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ORIGINAL PAPER

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Mutation analysis of the different *tfd* genes for degradation of chloroaromatic compounds in *Ralstonia eutropha* JMP134

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Abstract *Ralstonia eutropha* JMP134 possesses two sets of similar genes for degradation of chloroaromatic compounds, *tfdCDEFB* (in short: tfd_I cluster) and $tfdD_{II}C_{II}E_{II}F_{II}B_{II}$ (tfd_{II} cluster). The significance of two sets of *tfd* genes for the organism has long been elusive. Here, each of the *tfd* genes in the two clusters on the original plasmid pJP4 was replaced by double recombination with a gene fragment in which a kanamycin resistance gene was inserted into the respective *tfd* gene's reading frame. The insertion mutants were all tested for growth on 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA), and 3-chlorobenzoate (3-CBA). None of the $tfdD_{II}C_{II}E_{II}F_{II}B_{II}$ genes appeared to be essential for growth on 2,4-D or on 3-CBA. Mutations in *tfdC*, *tfdD* and *tfdF* also did not abolish but only retarded growth on 2,4-D, indicating that they were redundant to some extent as well. Of all *tfd* genes tested, only *tfdE* and *tfdB* were absolutely essential, and interruption of those two reading frames abolished growth on 2,4-D, 3-CBA (*tfdE* only), and MCPA completely. Interestingly, strains with insertion mutations in the tfd_I cluster and those in $tfdD_{II}$, $tfdC_{II}$, $tfdE_{II}$ and $tfdB_{II}$ were severely effected in their growth on MCPA, compared to the wild-type. This indicated that not only the tfd_I cluster but also the tfd_{II} cluster has an essential function for *R. eutropha* during growth on MCPA. In contrast, insertion mutation of $tfdD_{II}$ resulted in better growth of *R. eutropha* JMP134 on 3-CBA, which is most likely due to the prevention of toxic metabolite production in the absence of $TfdD_{II}$ activity.

Keywords *Ralstonia eutropha* JMP134 · *tfd* genes · Insertion mutagenesis · 2,4-Dichlorophenoxyacetic acid · 2-Methyl-4-chlorophenoxyacetic acid · 3-Chlorobenzoate

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Introduction

Ralstonia eutropha JMP134 (pJP4) was originally isolated from an unspecified soil sample based on its ability to use the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) as sole carbon and energy source (Don and Pemberton 1981; Don et al. 1985). The strain can grow on various other haloaromatic compounds, such as 3-chlorobenzoate (3-CBA) (Don et al. 1985) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (Pieper et al. 1988). The initial degradation steps of 2,4-D and MCPA involve an oxidation at the ether bond by means of an α-ketoglutarate dependent dioxygenase (encoded by *tfdA*), which leads to the release of glyoxylate and the formation of 2,4-dichlorophenol and 2-methyl-4-chlorophenol, respectively (Streber et al. 1987; Pieper et al. 1988) (Fig. 1). The chlorophenols from 2,4-D and MCPA are then oxidized by a hydroxylase (encoded by *tfdB* and possibly also by $tfdB_{II}$) to form 3,5-dichloro- and 3-methyl-5-chlorocatechol, respectively. 3-CBA is oxidized at the 1,2- or 1,6-position by a benzoate dioxygenase and then dehydrogenated to form approximately two-thirds 3-chloro- and one-third 4-chlorocatechol (Pieper et al. 1993). *tfdA* and *tfdB* are located on the plasmid pJP4 (Streber et al. 1987; Perkins et al. 1990), whereas the genes for conversion of 3-CBA to chlorocatechol are presumed to be chromosomally located (Laemmli et al. 2002). Although different enzymes are used in the initial degradation steps, metabolism of 2,4-D, MCPA, and 3-CBA all proceeds via chloro- (methyl-) substituted catechols (Fig. 1). The chloro- (methyl-) catechols are transformed by the so-called modified *ortho*-cleavage pathway enzymes, which

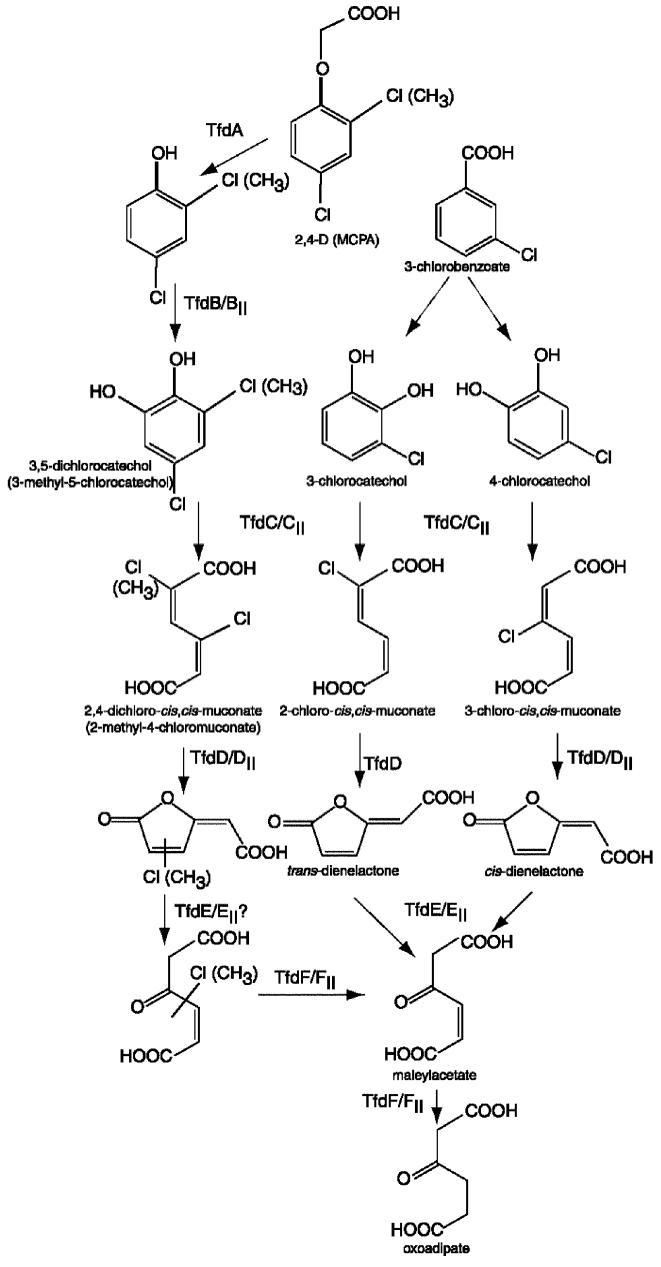

Fig. 1 Metabolic pathways for 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 3-chlorobenzoate by *Ralstonia eutropha* JMP134 (pJP4). Individual intermediates in each of the pathways are displayed as far as known, with assignment of the respective Tfd-enzymes catalyzing the reaction. For simplicity, not all intermediate steps in muconate conversion are drawn. The pathway for MCPA degradation follows that of 2,4-D and the position of the methyl group in MCPA and its intermediates is shown within *parentheses*. Details of the reactions catalyzed by TfdE and $TfdE_{II}$ with chloro- or methyldienelactones are not known. *TfdA* α-Ketoglutarate-dependent dioxygenase, *TfdB(B_{II})* chlorophenol hydroxylase, *TfdC(C_{II})* chlorocatechol 1,2-dioxygenase, *TfdD(D_{II})* chloromuconate cycloisomerase, *TfdE(E_{II})* dienelactone hydrolase, *TfdF(F_{II})* maleylacetate reductase

are also encoded on plasmid pJP4. Interestingly, pJP4 harbors two homologous sets of genes encoding the transformation of chloro- (methyl-) phenol via chloro- (methyl-) catechols through the modified *ortho*-cleavage pathway. These genes are called *tfdCDEFB* (in short, the tfd_I cluster) and $tfdD_{II}C_{II}E_{II}F_{II}B_{II}$ (in short, tfd_{II}).

Initially, only the *tfdCDEFB* genes had been identified through transposon mutagenesis to be responsible for growth on 2,4-D (Don et al. 1985). Long afterwards, the $tfdD_{II}C_{II}E_{II}F_{II}B_{II}$ cluster was identified by DNA sequencing (Laemmli et al. 2000). Both gene clusters lay in a 22-kb region on plasmid pJP4 (Fig. 2), and it was proposed that the second cluster had been more recently inserted on pJP4 by the action of a transposable element formed by one intact and one partial copy of IS*JP4* (Leveau and van der Meer 1997). The potential role of the second gene cluster for *R. eutropha* has become subject of a debate (Laemmli et al. 2000; 2002; Pérez-Pantoja et al. 2000; Plumeier et al. 2002; Schlömann 2002). Heterologous expression studies showed that all genes from the tfd_{II} cluster produce active enzymes in *Escherichia coli,* with enzymatic activities expected based on sequence similarities to their counterparts in the tfd_I cluster (Laemmli et al. 2000). Transcription studies indicated that the genes from the tfd_I and from the tfd_{II} cluster are actively transcribed during growth on 2,4-D (Leveau et al. 1999). More recently, Plumeier et al. (2002) separated and partially purified the enzyme fractions from *R. eutropha* JMP134 grown on 2,4-D and demonstrated that the cluster II enzymes were indeed synthesized, except perhaps $TfdE_{II}$, the dienelactone hydrolase, which contributed to only 5% of dienelactone hydrolase activity in cell extracts. The relative activities of the chlorocatechol 1,2-dioxygenase $TfdC_{II}$ compared to TfdC, and of the chloromuconate cycloisomerase $TfdD_{II}$ compared to TfdD in 2,4-D grown cells amounted to 20%, and that of $TfdF_{II}$ compared to TfdF even to 50% (Plumeier et al. 2002). No hydroxylase activity (by TfdB or $TfdB_{II}$) was purified in this study. These data allowed the conclusion that *R. eutropha* JMP134 uses both *tfd* clusters during growth on 2,4-D, even though one or more 2,4-D-degrading enzymes may actually be redundant. To draw more conclusive evidence about the necessity, specificity, and differences between the two *tfd* clusters, each of the *tfd* clusters was cloned individually in a *R. eutropha* strain devoid of plasmid pJP4, such as JMP222 (Pérez-Pantoja et al. 2000) and JMP289 (Laemmli et al. 2002). Since these *R. eutropha* strains lack *tfdA* and *tfdB*, they cannot grow on 2,4-D, but can convert 3-CBA to 3-chloro- and 4-chlorocatechol (CC) (Fig. 1), and CC metabolism could thus be tested by introducing the tfd_I or tfd_{II} cluster. The results of those studies showed that *R. eutropha* JMP222 and 289 carrying a plasmid containing the tfd_I cluster under control of *tfdR* could grow on 3-CBA (Leveau and van der Meer 1996; Pérez-Pantoja et al. 2000; Laemmli et al. 2002). However, *R. eutropha* JMP289 with the tfd_{II} cluster did not grow on 3-CBA as sole carbon source (Laemmli et al. 2002). This was attributed to an inability of $TfdD_{II}$ to convert 2-chloromuconate, to the formation of possible toxic intermediates, and to a very low activity of $TfdE_{II}$ (Laemmli et al. 2002; Plumeier et al. 2002; Pérez-Pantoja et al. 2003). Others have reported that *R. eutropha* JMP222 provided with the tfd_{II} cluster could grow on 3-CBA (Pérez-Pantoja et al. 2000), but no

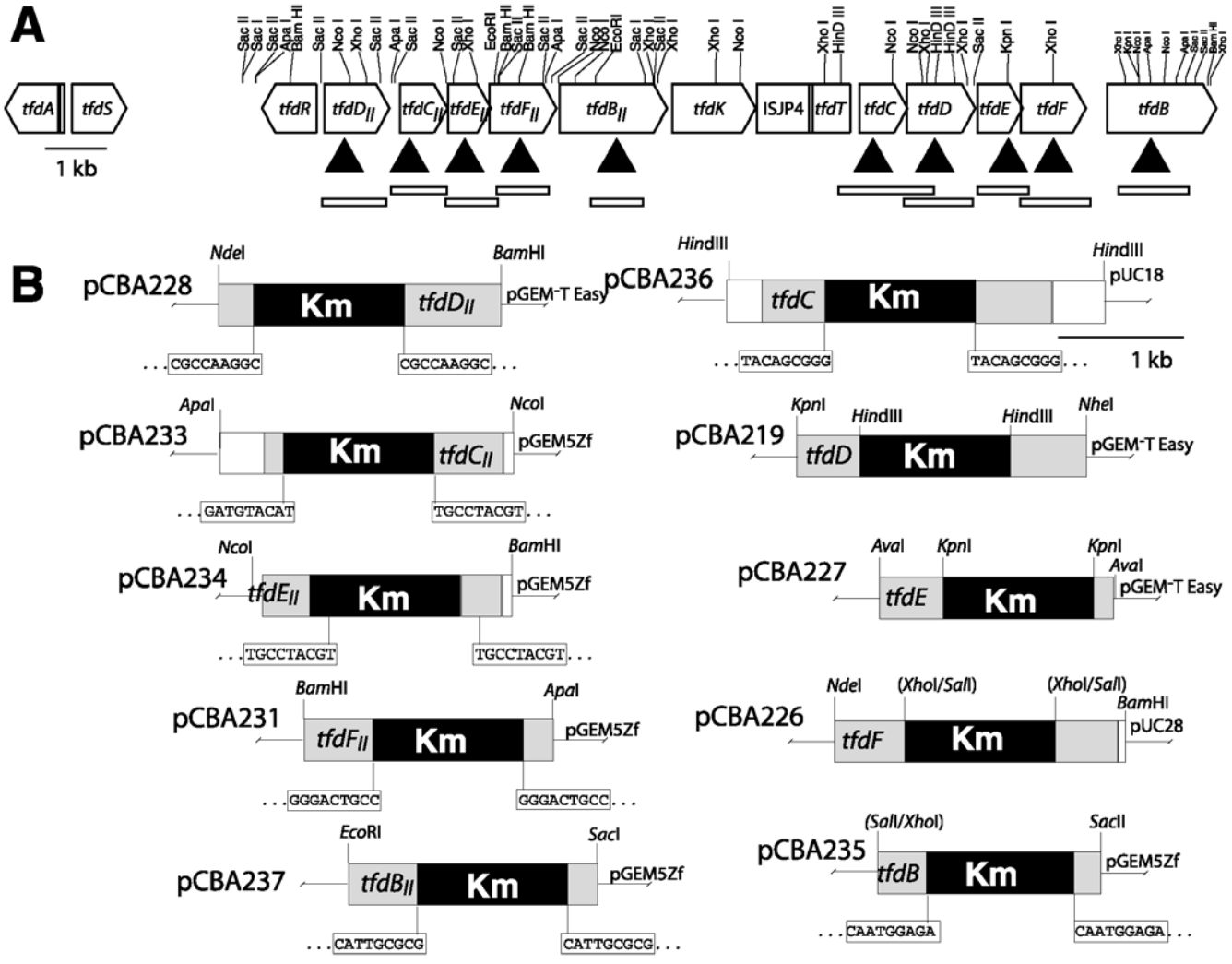

Fig. 2 **A** The location, orientation, and size of all known *tfd* genes on plasmid pJP4, including relevant restriction sites and fragments used for cloning (depicted as white *bars*). *Black triangles* indicate the approximate kanamycin gene insertion sites. **B** Maps and designation of the plasmids used in this study for construction of the *tfd* knockout mutants, and the exact insertion sites of the kanamycin gene (exemplified as a *black box*). *Gray zones* indicate coding area of the target gene, *white zones* that of non-target coding areas present on the same cloned fragment. Nucleotides duplicated by the kanamycin gene in vitro insertion are *boxed*

satisfactory explanation has been given for the different behavior of strains JMP222 and JMP289.

One disadvantage of the studies with separately cloned *tfd* clusters in *R. eutropha* was that only functional differences with respect to 3-CBA and its central intermediates 3-CC and 4-CC could be investigated. The objective of the present study was to analyze the function of each *tfd* gene in either gene cluster with respect to metabolism of 2,4-D and MCPA. This would reveal whether functional differences exist between the gene products of the two *tfd* clusters with respect to metabolism of chlorophenols, 3,5-dichloro- and 3-methyl-5-chlorocatechol. In order to achieve this, mutants of *R. eutropha* JMP134 were constructed by directly inactivating each gene of the tfd_I and tfd_{II} cluster on the original pJP4 plasmid. The growth behavior of these mutants on 2,4-D, 3-CBA and MCPA was determined and further confirmed by expression analysis of the *tfd* genes in the knockout mutants through RNA hybridizations and by enzyme activity measurements.

Materials and methods

Bacterial strains and cultivation conditions

R. eutropha JMP134 (pJP4) uses 2,4-D, 3-CBA, and MCPA as sole carbon and energy sources (Don and Pemberton 1981; Don et al. 1985). *Escherichia coli* DH5α (Sambrook et al. 1989) was used for cloning purposes. *E. coli* cultures were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml), kanamycin (100 µg/ml), or tetracycline (10 µg/ml).

R. eutropha JMP134 and mutant cultures were grown at 30 °C in nutrient broth (Biolife, Milan, Italy), one-tenth strength nutrient broth, or in *Pseudomonas* type 21C mineral medium (Gerhardt et al. 1981) supplemented with either 10 mM fructose, 2.5 mM 2,4-D, 3 mM 3-CBA, or 3 mM MCPA, and kanamycin (100 µg/ml) if necessary. Growth experiments were carried out in triplicate in 200-ml Erlenmeyer flasks. Growth was monitored by measuring the turbidity of a culture sample at 546 nm (OD_{546}) against medium, or in 96-well microtiter plates. In case of colorization of the medium after growth on chloroaromatic compounds, the OD_{546} of the spent

Table 1 Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pUC28		
pGEM-T-Easy		Promega
pGEM5Zf		Promega
pRME1	Kanamycin resistance gene of Tn*903*, flanked by symmetrical restriction sites	(Harayama et al. 1986)
pCBA10	1.4-kb *Hin*dIII fragment of pCBA4 containing *tfdC* and part of *tfdD*	This work
pCBA83III	1 kb *Apa*I-*Nco*I fragment of pCBA83 (Leveau et al. 1999) in pGEM5Zf, containing $tfdC_{II}$	(Laemmli et al. 2000)
pCBA83IV	0.8-kb *Nco*I-*Bam*HI fragment of pCBA83 in pGEM5Zf, containing $tfdE_{II}$ and a small part of $tfdF_{II}$	(Laemmli et al. 2000)
pCBA84III	0.5-kb *Bam*HI-*Apa*I fragment of pCBA84 in pGEM5Zf, containing an internal piece of $tfdF_{II}$	(Laemmli et al. 2000)
pCBA96	0.85-kb *Eco*RI-*Sac*I fragment of pCBA84 in pGEM7Zf, containing an interal $tfdB_{II}$ fragment	(Leveau et al. 1999)
pCBA102	0.4-kb *Dra*I-*Xho*I fragment of *tfdB* in pGEM5Zf	(Leveau et al. 1999)
pCBA127	$tfdD_I$ in pGEM-T-Easy	(Laemmli et al. 2002)
pCBA219	1.2-kb *Hin*dIII fragment of pRME1 into the internal *Hin*dIII site in *tfdD* of pCBA127	This work
pCBA221	$tfdF_I$ ORF amplified by PCR into pGEM-T-Easy	This work
pCBA225	Open reading frame of *tfdE* amplified by PCR and cloned into pGEM-T-Easy	This work
pCBA226	1.2-kb *Kpn*I fragment of pRME1 into the internal *Kpn*I site in *tfdE* of pCBA225	This work
pCBA227	1.2-kb *Sal*I fragment of pRME1 into the internal *Xho*I site in *tfdF* of pCBA221	This work
pCBA231	EZ::TN <KAN-2>insertion into $tfdF_{II}$ on pCBA84III	This work
pCBA233	EZ::TN <KAN-2>insertion into $tfdC_{II}$ on pCBA83III	This work
pCBA234	EZ::TN <KAN-2>insertion into $tfdE_{II}$ on pCBA83IV	This work
pCBA235	EZ::TN <KAN-2>insertion into *tfdB* on pCBA102	This work
pCBA236	EZ::TN <KAN-2>insertion into *tfdC* on pCBA10	This work
pCBA237	EZ::TN <KAN-2>insertion into $tfdB_{II}$ on pCBA96	This work

medium was measured separately. These growth experiments were repeated four times with independently grown precultures. Growth of *R. eutropha* mutants on the various substrates was also determined in 96-well polystyrene plates. Five µl of a preculture grown on nutrient broth with kanamycin was diluted in 250 µl medium in a plate well. Cultures were inoculated in six-fold repetition on each of the four growth substrates (one-tenth strength nutrient broth, minimal medium plus 2,4-D, MCPA, or 3-CBA) or on minimal medium alone, and on three different plates, which were started with an 8-h time difference. The 96-well plates were closed with a lid that was taped extensively with Parafilm to avoid evaporation and were incubated at 30 °C with 200 rpm rotation. Growth of the cultures was followed by measuring the OD_{546} in a Rainbow Microwell plate reader. Growth experiments in 96-well plates were repeated at least twice for each mutant independently. After growth in 96-well plates, the cultures were checked by PCR for the correct insertion mutation (see below).

DNA and RNA manipulations

Plasmid isolations from *E. coli* DH5α, genomic DNA isolation, transformations, and other DNA manipulations were carried out according to established procedures (Sambrook et al. 1989). Restriction enzymes and other DNA modifying enzymes were obtained from Amersham Life Science (Cleveland, Ohio, USA), Sigma, or GIBCO/BRL Life Technologies (Gaithersburg, Md., USA) and used according to the specifications of the manufacturer. Oligonucleotides for the PCR were obtained from Microsynth GmbH (Balgach, Switzerland). The PCR mixtures contained 200 pmol of each primer per ml, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.05% (v/v) W-1, 2 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, and 30 U *Taq* DNA polymerase (Sigma) per ml. DNA was sequenced as described previously (Ravatn et al. 1998), and nucleotide sequences were analyzed with the DNASTAR software (DNASTAR Inc., Madison, Wis., USA). Genomic DNA isolated from *R. eutropha* JMP134 and its mutants was blotted by hydrochloric acid depurination and vacuum-transferred as described by the manufacturer of the VacuGene XL blotting system (Pharmacia, Uppsala, Sweden).

In order to test for polar effects of the inserted kanamycin resistance gene, RNA was isolated from cell pellets of *R. eutropha* JMP134 and its mutants as described previously (Baumann et al. 1996). Cultures for RNA isolation were grown on 10 mM fructose at 30 °C to an OD_{546} of between 0.8 and 0.9, and induced by adding 3-CBA to a final concentration of 1 mM. Induction continued for 2 h, after which the cells were immediately recovered by centrifugation. DNase I-treated RNA samples were spotted on Hybond N+ membranes (Amersham) and hybridized with biotin-labeled antisense RNAs for each of the *tfd* ORFs, except *tfdA* and $tfdD_{II}$, as described elsewhere (Leveau et al. 1999).

Plasmids

For each of the *tfdCDEFB* or $tfdD_{II}C_{II}E_{II}F_{II}B_{II}$ genes, a gene displacement plasmid was constructed containing the gene to be interrupted and the kanamycin resistance gene within it. Since the genes were interrupted with the kanamycin resistance gene only and not by classical transposon mutagenesis, the risk for polar effects was minimized. The plasmids used for construction of the *tfd* gene fragments are listed in Table 1. The location of the different insertions is depicted in Fig. 2. At least 300 bp of the target gene were included on each side of the inserted kanamycin resistance gene in order to facilitate recombination in *R. eutropha* JMP134.

In three cases (*tfdD*, *tfdE*, and *tfdF*) the kanamycin gene was inserted through directional cloning. The kanamycin resistance gene was recovered as a 1.2-kb *Hin*dIII fragment from plasmid pRME1

and inserted into an internal *Hin*dIII restriction site of *tfdD* (in plasmid pCBA127, yielding pCBA219). Similarly, a 1.2-kb *Kpn*I fragment of pRME1 was used to interrupt *tfdE* at its internal *Kpn*I site (in pCBA225, yielding pCBA226). The *tfdF* gene was interrupted by using the internal *Xho*I site, into which the 1.2-kb *Sal*I fragment of pRME1 was inserted (in pCBA221, yielding pCBA227). For the other *tfd* genes, plasmids containing specific gene fragments were in vitro mutagenized using the EZ::TN <KAN-2>insertion kit (Epicentre Technologies, Madison, Wis., USA). After transformation into *E. coli* and subsequent plasmid isolations, the insertion sites of the kanamycin resistance gene were verified on agarose gel and by DNA sequencing, using primers facing outwards from the kanamycin gene (according to instructions of the supplier). This resulted in plasmids pCBA231, 233, 234, 235, 236, and 237 (Fig. 2, Table 1).

Construction of gene displacement mutants of *R. eutropha* JMP134

All *tfd* knockout plasmids were transformed individually into *R. eutropha* JMP134 by electroporation. To obtain electrocompetent *R. eutropha* JMP134, the strain was inoculated (1:50) from a freshly grown culture on 2,4-D in 5 ml nutrient broth and grown overnight. From this culture, 0.25 ml were used to inoculate 50 ml nutrient broth. The latter culture was grown at 30 °C to an OD_{546} of 0.5. Subsequently, the cells were placed on ice for 30 min and centrifuged for 15 min at 4,300×*g* and 4 °C. The cell pellet was resuspended with 40 ml of 10% (v/v) ice-cold glycerol/demineralized water solution and the cells were again centrifuged. The pellet was then resuspended in 20 ml of 10% ice-cold glycerol solution, the cells again centrifuged, and the cell pellet resuspended in 200 µl ice-cold 10% glycerol. Prior to electroporation, 40 µl competent cells were mixed with 2 µl plasmid DNA (containing approximately 200 ng DNA) and placed on ice for 1 min. The settings for the electroporation were 25 µF, 200 Ω, and 2.3 kV. Immediately following the pulse, 1 ml nutrient broth was added to the cells, and the cell suspension was removed from the cuvette, transferred to a 12-ml sterile vial, and incubated at 30 °C with shaking. One third of this cell suspension was plated on nutrient agar containing kanamycin for selection after 2 h and the rest after overnight incubation. Typically, between 10 and 50 kanamycin-resistant *R. eutropha* colonies appeared from 200 ng plasmid DNA after 36 h at 30 °C. Single and double recombinants were differentiated by checking for the presence or absence of vector DNA by screening for ampicillin resistance (at 50 µg/ml) or sensitivity. All suspected double recombinants were checked by Southern blotting and by PCR with relevant primers (sequences available on request).

Induction of *tfd* gene expression in *R. eutropha* JMP134 and mutants

R. eutropha JMP134 and a few selected mutants were grown at 30 °C in *Pseudomonas* minimal medium supplemented with 10 mM fructose to an OD_{546} of 0.8–0.9. Induction was achieved by adding 3-CBA, 2,4-D, or MCPA to a final concentration of 1 mM, and strains were incubated for an additional 2 h at 30 °C. Cells were then centrifuged for 15 min at 4,300×*g* (4 °C), washed with 40 ml washing buffer (20 mM Tris-HCl, pH 7) and resuspended in 1 ml washing buffer. The cell suspensions was disrupted by sonication (Branson Sonifer 450; SCAN AG, Basel, Switzerland). One-ml cell suspensions were sonicated (eight times for 5 s each) at an output of 30–40 W, with at least a 1-min pause between pulses. Cell suspensions were then centrifuged for 30 min (4 °C) at 15,000×*g*. The resulting supernatants, referred to as cell extracts, were transferred to a fresh tube and used in enzyme activity assays. Protein concentrations in the cell extracts were determined as described by Bradford (1976), using bovine serum albumin as standard. The enzyme assays were carried out by spectrophotometric methods as described previously (Laemmli et al. 2000). The substrates tested included 3-chloro- (3-CC) and 3,5-dichlorocatechol (3,5-DCC) for chlorocatechol 1,2-dioxygenase, 3-chloromuconate (3-CM) for chloromuconate cycloisomerase, *cis*-dienelactone for dienelactone hydrolase, and maleylacetate for maleylacetate reductase.

Chemicals

3-CC (purity 99%) and 3,5-DCC (purity 99%) were purchased from Promochem (Wesel, Germany). 3-Chloromuconate, *cis*- and *trans*-dienelactone were the kind gift of Dr. Walter Reineke (Bergische Universität-Gesamthochschule Wuppertal, Wuppertal, Germany). Maleylacetate was prepared by alkaline hydrolysis of *cis*-dienelactone (Evans et al. 1971) by mixing 1 ml of 5 mM *cis*-dienelactone with 7.5 µl of 2 N NaOH and incubating for 15 min at room temperature. 2,4-D, 3-CBA and MCPA were purchased from Fluka Chemie (Buchs, Switzerland).

Results

Transcription of *tfd* in the insertion mutants

In order to study possible differences and redundancies in the enzymes of the two modified *ortho*-cleavage pathways encoded by the *tfd* gene clusters I and II, the genes were inactivated individually on plasmid pJP4 in *R. eutropha* JMP134 by insertion mutagenesis with a kanamycin resistance gene. Recombination onto plasmid pJP4 in *R. eutropha* was relatively effective, and for all targeted *tfd* genes double recombinants were obtained at an approximate frequency of 20% of all primary kanamycin resistant transformants. Correct insertion of the kanamycin gene was verified by PCR and Southern hybridization (data not shown). In order to determine whether expression of the *tfd* genes within the JMP134 mutants was impaired by the insertion of the kanamycin gene (polar effects), the abundance of *tfd* mRNA in the different mutants after induction with 3-CBA was analyzed by dot-blot hybridization of total RNA using anti-sense RNA probes for each of the *tfd* genes, except $tfdD_{II}$, *tfdA* and *tfdR*/*S*. Hybridization signals of different RNA dilutions and after different exposure times revealed that insertion of the kanamycin gene did not largely effect expression of the *tfd* genes and that, upon induction with 3-CBA, expression of nearly all of the *tfd* genes in the knockout mutants resembled that of the wild-type (not shown). Except for the relatively poor expression of *tfdF* in strain JMP134 (*tfdD*::Km), there were very few clear differences in expression. However, the difference in strain JMP134 (*tfdD*::Km) could not directly be attributed to insertion of the kanamycin resistance gene, since the expression of *tfdE* in strain JMP134 (*tfdD*::Km) did not seem to be influenced. Thus, it was concluded that the effects of gene displacement by insertion of the kanamycin resistance gene were due to inactivation of that particular *tfd* gene and not to polar effects on other *tfd* genes.

Growth on 3-CBA, 2,4-D and MCPA by *tfd* knockout mutants of strain JMP314 (pJP4)

All insertion mutants were analyzed for growth on fructose (not shown), 3-CBA, 2,4-D, or MCPA as sole carbon and energy source (Fig. 3, Table 2). Growth of the wild-type strain JMP134 (pJP4), which uses all three compounds as sole carbon and energy source, was used as reference. In-

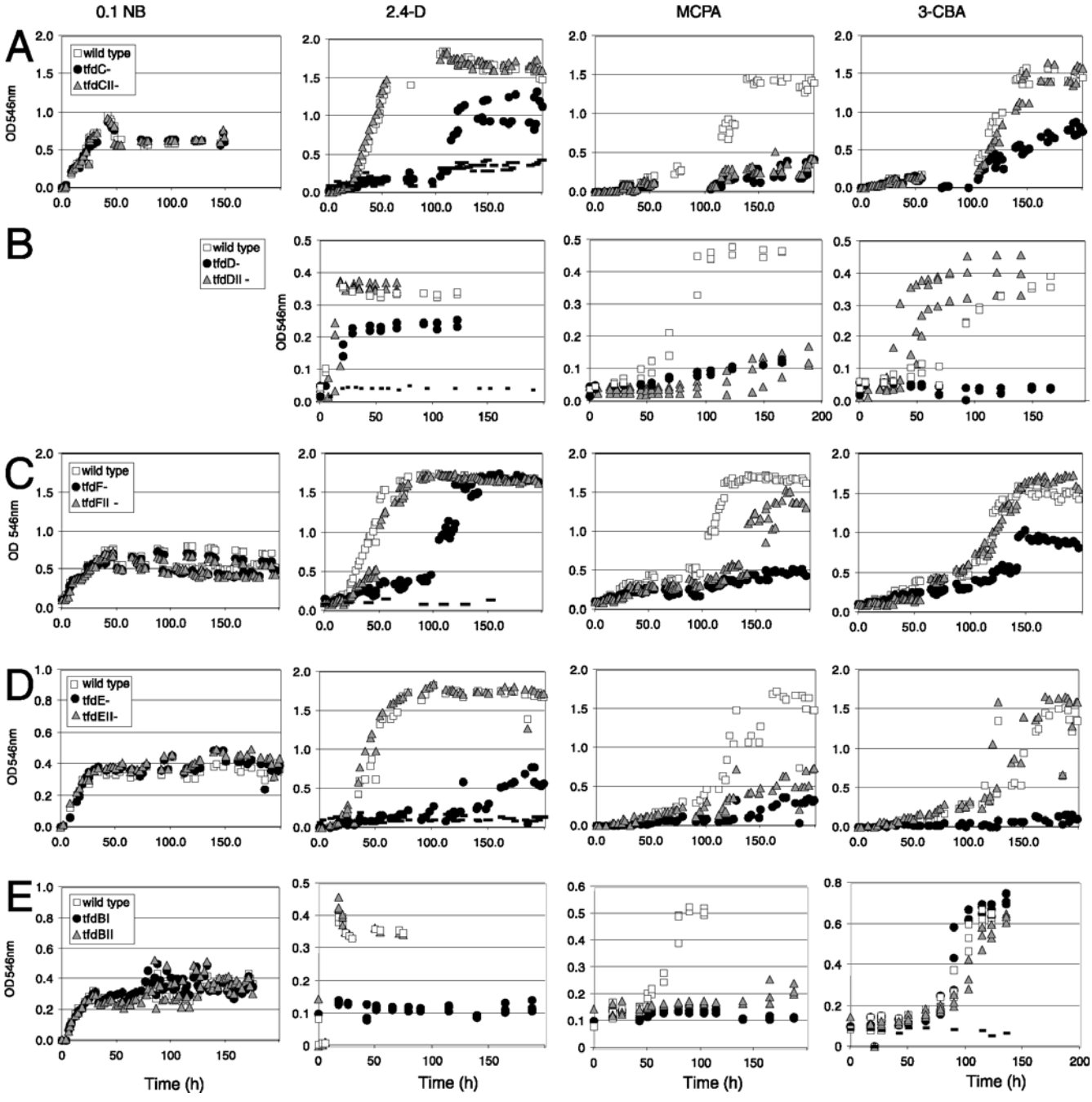

Fig. 3A–E Growth of *R. eutropha* JMP134 (pJP4) and the *tfd* insertion mutants on one-tenth strength nutrient broth or minimal medium supplemented with either 2.5 mM 2,4-D, 3 mM MCPA, or 3 mM 3-CBA. Growth was measured as turbidity at 546 nm, but cannot be compared between **A**, **C**, **D** and **E** NB (experiment in microwell plates), and **B** and **E** 2,4-D/MCPA/3-CBA (Erlenmeyer flasks), due to differences in the optical translucence of the microwell plates and cuvettes used to measure samples from the Erlenmeyer flasks and the different lengths of the optical paths. Data points are means from triplicate measurements of the same incubation series (shown without error bar), but plotted for all independent incubations simultaneously, thus resulting in a 'stuttering' curve. *Open squares* Wild-type JMP134, *closed circles* mutation in tfd_I cluster, *gray triangles* mutation in tfd_{II} cluster gene, *stripes* control incubation with minimal medium only. **A** *tfdC* and $tfdC_{II}$; **B** *tfdD* and $tfdD_{II}$; **C** *tfdF* and $tfdF_{II}$; **D** *tfdE* and $tfdE_{II}$; **E** *tfdB* and $tfdB_{II}$

sertion of the kanamycin gene did not lead to measurable growth retardation in any of the mutants on one-tenth strength nutrient broth in batch cultures (Fig. 3). All mutants also grew well on fructose, indicating that no auxotrophy was generated by accident. The effect of the mutations was different for each of the three chlorinated carbon sources. None of the mutations in *tfd* cluster II genes affected growth of *R. eutropha* on 2,4-D. By contrast, mutations in *tfdC*, *tfdD*, and *tfdF* resulted in growth retardation on 2,4-D, but the cells recovered after a longer lag phase and/or to a lower yield, indicating that the activities of the TfdC, TfdD, and TfdF enzymes can be partially exchanged by $TfdC_{II}$, $TfdD_{II}$, and $TfdF_{II}$, respectively. Mutations in *tfdE* and *tfdB*, however, completely prevented

Table 2 Phenotype of *tfd* mutants in *Ralstonia eutropha* JMP134, summarized for all independent growth experiments. *2,4-D* 2,4-Dichlorophenoxyacetic acid, *MCPA* 2-methyl-4-chlorophenoxyacetic acid, *3-CBA* 3-chlorobenzoate. +++ Faster than wild-type growth, ++ equally fast and equal yield, + less fast and less yield, ± very slight growth, – non-significant growth, *E* essential for growth, *NE* non-essential, *R* redundant (function can partially be fulfilled by counterpart), *I* inhibitory

Mutant	Substrate					
	2,4-D		MCPA		3-CBA	
	Phenotype	Character	Phenotype	Character	Phenotype	Character
$tfdD_{II}$	++	N.E.	±	E	+++	I
$tfdC_{II}$	++	N.E.	–	E	++	N.E.
$tfdE_{II}$	++	N.E.	±	E	++	N.E.
$tfdF_{II}$	++	N.E.	+	R	++	N.E.
$tfdB_{II}$	++	N.E.	±	E	++	N.E.
tfdC	+	R	–	E	+	R
tfdD	+	R	±	E	–	E
tfdE	–	E	–	E	–	E
tfdF	+	R	±	E	+	R
tfdB	–	E	–	E	++	N.E.

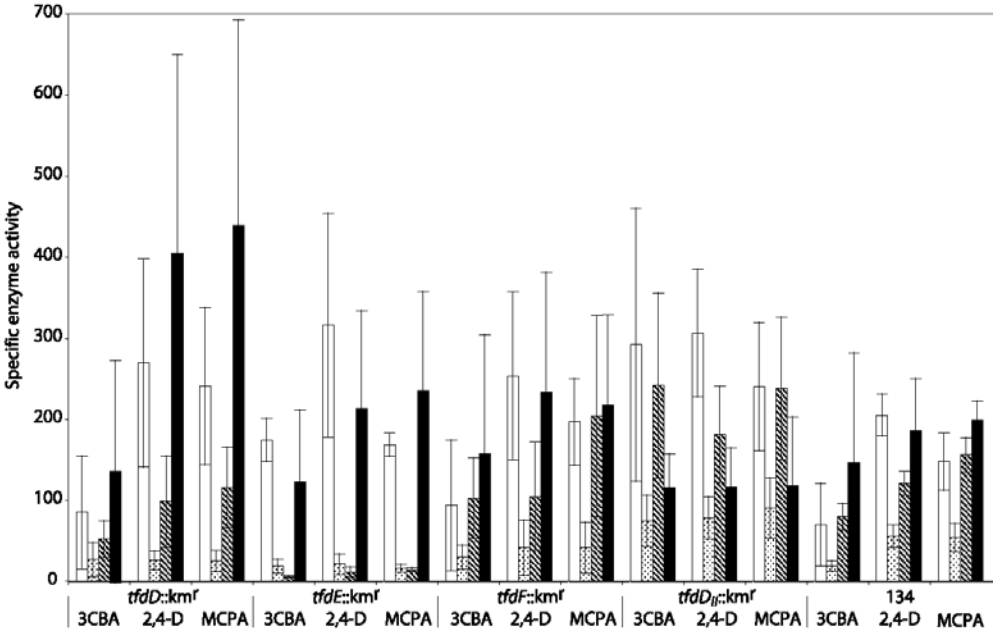

Fig. 4 Specific enzyme activities encoded by the tfd_I and tfd_{II} genes measured in cell extracts of *R. eutropha* JMP134 and several insertion mutants. 1 mU corresponds to 1 nmol of substrate disappearance or product formation per min. *Open bars* Chlorocatechol 1,2-dioxygenase, *stippled bars* chloromuconate cycloisomerase, *hatched bars* dienelactone hydrolase, *black bars* maleylacetate reductase

growth on 2,4-D. In comparison to a minimal medium only control, these mutants did not grow significantly or consistently on 2,4-D. Therefore, *tfdE* and *tfdB* appear to be essential for growth of *R. eutropha* on 2,4-D (Table 2).

A very different behavior was found when *R. eutropha* mutants were incubated with MCPA. Compared to growth of the wild-type strain on MCPA, the growth rates and yields of all of the mutants, except for $tfdF_{II}$, were severely retarded (about 20% of wild-type turbidity after 200 h incubation). The mutant with the $tfdF_{II}$ insertion could still grow on MCPA, but with a considerably longer lag phase, at a slightly lower rate, and with a lower yield (80% of the turbidity of the wild-type culture after 200 h). This was surprising and suggests that either the gene copy number or subtle differences in substrate range between the Tfd enzymes encoded by cluster I and cluster II are important for growth on MCPA. The effect of the different mutations for growth on 3-CBA was more variable. In general, even wild-type *R. eutropha* grew slower and after a longer lag-phase with 3-CBA than with 2,4-D or MCPA. Most mutations in *tfd* cluster II genes did not affect growth rate and yield on 3-CBA, except for $tfdD_{II}$. The $tfdD_{II}$ mutant of *R. eutropha* JMP134 (pJP4) actually grew more consistently and faster on 3-CBA compared to the wild-type strain. Mutations in *tfdC* and *tfdF* did not completely abolish growth on 3-CBA, whereas those in *tfdD* and *tfdE* did. Mutations in *tfdB* or $tfdB_{II}$ did not affect growth on 3-CBA.

Activity of the gene products from the *tfdI* and *tfdII* cluster in mutants of strain JMP134

The activities of the chlorocatechol pathway enzymes were measured in cell extracts of several mutant *R. eutropha* strains as well as of wild-type JMP134 upon induction with 3-CBA, 2,4-D, or MCPA. The three induction substrates were chosen for comparison to the substrates used in the growth experiments. No significant differences in induction level were observed with respect to the induc-

tion substrate (3-CBA, 2,4-D, or MCPA) (Fig. 4), although 3-CBA tended to be the least efficient inducer. However, differences were observed between the strains. In all cell extracts tested, chlorocatechol 1,2-dioxygenase activity (TfdC and $TfdC_{II}$) varied between 100 and 300 mU·(mg·protein)$^{-1}$ and maleylacetate reductase activity (TfdF and $TfdF_{II}$) between 150 and 400 mU·(mg·protein)$^{-1}$ depending on the strain and the induction substrate used. *R. eutropha* strain *tfdD$_{II}$*::Km had the overall highest chloromuconate cycloisomerase (TfdD) and dienelactone hydrolase (TfdE) activities – around 80 mU·(mg·protein)$^{-1}$ and 200–250 mU·(mg·protein)$^{-1}$, respectively, but maleylacetate reductase activity was somewhat reduced. In contrast, *R. eutropha* with an insertion in *tfdE* displayed surprisingly low chloromuconate cycloisomerase (TfdD and $TfdD_{II}$) and dienelactone hydrolase activity ($TfdE_{II}$). Cell extracts of this strain contained chloromuconate cycloisomerase activities ranging between 16 and 21 mU·(mg·protein)$^{-1}$ and no significant dienelactone hydrolase activities (at the level of our detection limit). The absence of dienelactone hydrolase activity ($TfdE_{II}$) would imply that *tfdE$_{II}$* was either not translated or produced a dysfunctional or very unstable protein, since $TfdE_{II}$-activity was measured before in *E. coli* (Laemmli et al. 2000).

Discussion

In terms of its evolution, it was convincingly shown that the *tfd$_{II}$* gene cluster had been inserted into an ancestor *tfd$_{I}$* cluster by means of transposition (Leveau and van der Meer 1997). Hence, the remaining problem has been to demonstrate that the *tfd$_{II}$* gene cluster has a specific value for the strain in terms of chloroaromatic degradation, or else it would have disappeared or genetically changed. Several different approaches, i.e. heterologous cloning in *E. coli*, enzyme purification, transposon mutagenesis, and cloning in *R. eutropha*, were previously taken and it was concluded that the genes from the *tfd$_{II}$* cluster are expressed in *R. eutropha* JMP134 (pJP4) (Leveau et al. 1999; Plumeier et al. 2002), but without obvious selective advantage for the microorganism. Several possible advantages were postulated, such as double gene dosage and thus a higher expression level of the chlorocatechol degradative enzymes (Pérez-Pantoja et al. 2000, 2003; Plumeier et al. 2002), the presence of a 2,4-D uptake system in the *tfd$_{II}$* cluster (Leveau et al. 1998), and of the regulatory genes *tfdR* and *tfdS* (Leveau and van der Meer 1996; Laemmli et al. 2002). Clear negative effects of the *tfd$_{II}$* cluster were also found. For example, the $TfdD_{II}$ enzyme appeared to be incapable of converting 2-CM, one of the possible intermediates in the metabolism of 3-CC (Laemmli et al. 2000; Pérez-Pantoja et al. 2000; Laemmli et al. 2002; Plumeier et al. 2002), which limits growth of *R. eutropha* strains carrying the *tfd$_{II}$* gene cluster on a plasmid on 3-CBA (Laemmli et al. 2002). It could also be shown that 3-CC and 4-CC exert toxic effects on *R. eutropha* when not metabolized rapidly enough (Pérez-Pantoja et al. 2003). Hence, too low expression levels of the chlorocatechol-1,2-dioxygenase TfdC or $TfdC_{II}$ leading to higher than normal 3-CC and 4-CC metabolite production will inhibit growth.

In order to further study the function of each of the *tfd* genes for *R. eutropha* JMP134 (pJP4), we did not use the previously practiced approach of cloning either the *tfd$_{I}$* or *tfd$_{II}$* cluster (or hybrid clusters) on a plasmid in a pJP4-free strain of *R. eutropha*. With these strains it was only possible to test intracellular conversion of chlorocatechols, which would be formed by growing the cells on 3-CBA. Therefore, we constructed mutants of the wild-type strain JMP134 by interrupting each of the *tfd* genes on pJP4 (except *tfdA*, *tfdR*, and *tfdS*) by homologous recombination and gene replacement with a kanamycin resistance gene. The knockout strains were tested for their ability to grow on 2,4-D, 3-CBA, or MCPA as sole carbon and energy source. Compared to the growth behavior of the wild-type strain JMP134, four distinct phenotypes were observed for the knockout mutants: (1) no or very slight growth, (2) less optimal growth than the wild-type strain, (3) growth equal to that of the wild-type strain, and (4) more optimal growth than the wild-type. Based on these phenotypes, all of the *tfd* genes were categorized with respect to their function for metabolism of 2,4-D, 3-CBA and MCPA into essential, not essential, inhibitory, and redundant genes (Table 2). The outcome of these studies was that *tfdE* and *tfdB* are the only essential genes in the *tfd* clusters, since their disruption abolished growth on all three substrates (for *tfdE*) or on 2,4-D and MCPA (for *tfdB*, since TfdB activity is not required for growth on 3-CBA). Apparently, the "counterparts" of *tfdE* and *tfdB* (*tfdE$_{II}$* and *tfdB$_{II}$*) do not replace their functions in *R. eutropha*. No dienelactone hydrolase activity could be measured in induced cell extracts of *R. eutropha* with the *tfdE* interruption, which is in agreement with the lack of observed growth of this strain. Very low $TfdE_{II}$ activity had also been detected in cell extracts of *R. eutropha* JMP134 (pJP4) pregrown on 2,4-D (Plumeier et al. 2002). Since *tfdE$_{II}$* mRNA could be detected at normal levels in *R. eutropha* with the *tfdE*::Km insertion, and since we had previously shown that expression of *tfdE$_{II}$* in *E. coli* (from an optimal promoter and translational fusion system) results in a functional dienelactone hydrolase able to catalyze the conversion of *cis*- and *trans*-dienelactone (Laemmli et al. 2000), we conclude that for some reason the *tfdE$_{II}$* mRNA is either not or very weakly translated in *R. eutropha* or results in a very unstable protein. In this respect, it was surprising to find that the *tfdE$_{II}$* insertion mutant was not able to grow on MCPA, suggesting that weak $TfdE_{II}$ activity is still important for growth on that substrate.

In contrast to the results obtained for *tfdE* and *tfdB*, the *tfdC*, *tfdD* and *tfdF* gene products were not absolutely essential for chloroaromatic growth. For example, *tfdD* expression was only essential for growth on 3-CBA, which can be explained by the fact that the counterpart enzyme, $TfdD_{II}$, is unable to catalyze the conversion of 2-CM, an intermediate in 3-CBA metabolism (Fig. 1), to *trans*-dienelactone (Laemmli et al. 2002; Plumeier et al. 2002). When tested on 2,4-D, the *tfdD* gene was not essential for

growth, but led only to growth retardation and lower yield. Apparently, the *tfdD$_{II}$* gene product is sufficiently active with 2,4-dichloromuconate (Fig. 1) to allow growth on 2,4-D. The *tfdC* and *tfdF* genes turned out to be redundant for growth on 2,4-D as well, and insertion mutants grew with a significantly longer lag phase, but not with a slower growth rate. However, both insertion mutants did have difficulties in growing on 3-CBA. This demonstrates that, depending on the growth substrate, the *tfdC$_{II}$* and *tfdF$_{II}$* gene products can take over the role of their counterparts TfdC and TfdF.

On MCPA, all *tfd$_I$* cluster mutants were essentially unable to grow or grew only very slightly. However, also cells carrying an insertion mutations in the *tfd$_{II}$* gene cluster, except for the *tfdF$_{II}$* insertion mutant, grew at best very slowly on MCPA. Since the wild-type strain grew well on MCPA, and since all mutants displayed different phenotypes on 2,4-D and 3-CBA, their growth behavior on MCPA cannot be an artifact. Hence, this demonstrated that the gene products of *tfd* cluster II play an important role in growth on MCPA and is thus a clear indication for a positive effect for *R. eutropha* JMP134 of carrying the *tfd$_{II}$* gene cluster for chloroaromatic degradation: a widening of the substrate range. While several hypotheses can be raised for the behavior of the mutants of MCPA, they are not completely satisfactory at this moment. First of all, the expression level of the *ortho*-cleavage pathway enzymes could become too low with only one of the gene copies present. This, however, does not seem to be the case, since the level of enzyme activities in mutant *R. eutropha* cultures induced with MCPA were not particularly different from those induced with 3-CBA or 2,4-D, nor from the wild-type (Fig. 4). The second hypothesis is that different substrate specificities of the enzymes from both clusters are needed for MCPA degradation. This, however, would imply that MCPA metabolism follows different pathways, which is not impossible but for which no further evidence exists at the moment (Pieper et al. 1988).

The most pressing problem for *R. eutropha* JMP134 (pJP4) is that expression of *tfdD$_{II}$* is not favorable for growth on 3-CBA. We conclude this from the finding that *tfdD$_{II}$* knockouts grew even more consistently than the wild-type strain on 3-CBA, which indicates that TfdD$_{II}$ activity during metabolism of 3-CBA is inhibitory to the cells, even when TfdD is active. Our results with the *tfdF$_{II}$* insertion mutant are slightly in contrast to those obtained by others. For example, Ledger et al. (2002) constructed a *tfdF$_{II}$* insertion mutant in a strain of *R. eutropha* JMP134 (pJP4) with the additional *xylXYZ* genes. This mutant could not grow on 3,5-dichlorobenzoate, which was taken as an indication for the necessity of *tfdF$_{II}$* in the conversion of 3,5-dichlorocatechol. 3,5-Dichlorocatechol is also an intermediate in 2,4-D degradation. We presently cannot explain this difference. Seibert et al. (1993) purified the chloromaleylacetate reductase activity from *R. eutropha* JMP134 which consisted of the TfdF$_{II}$ protein, suggesting that the TfdF$_{II}$ protein was solely responsible for this activity and thus essential for growth on chloroaromatic compounds. However, more recently, Plumeier et al. (2002) purified both TfdF and TfdF$_{II}$ from *R. eutropha* JMP134 (pJP4) grown on 2,4-D, and showed that each enzyme contributes to about half of the total maleylacetate reductase activity. Hence, insertion mutants in either *tfdF* or *tfdF$_{II}$* would still be able to grow on 2,4-D.

The results of the present study have helped to elucidate further subtle differences in the role of the *tfd$_I$* and *tfd$_{II}$* gene clusters for chloroaromatic degradation by *R. eutropha* JMP134 (pJP4). From an evolutionary standpoint, the presence of two orthologous gene clusters on plasmid pJP4 is interesting and exemplary for various other microbial systems, and is always assumed to have some selective advantage. Summarizing all previous data, would lead to the conclusion that indeed some functional differences exist, but they also mostly argue against keeping the *tfd$_{II}$* cluster. Our previous hypothesis had therefore been that maintaining the configuration of two *tfd* clusters on plasmid pJP4 must somehow provide a selective disadvantage of deleting the *tfd$_{II}$* part (Laemmli et al. 2002). Deletion of the *tfd$_{II}$* cluster (most easily envisioned through recombination between the ends of the IS*JP4* elements) would result in loss of the *tfdR* and *tfdS* regulatory genes, which would affect efficient chloroaromatic degradation. However, as was discovered in the present study, there is another clear metabolic advantage for keeping the *tfd$_{II}$* cluster genes, namely, the degradation of MCPA.

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