values were calculated after subtraction of the OD_{600} determined for the cell-free supernatants from cultures after centrifugation of cells. The effect of chlorocatechols produced from 3-CB in cells growing in benzoate was studied by determining the viability in a 5 mM benzoate culture of strain JMP222 or JMP222(pBBRCI) to which 1 mM 3-CB was added at the late exponential phase and comparing the results with the results for a control which received no 3-CB. Samples were taken every 2 to 6 h for up to 24 h, and appropriate dilutions were plated on LB agar plates to determine the CFU.

Enzyme activity assays. For enzyme activity assays, cells were grown in minimal medium containing 3 mM benzoate until the late exponential growth phase and were induced with 1 mM 3-CB for 3 h. Crude extracts were prepared and the activities of chlorocatechol 1,2-dioxygenase (TfdC) and chloromuconate cycloisomerase (TfdD) were measured as reported previously (27).

HPLC analysis. Accumulation of chlorinated compounds was determined by high-performance liquid chromatography (HPLC) analysis by using cell-free supernatants from cell suspensions grown in 5 mM benzoate and induced for at least 2 h with 0.5 mM 3-CB. Cells were pelleted, washed twice with minimal medium, and resuspended in minimal medium to an OD_{600} of 0.5. 3-CB (0.2 mM) was added, and the cells were incubated at 30°C in an orbital shaker. Samples (10 μl) from cell-free supernatants were taken at different times and injected into a Shimadzu LC-10AD liquid chromatograph system equipped with an SC125 Lichrospher 5 μm column (Bischoff, Leonberg, Germany). A methanol-H_2O (60:40) mixture containing 0.1% (vol/vol) phosphoric acid was used as the solvent at a flow rate of 1 ml min^{-1}. The column effluent was monitored simultaneously at 210, 260, and 270 nm with an SPD-M10A diode array detector. The retention volumes were as follows: 2-chloro-*cis*,*cis*-muconate, 0.7 ml; 3-chloro-*cis*,*cis*-muconate, 0.5 ml; 3-CB, 4.6 ml; 3-CC, 1.4 ml; and 4-CC, 1.8 ml.

RESULTS

Growth in 3-CB of *R. eutropha* JMP222 derivatives containing the $tfdC_ID_IE_IF_I$ or $tfdD_{II}C_{II}E_{II}F_{II}$ gene cluster. Derivatives containing about 10 copies (37) of $tfdR$-$P_{tdf\text{-}I}$ $tfdC_ID_IE_IF_I$ [*R. eutropha* JMP222(pBBR1M-I)] or $tfdR$-$P_{tdf\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$ [*R. eutropha* JMP222(pBBR1M-II)] were previously reported to grow in liquid cultures with different concentrations of 3-CB as the sole carbon and energy source (27) (Fig. 2). The effect of the presence of single copies of these *tfd* gene modules was also tested. The mini Tn*5*-derived suicide plasmid vector pBSL202 was used to insert $tfdR$-$P_{tdf\text{-}I}$ $tfdC_ID_IE_IF_I$ (pR1TFD), $tfdR$-$P_{tdf\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$ (pR2TFD), or both gene clusters (pR12TFD) into the chromosome of strain JMP222, as recently reported (23). The presence of each gene module in the chromosome of JMP222 transconjugants was confirmed by PCR and Southern blot analysis (23). None of the transconjugants, including strains JMP222::$tfdR$-$P_{tdf\text{-}I}$ $tfdC_ID_IE_IF_I$ (JMP222::R1TFD), JMP222::$tfdR$-$P_{tdf\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$ (JMP222::R2TFD), and JMP222::$tfdR$-$P_{tdf\text{-}I}$ $tfdC_ID_IE_IF_I$-$tfdR$-$P_{tdf\text{-}II}$ $tfdD_{II}C_{II}E_{II}F_{II}$ (JMP222::R12TFD), was able to proliferate in liquid cultures containing 0.5 to 10 mM 3-CB (Fig. 2 and results not shown). The possibility of a position effect of the inserted *tfd* gene clusters was eliminated because all transconjugants showed the same growth behavior in 3-CB. Determination of the copy numbers of *tfd* genes in selected transconjugants, based on a previously described procedure (37), indicated that there was single-copy gene dosage. The inability of derivatives harboring single copies of the *tfd* gene clusters to grow in 3-CB may have been due to differences in enzyme activity compared with the activities of derivatives containing multiple copies. Therefore, the specific activities of TfdC and TfdD were determined in crude extracts prepared from cells grown in benzoate and induced with 3-CB. The TfdC specific activities of the derivatives harboring chromosomal insertions were 4- to 10-fold lower than those observed for strain JMP222 containing multiple plasmid copies of each *tfd* gene module or the wild-type strain (Table 2). The same pattern was observed with chloromuconate cycloisomerase specific activities (data not shown). These results also indicate that the *tfd* genes were active in each derivative.

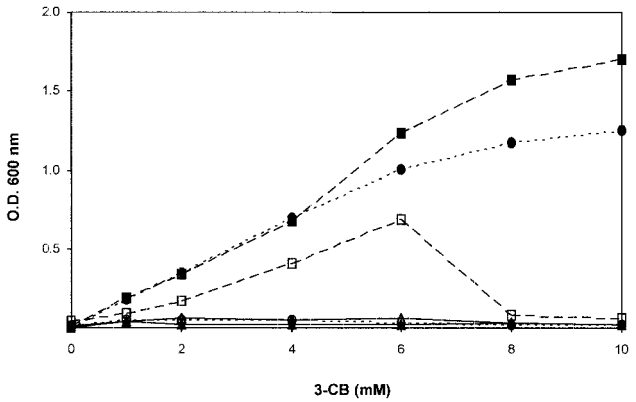

FIG. 2. Growth with different 3-CB concentrations of *R. eutropha* derivatives harboring different copy numbers of *ortho* ring cleavage pathway *tfd* gene modules. Symbols: ■, *R. eutropha* JMP222(pBBR1M-I); □, *R. eutropha* JMP222(pBBR1M-II); ▲, *R. eutropha* JMP222::R1TFD; △, *R. eutropha* JMP222::R2TFD; ●, *R. eutropha* JMP222::R1TFD(pBBRCI); ○, *R. eutropha* JMP222::R1TFD(pBBRPI). OD_{600} was measured at the stationary phase. The values are means based on triplicate experiments. The deviations (not shown for clarity) were less than 5 to 10%.

Multiple copies of the $tfdC_I$ gene prevent toxicity of chlorocatechols and stimulate derivatives of *R. eutropha* JMP222 to grow in 3-CB. Low specific activities of enzymes that catalyze rate-limiting steps should produce slow growth but do not necessarily mean that there is a lack of growth phenotypes. Therefore, we assumed that lower enzyme activities of deriv-

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TABLE 2. Chlorocatechol 1,2-dioxygenase specific activities in crude extracts of *R. eutropha* strains grown in benzoate and induced with 3-CB

R. eutropha strain	Chlorocatechol 1,2-dioxygenase sp act (U/mg)[a]
JMP134(pJP4)	0.24 ± 0.01
JMP222(pBBR1M-I)	0.71 ± 0.03
JMP222(pBBR1M-II)	0.22 ± 0.02
JMP222::R1TFD	0.06 ± 0.01
JMP222::R2TFD	0.02 ± 0.01
JMP222::R1TFD(pBBRCI)	0.80 ± 0.07
JMP222::R1TFD(pBBRPI)	0.08 ± 0.07
JMP134(pJP4Δ*tfdC*$_I$)	0.11 ± 0.02
JMP134(pJP4Δ*tfdC*$_{II}$)	0.17 ± 0.02

[a] Chlorocatechol 1,2-dioxygenase activity was assayed with 3,5-dichlorocatechol. The values are averages of two determinations.

atives of strain JMP222 harboring chromosomal copies of the *tfd* gene clusters could produce an accumulation of toxic intermediates. Chlorocatechols are compounds that affect bacterial growth (35, 36). We hypothesized that chlorocatechols accumulate and produce toxic effects in *R. eutropha*, and this possibility was studied. The accumulation of chlorocatechols after exposure of different JMP222 derivatives to 3-CB was determined by HPLC analysis. Strain JMP222 quantitatively transformed 0.2 mM 3-CB into 3-CC and 4-CC (Fig. 3a). These chlorocatechols are not transformed further because this strain lacks any chlorocatechol-degrading genes. A characteristic deep brown color was clearly observed after extended incubation. Strain JMP222::R1TFD accumulated about 50% of the added 3-CB as chlorocatechols (Fig. 3b). Because of the presence of the *tfd* gene cluster in the chromosome, this strain was capable of further transformation of chlorocatechols. However, due to the poor activity of TfdD$_I$ with 2-chloromuconate (20, 41), about one-third of the 3-CB added was excreted as

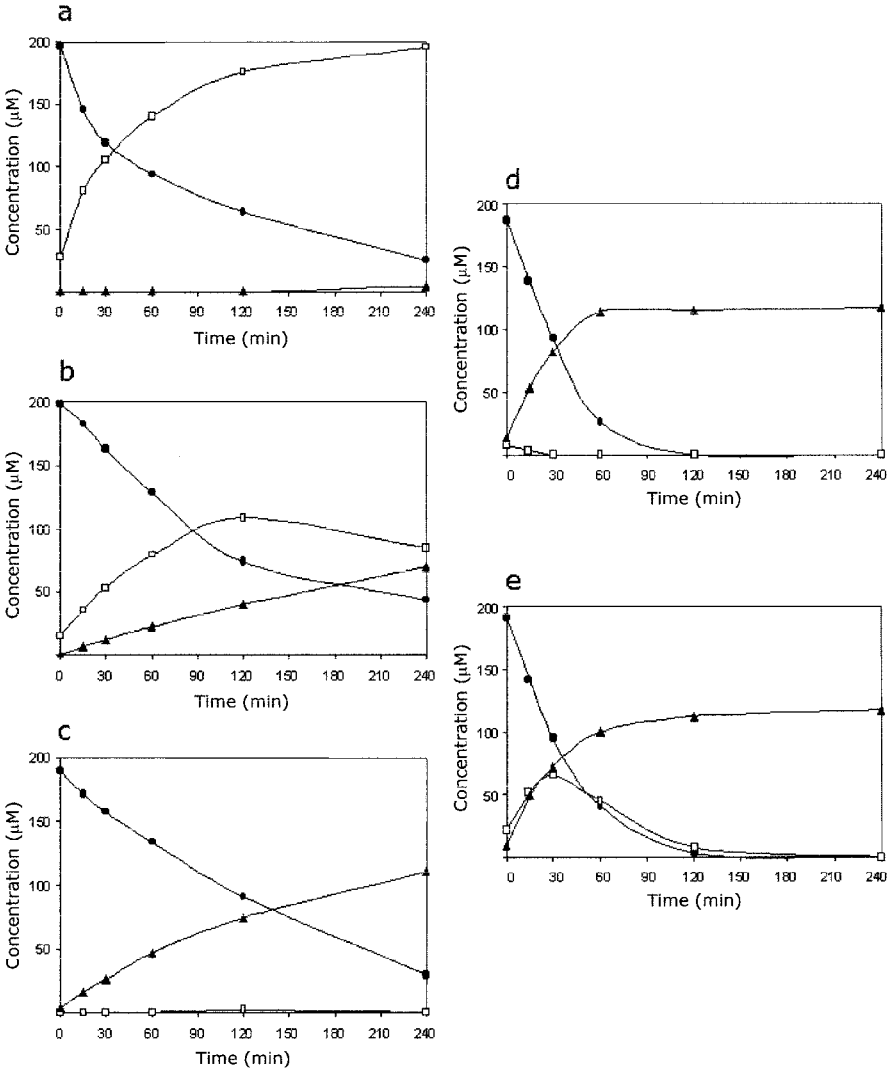

FIG. 3. Accumulation of chlorocatechols and 2-chloromuconate from 3-CB. Chlorinated intermediates were detected by HPLC by using samples of supernatants after incubation of 0.2 mM 3-CB with preinduced cell suspensions (OD_{600}, 0.5) of strains JMP222 (a), JMP222::R1TFD (b), JMP222::R1TFD(pBBRCI) (c), JMP134(pJP4) (d), and JMP134::X(pJP4) (e). Symbols: ●, 3-CB; □, 3-CC plus 4-CC; ▲, 2-chloromuconate.

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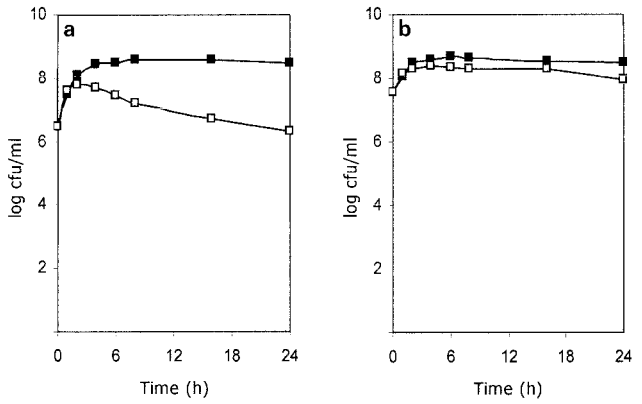

FIG. 4. Effect of 3-CB on cell viability of *R. eutropha* JMP134 derivatives. Cell suspensions of strain JMP222 (a) or JMP222 (pBBRC1) (b) previously grown in 5 mM benzoate were exposed (□) or not exposed (■) to 1 mM 3-CB, and samples were analyzed to determine the number of CFU at different times. The values are averages based on two replicates.

2-chloromuconate (Fig. 3b). To study if accumulation of chlorocatechols occurred because of a limiting specific activity of chlorocatechol 1,2-dioxygenase, the gene dosage of $tfdC_I$ was increased by introducing pBBRCI into strain JMP222::R1TFD. In this derivative, harboring about 10 copies of the $tfdC_I$ gene per cell, no accumulation of chlorocatechols was detected (Fig. 3c), and the 2-chloromuconate accumulation increased to one-half of the 3-CB carbon input. Strain JMP222::R1TFD containing the control plasmid pBBRPI, encoding *tfdR* plus the upstream noncoding region of $tfdC_I$, did accumulate chlorocatechols and 2-chloromuconate (data not shown) at rates and quantities similar to those of strain JMP222::R1TFD, showing that the effect of pBBRCI is not due to activation of the chromosomal copy of the $tfdC_I$ gene driven by the higher *tfdR* gene dosage.

To study the possibility that accumulation of chlorocatechols intoxicates *R. eutropha* cells, the MIC of the two chlorocatechols produced during growth in 3-CB (Fig. 1a) was determined. The MIC of chlorocatechols for growth of *R. eutropha* JMP222 in 5 mM benzoate was 40 μM. The MIC for strain JMP134 was higher, 200 μM. When LB broth was used, the MICs were 200 and 500 μM for strains JMP222 and JMP134, respectively. No significant differences between the effect of 3-CC and the effect of 4-CC were found. The effect of in situ formation of chlorocatechols on cell viability, measured by determining the CFU, was also tested. With cells of strain JMP222 growing on 5 mM benzoate and exposed to 1 mM 3-CB, a 2-order-of-magnitude decrease in cell viability was observed (Fig. 4a). When the same experiment was performed with strain JMP222(pBBRCI), harboring multiple copies of $tfdC_I$, no effect on cell viability was observed (Fig. 4b). These observations strongly indicate that 3-CC and 4-CC actually provoke a toxic effect in *R. eutropha* derivatives.

If multiple copies of the $tfdC_I$ gene prevent accumulation of toxic chlorocatechols in *R. eutropha* derivatives that do not grow in 3-CB because they possess only a chromosomal copy of the chlorocatechol-degrading *tfd* genes, the presence of several copies of $tfdC_I$ in such derivatives should also allow growth in 3-CB. Accordingly, strain JMP222::R1TFD(pBBRC1) could grow in 1 to 10 mM 3-CB as well as strain JMP222(pBBR1M-I) (Fig. 2), but it grew slowly. The presence of pBBRC1 provided levels of chlorocatechol 1,2-dioxygenase similar to that observed when the complete $tfdC_ID_IE_IF_I$ gene module in plasmid pBBR1M-I was present (Table 2). This growth-proficient effect was due to the *tfdR*-driven expression of $tfdC_I$ and not to expression of the chloromuconate cycloisomerase activity encoded by the truncated $tfdD_I^{\nabla}$ gene (data not shown). This effect was also not due to expression (driven by a higher *tfdR* gene dosage) of any chromosomally encoded enzyme activity using chlorocatechols, because strain JMP222::R1TFD(pBBRPI) was not able to grow in 3-CB (Fig. 2) and did not express higher levels of chlorocatechol 1,2-dioxygenase (Table 2).

Imbalance between chlorocatechol-producing and chlorocatechol-consuming reactions in *R. eutropha* JMP134(pJP4) affects growth in 3-CB. As the level of chlorocatechol 1,2-dioxygenase activity is important for growth of *R. eutropha* in 3-CB, inactivation of either of the two *tfdC* genes present in the wild-type strain should affect the ability to grow in 3-CB. To explore this possibility, $tfdC_I$ or $tfdC_{II}$ gene mutants were constructed in strain JMP134(pJP4) by using a procedure that inactivates genes by allelic exchange, followed by nearly precise excision of an antibiotic resistance gene (7). Inactivation of the *tfdC* genes was demonstrated by enzyme assays (Table 2). The derivative with the inactivated $tfdC_I$ gene completely lost the ability to grow in 3-CB (Fig. 5), whereas the mutant with the inactivated $tfdC_{II}$ gene exhibited a less severe effect during growth with this compound (Fig. 5). Both derivatives fully recovered the ability to grow in 3-CB when plasmid pBBRCI was present (data not shown).

Results described above indicate that a decrease in chlorocatechol-transforming activity impairs growth in 3-CB. Therefore, an increase in a chlorocatechol-producing enzyme activity should also have a negative effect on growth with this substrate. To test this possibility, the pWW0-encoded toluate dioxygenase system was introduced into derivatives of strains JMP134(pJP4) and JMP222. The toluate dioxygenase system (*xylS-xylXYZL* gene cluster) encodes the broad-substrate-range

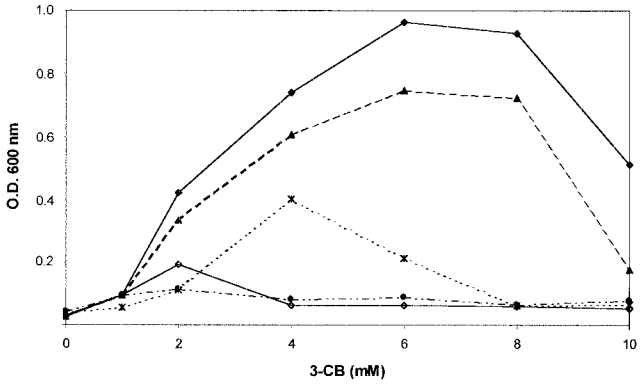

FIG. 5. Growth with different 3-CB concentrations of *R. eutropha* derivatives harboring *xyl* genes or inactivated *tfdC* genes. Symbols: ◆, *R. eutropha* JMP134(pJP4); ◇, *R. eutropha* JMP134::X(pJP4); ●, *R. eutropha* JMP134(pJP4-Δ$tfdC_I$); ∗, *R. eutropha* JMP134(pJP4-Δ$tfdC_{II}$); ▲, *R. eutropha* JMP134::X(pJP4-F3). OD_{600} was measured at the stationary phase. The values are means based on triplicate experiments. The deviations (not shown for clarity) were less than 5 to 10%.

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benzoate 1,2-dioxygenase (*xylXYZ*) and the 1,2-dihydro-1,2-dihydroxytoluate dehydrogenase (*xylL*), regulated by the transcriptional activator *xylS* (Fig. 1a). This enzyme system efficiently catalyzes the conversion of 3-CB to 3-CC and 4-CC (33). By using pSPM100, a pUT derivative containing the *xylS-xylXYZL* genes, several *R. eutropha* transconjugants containing chromosomal insertions of the *xyl* gene cluster were obtained (23). One of the derivatives, strain JMP134::X(pJP4), exhibited significantly less growth in 3-CB than the wild-type strain (Fig. 5). The possibility of a position effect of the *xyl* gene cluster insertion was eliminated because all of the approximately 100 transconjugants tested behaved similarly; this included some transconjugants that contained the insertion of *xyl* genes in the pJP4 backbone. As expected, transient accumulation of chlorocatechols was detected only in strain JMP134::X(pJP4), whereas 2-chloromuconate accumulated almost equally in the wild-type strain and the derivative containing *xyl* genes (Fig. 3d and e). Although adequate expression of XylXYZL activities was observed in strain JMP134::X(pJP4) (23; unpublished data), expected differences in the rates of 3-CB degradation between this strain and strain JMP134 were not detected by using cell suspensions and HPLC analysis and, therefore, are not visible in Fig. 3. Impairment of growth in 3-CB was also observed for strain JMP222::X(pBBR1M-I) and, especially, for strain JMP222::X(pBBR1M-II) (data not shown). The negative effect of introduction of an additional chlorocatechol-producing enzyme activity in strain JMP134 (pJP4) should be balanced by a simultaneous increase in a chlorocatechol-consuming activity. This idea was explored by introducing the *xyl* gene cluster into strain JMP134 harboring pJP4-F3. This pJP4 derivative has a long duplication that includes both the $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$ gene clusters and, therefore, has increased TfdC enzyme activity (5). Accordingly, strain JMP134::X(pJP4-F3) grew in 3-CB much better than strain JMP134::X(pJP4) grew, at levels fairly similar to those of the wild-type strain (Fig. 5).

DISCUSSION

In this study we showed that the ability of *R. eutropha* JMP134(pJP4) to grow in 3-CB is affected by the gene dosage of the chlorocatechol-degrading *tfd* gene clusters, $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$. Here we provide evidence that impairment of growth is primarily due to accumulation of toxic chlorocatechols when a low *tfdCDEF* gene dosage is present. In addition to the effect of the $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$ modules (5, 23, 27, 37; this study), a growth-proficient effect due to an increase in the copy number of chlorocatechol degradation gene clusters has been shown for the *clc* element of *Pseudomonas* sp. strain B13 (31) and the *tcbCDEF* gene cluster of *Pseudomonas* sp. strain P51 (17). In the latter cases, no explanation was provided.

Lower gene dosages of *tfd* gene clusters result in lower Tfd enzyme activities. It is evident that activities encoded by the chlorocatechol degradation genes can become rate limiting. The transformation of 2-chloromuconate has been described previously as a rate-limiting step during degradation of 3-CB by the wild-type strain (28). Both $TfdD_I$ (20, 41) and, more drastically, $TfdD_{II}$ (22, 27, 30) are only poorly suited to transform this substrate. Moreover, *cis*-dienelactone transformation is rate limiting in strains containing only $tfdD_{II}C_{II}E_{II}F_{II}$ (22, 27, 30). Theoretically, accumulation of a metabolite should produce lower growth rates and/or growth yields (as in the cases mentioned above) but not necessarily a lack of growth, as reported here for *R. eutropha* derivatives with single copies of the *tfd* modules. This is the case for the growth yield of strain JMP222 harboring each *tfd* gene module. Strain JMP222 harboring pBBR1M-II and, therefore, encoding a TfdD enzyme activity which is unable to metabolize 2-chloromuconate (22, 30) produced one-half the growth yield produced by strain JMP222 harboring pBBR1M-I, in which the level of the TfdD enzyme is low but the enzyme is active (Fig. 2). However, if the accumulated metabolite is toxic, a significant loss of the ability to grow, especially at high concentrations, can be expected. As the wild-type strain is able to grow in 3-CB while it accumulates significant amounts of 2-chloromuconate, the possible toxic effect of this metabolite, if there is any, should not be strong enough to prevent growth in 3-CB. In contrast, toxicity of chlorocatechols for bacterial cells has been reported for several organisms (1, 10, 13, 35, 36), including *R. eutropha* (this study). Therefore, the presence of adequate levels of chlorocatechol-consuming enzyme activities should be essential for growth in 3-CB (Fig. 6) and other chloroaromatic compounds as well. These levels should be high enough to prevent chlorocatechol accumulation and avoid a death rate higher than the growth rate. Although some activity with chlorocatechols has been reported for the chromosomally encoded catechol 1,2-dioxygenase (29), transformation of chlorocatechols into the corresponding chloromuconates is performed mainly by the chlorocatechol 1,2-dioxygenase encoded by the $tfdC_I$ and $tfdC_{II}$ genes. The activity of $TfdC_I$ with 3-CC or 4-CC is about two to three times higher than that of $TfdC_{II}$ (27, 30). In this context, it is interesting to propose that differences in TfdC activity are the main reason for impairment of growth at high 3-CB concentrations for *R. eutropha* JMP222 harboring $tfdC_ID_IE_IF_I$ or $tfdD_{II}C_{II}E_{II}F_{II}$ (27) (Fig. 2). Although differences in growth rates between the two types of derivatives may be explained in part by differences in the corresponding TfdD and TfdE activities (21, 22, 27, 30), derivatives possessing the more active $TfdC_I$ enzyme exhibit higher growth yields and use higher concentrations of 3-CB than derivatives with the less active $TfdC_{II}$ enzyme. The catabolic phenotypes of the $tfdC_I$ and $tfdC_{II}$ mutants agree perfectly with their differential contributions to chlorocatechol turnover (Fig. 5). The growth-proficient effects of the presence of multiple copies of the $tfdC_I$ gene (pBBRCI) in strains harboring pJP4Δ$tfdC_I$, pJP4Δ$tfdC_{II}$, or chromosomal insertions of a *tfd* gene cluster strongly support the idea that chlorocatechol-consuming reactions play the main role in the catabolic performance of *R. eutropha* derivatives with 3-CB. Recent evidence indicates that relatively small differences in TfdC activity result in significant differences in the ability to grow in 3-CB, as observed when growth of the wild-type strain JMP134(pJP4) in 3-CB was compared with growth of strain JMP134-F3 having a duplication of most *tfd* genes (5). Integration of a single copy of pJP4 into the chromosome of strain JMP134, which normally harbors about five copies of pJP4 per genome, did not allow growth of this derivative in 3-CB (37). This finding further supports the observation that a single copy of both *tfd* gene modules is not enough for growth in chlorobenzoate, as also observed for

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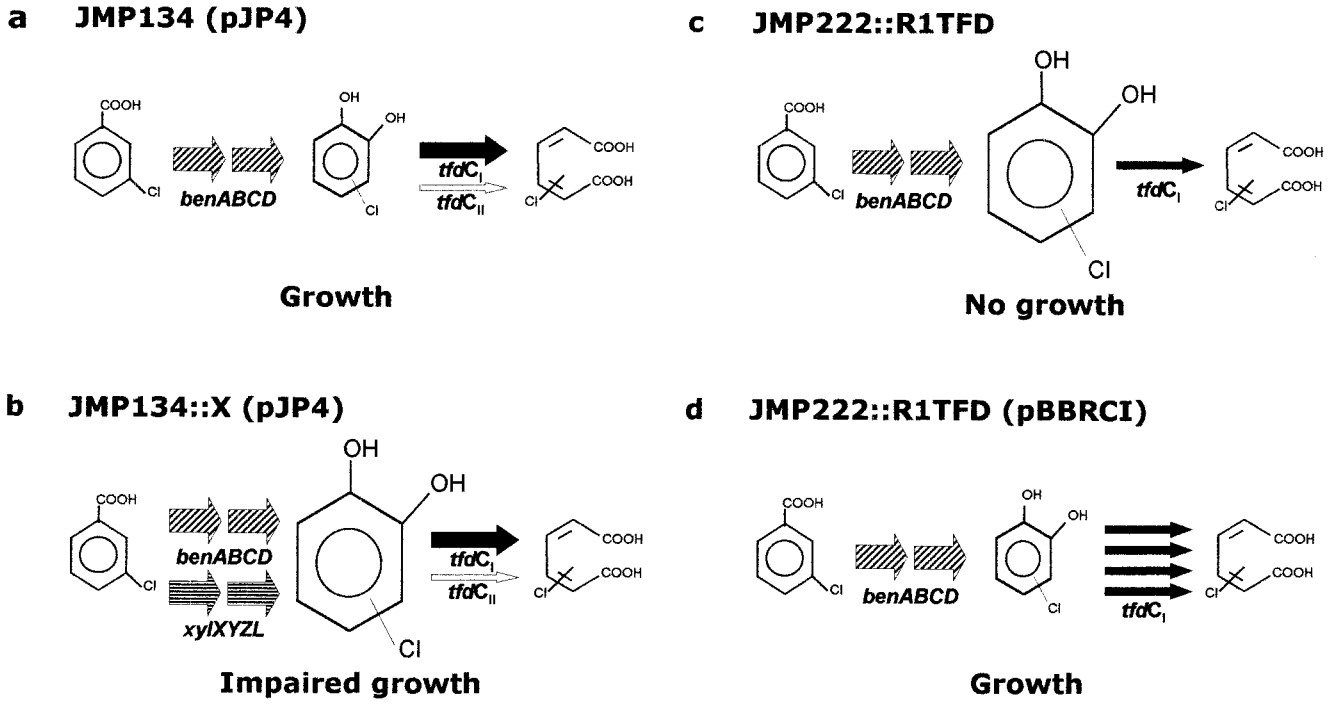

FIG. 6. Imbalance between chlorocatechol-producing and chlorocatechol-consuming reactions results in accumulation of toxic metabolites and in an inability to grow in 3-CB. The relative amounts of chlorocatechols, indicated by the different sizes of molecule diagrams, and the growth phenotypes in 3-CB are shown for strains JMP134(pJP4) (a), JMP134::X(pJP4) (b), JMP222::R1TFD (c), and JMP222::R1TFD(pBBRCI) (d). The arrow thickness indicates the relative specific activity of TfdC proteins.

strain JMP222::R12TFD (this study). These observations clearly indicate that two- to fivefold differences in TfdC activity have a dramatic effect on growth with 3-CB. A threshold copy number higher than two was determined for the *clc* element to allow growth of *Pseudomonas putida* F1 in chlorobenzene (31), and at least two copies of the *tcb* gene cluster are required for growth of *P. putida* KT2442 in 3-CB (17). In this context, it is worth mentioning that it has been speculated that the main reason for the presence of the *tfdR-tfdD*$_{II}$*C*$_{II}$*E*$_{II}$*F*$_{II}$ gene cluster in pJP4 is a requirement for an active regulatory gene (*tfdR*) for *tfd* genes (21, 22). However, results reported here indicate that the presence of two *tfd* gene modules for chlorocatechol degradation that code for the same functions in the pJP4 plasmid, a very unusual case in chloroaromatic compound degradation (38, 40), might also be due to a requirement for higher TfdC activity levels.

Limiting activities of TfdC enzymes may produce not only toxic effects due to accumulation of chlorocatechols but also less expression of *tfd* genes due to lower concentrations of chloromuconates, the inducer molecules for *tfd* genes (24). Therefore, it can be argued that differences in growth in 3-CB may be due to differential expression of *tfd* genes and not to toxic effects of chlorocatechols. However, the available evidence indicates that limited *tfd* expression is a less important factor in the accumulation of chlorocatechols than copy number changes are. Accumulation of chlorocatechols does not necessarily mean no formation of chloromuconates in strains having *tfdC* genes. The low or null activities of TfdD proteins with 2-chloromuconate result in accumulation of inducer levels that are up to 50% of the 3-CB input (Fig. 3), which are enough for induction, as has been shown for chlorocatechol-degrading gene systems (26; L. Guzmán and B. González, unpublished results). On the other hand, strains JMP134 and JMP134::X(pJP4) accumulate essentially the same levels of chloromuconates (Fig. 3d and e) and, therefore, presumably have similar levels of activation of *tfd* gene expression. However, only strain JMP134::X(pJP4) accumulates chlorocatechols because it has a higher copy number of chlorocatechol-producing genes. In addition, when a single copy of the *tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ gene module, cloned under control of the P_{tac}/LacI heterologous regulatory system and therefore independent of chloromuconates, was introduced into strain JMP222, the cells expressed higher TfdC levels than strain JMP222::R1TFD expressed (4). These cells also produced chloromuconate at fairly normal levels but still did not grow in 3-CB (4). Similar results were obtained for JMP222 derivatives harboring the *tfdC*$_I$*D*$_I$*E*$_I$*F*$_I$ gene module under the control of another heterologous regulatory system, P_{sal}/NahR (C. Varela, R. Céspedes, and B. González, unpublished results).

If chlorocatechol-consuming conversions are important for accumulation of the intermediates, chlorocatechol-producing reactions should also play a role. *R. eutropha* strains possess a chromosomally encoded benzoate dioxygenase system that is fairly active with 3-CB (28, 33). In fact, when strain JMP222 (but not strain JMP134) was exposed to 3-CB, chlorocatechols rapidly accumulated (Fig. 3). This behavior has also been reported for another pJP4-free strain JMP134 derivative, strain JMP289 (25). Chlorocatechol production should be essentially the same in derivatives harboring single and multiple copies of the chlorocatechol-degrading genes, thus leading to greater

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accumulation of chlorocatechols if single copies of *tfd* genes are present (Fig. 6a and c). Such an imbalance is corrected when additional copies of a *tfdC* gene are provided (Fig. 6d). In this context, it was interesting to study the effect of the introduction into *R. eutropha* of an additional enzyme system producing chlorocatechols from 3-CB, i.e., the *xylS-xylXYZL* system from plasmid pWW0. The plasmid-encoded activity reported for *P. putida* mt-2 has an activity with 3-CB that is higher than the chromosomally encoded *R. eutropha* enzyme activity (33). Our results clearly show that the *xyl* gene-encoded toluate dioxygenase system produces an imbalance in chlorocatechol turnover, leading to accumulation of chlorocatechols and impairment of growth in the presence of 3-CB (Fig. 6b).

The results reported here have significant implications concerning the evolution of chloroaromatic compound metabolism pathways and also construction of chloroaromatic compound-degrading bacterial strains. For a long time, it has been known that many aerobic chloroaromatic compound catabolic pathways consist of diverse peripheral reactions that produce the corresponding chlorocatechols and central pathways which channel the chlorocatechols into the energy-producing intermediate metabolism (32, 34). In such a way, a microorganism can use a chloroaromatic compound as a sole carbon and energy source. Therefore, it has been assumed in several cases that acquisition of a chlorocatechol-degrading gene cluster by bacterial strains having peripheral, chlorocatechol-producing reactions may allow use of a specific chloroaromatic compound as a carbon source. There are several cases which show that this assumption is correct (32). However, there are also reports indicating that the expected growth property was not observed (8, 11, 12, 14, 16, 17, 32). In some of these cases, it has been proposed that impaired gene expression of catabolic genes in heterologous hosts is the reason for such failures (8, 11, 12, 16). Our results clearly suggest that intoxication due to unbalanced turnover of chlorocatechols should also be considered an important factor. In this context, it is worth noting that the catabolic plasmid pP51 codes for both the chlorocatechol-producing and chlorocatechol-consuming enzyme activities (39). Thus, if changes in copy number occur, the normal balance is not altered.

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