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Involvement of different transcriptional regulators in the differential expression of *tfd* genes in *Cupriavidus necator* JMP134.

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Running title: Genes *tfd* in *C. necator* JMP134

Summary. *Cupriavidus necator* JMP134 has been extensively studied because of its ability to degrade chloroaromatic compounds, including the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and 3-chlorobenzoic acid (3-CB), which is achieved through the pJP4-encoded chlorocatechol degradation gene clusters: $tfdC_ID_IE_IF_I$ and $tfdD_{II}C_{II}E_{II}F_{II}$. We found a different *tfd*-genes expression profile when *C. necator* cells were induced with 2,4-D or 3-CB. However, *in vitro* binding assays of the purified transcriptional activator TfdR showed similar binding to both *tfd* intergenic regions and *in vivo* studies of expression of transcriptional *lacZ* fusions for these intergenic regions confirmed these results. We investigated if other pJP4 plasmid or chromosomal regulatory proteins could contribute to the differences in the response of both *tfd* promoters to induction by 2,4-D and 3-CB. We found that the transcriptional regulators from the benzoate degradation pathway, CatR1 and CatR2, affect 3-CB and 2,4-D growth capabilities. We also found that ISJP4-interrupted protein TfdT decreases growth on 3-CB. In addition, an ORF with 34% amino acid identity with IclR-type transcriptional regulator members, located near to the tfd_{II} gene cluster module, modulates the 2,4-D growth capability. Taken together, these results suggest that *tfd* transcriptional regulation in *C. necator* JMP134 is far more complex than was previously thought and it involves proteins from different transcriptional regulator families. [**Int Microbiol** 2009; 12(2):XXX-XXX]

Key words: *Cupriavidus necator* · LysR transcriptional regulators · pJP4 catabolic plasmid · *tfd* catabolic genes

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Introduction

In the last century, a great variety of xenobiotic compounds have been released to the environment, leading to the question of how bacteria evolve regulated catabolic pathways that allow them to use these novel man-made products as carbon and energy source. *Cupriavidus necator* JMP134 is a model for aromatic compounds degradation in bacteria [31]. This bacterium can degrade a wide range of aromatic compounds including the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), 3-chlorobenzoate (3-CB), 4-fluorobenzoate, 2,4,6-trichlorophenol, 4-chloro-2-methylphenoxyacetate (MCPA), among many others [31]. The key catabolic abilities towards 2,4-D and 3-CB are encoded on the plasmid pJP4 [7, 41], by the two not completely isofunctional, *tfdC$_I$D$_I$E$_I$F$_I$* (*tfd-I*) and *tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$* (*tfd-II*), gene clusters. It has been suggested that this novel genetic organization in the catabolic plasmid pJP4 is explained by the evolution of specialized chloroaromatic degradation pathways [41]. Although the *tfd* genes and their catabolic enzymes has been widely investigated [12,15, 16, 17, 21, 29, 30, 31, 32, 33, 34, 38, 39, 46], knowledge on transcriptional regulation of the *tfd* genes is scarce and mostly deduced from studies made on similar systems from other catabolic operons. (Chloro)aromatic degradative operons studied so far [3, 28, 43] possess a regulatory element of the LysR family, located upstream and transcribed divergently from the regulated genes. In *C. necator* JMP134, the identical LysR family *tfdR* and *tfdS* genes [21, 41] are located upstream to module *tfd-II*, while another LysR family gene, *tfdT*, is located upstream to module *tfd-I*. The role of TfdR as regulator of the *tfd-I* module and the *tfdA* and *tfdB* genes has been reported [13]. There is evidence supporting that expression of *tfdC$_I$* gene is not activated by *tfdT* gene, because this regulatory element is ISJP4-inactivated and this function could be taken over by *tfdR* gene [18]. Both modules, cloned independently and regulated by *tfd*R gene, allowed chlorocatechol metabolism in *C. necator* derivatives growing on 3-CB [29]. A genetic approach has suggested that 2,4-dichloromuconate, the product of TfdC protein during metabolism of 2,4-D, may be the inducer that interacts with TfdR [9].

The purpose of this work was to determine if differential expression of *tfd* genes occurs in response to 2,4-D or 3-CB in *Cupriavidus necator* JMP134, and to assess the role of different transcriptional regulators in this phenomenon.

Materials and methods

Bacterial strains, culture conditions and chemicals. *Escherichia coli* strains were grown in Luria Bertani broth at 37°C. *C. necator* strains were grown at 30°C in minimal medium supplemented with 10 mM fructose or 5 mM benzoate. For selection of *E. coli* or *C. necator* recombinants, liquid cultures and agar plates were supplemented with ampicillin (0.2 mg/ml), spectinomycin (0.1 mg/ml), gentamicin (0.005 mg/ml), or streptomycin (1 mg/ml). 3-chlorocatechol, and 3,5-dichlorocatechol were purchased to Helix Biotechnology (Vancouver, British Columbia). *cis, cis*-muconate was purchased to Cellgene Corp. (Warren, NJ). 2-chloromuconate and 2,4-dichloromuconate were synthesized as described before [29].

General DNA manipulation. Restriction, ligation, and dephosphorylation reactions, purification and electroporation of DNA were performed by standard procedures [2]. Supercoiled plasmid DNA was obtained using QIAGEN Plasmid Mini Kit (Chatsworth, CA). Purification of restriction fragments was carried out with the GeneClean II kit (Bio 101, Vista, CA). Plasmid derivatives (Table 1) were introduced into *E. coli* or *C. necator* strains via electroporation.

TABLE 1. It can go online only, depending on the length of the paper.

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Cloning, overexpression and purification of TfdR-His protein. The *E. coli* BL21DE3 (pET14b) host-vector expression system (Studier *et al.*, 1990) was used to overexpress the *tfdR* gene. The *tfdR* gene was obtained by PCR amplification using pUCDP1 plasmid as template (primer pairs 2 and 9, Table 2). This PCR fragment was cloned into pGEM-T Easy vector (Promega Corp, Madison, WI) to give pG*tfdR*. The pG*tfdR* plasmid was digested with *Nde*I and *EcoR*I endonucleases and the 937 bp fragment was inserted into the same cloning sites of pET14b, yielding pET14b*tfdR*. This construct allows T7 promoter-driven expression of the His fusion protein TfdR-His.

The purification of TfdR protein with the six histidine tag on its N-terminus was carried out according to His-Bind Resin Manual, (Novagen, Madison, WI). Overexpression of TfdR was obtained when *E. coli* (pET14b*tfdR*) was grown in a 100 ml culture containing M9 medium plus 150 μg/ml ampicillin until middle exponential phase (ca. 4×10^8 cells/ml), and induced with 1 mM isopropyl-β-D-thiogalactoside for 3 h. The

cells were collected by centrifugation, washed and suspended in 20 ml 1× binding buffer. Cells were lysed by sonication and debris were removed by ultracentrifugation. The TfdR protein was purified under denaturing conditions following supplier's instructions, because the overexpressed protein formed inclusion bodies. SDS-PAGE analysis of the fraction eluted from the nickel affinity column showed a single polypeptide with a molecular mass corresponding to the predicted size of TfdR (32,000 Daltons; 296 aa). This polypeptide comprised more than 90% of the protein in the gel. Before its use the TfdR protein was renatured following the instructions of the suppliers.

TABLE 2 It can go online only, depending on the length of the paper.

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Construction of *lacZ* transcriptional fusions. Plasmids used in this study are indicated in Table 1. Six *lacZ* transcriptional fusions were constructed (Fig. 1). Five of these constructs contained either the *tfdT*/*tfdC*$_I$ ($P_{tfd\text{-}I}$) or *tfdR*/*tfdD*$_{II}$ ($P_{tfd\text{-}II}$) intergenic region cloned with a complete *tfdC*$_I$ gene plus a truncated *tfdD*$_I$ gene, or a truncated *tfdC*$_I$ gene. Constructs also contained or not the *tfdR* gene cloned divergently from the promoter region. A 958 bp fragment containing *tfdR* gene was obtained with primer pairs 1 and 2 (primer pair sequences are listed in Table 2), using pUCDP1 DNA as template and cloned in pGEM, to give pGR. The 328 bp fragment containing a truncated *tfdC*$_I$ gene plus its upstream promoter region was obtained with primer pairs 3 and 4: with pJRC48 DNA as template and cloned in pGEM, to give pGC'. A 1220 bp fragment containing the complete *tfdC*$_I$ gene plus its upstream promoter region, and the first 250 bp of the *tfdD*$_I$ gene was generated using primer pairs 3 and 5, with pJRC48 DNA as template, and cloned in pGEM, to give pGCD'. The fragment containing the complete *tfdC*$_I$ gene plus the first 250 bp of *tfdD*$_I$ gene was generated using primer pairs 5, and 6, with pJRC48 DNA as template and cloned in pGEM, to give pGCD'1. The 1131 bp fragment containing the complete *tfdR* gene and its promoter ($P_{tfd\text{-}II}$) was obtained using primer pairs 1, and 7, with pUCDP1 DNA as template and cloned in pGEM, to give pGRI. The 220 bp fragment containing $P_{tfd\text{-}II}$ was generated using primer pairs 7, and 8, with pUCDP1 DNA as template, to give pGI. The approximate location of each primer pair and PCR product is indicated in Fig. 1. The construction containing the $P_{tfd\text{-}I}$ promoter and a truncated *tfdC*$_I$ gene plus the *tfdR* gene divergently positioned was obtained by cloning the 340 bp *Nde*I fragment of pGC' into pGR, to give pGRC'. The construction with the $P_{tfd\text{-}I}$ promoter, the *tfdC*$_I$ gene and a truncated *tfdD*$_I$ gene, plus *tfdR* gene, was obtained by cloning the 1,235 bp *Nde*I fragment of pGCD' into pGR, to give pGRCD'.

The construction with the $P_{tfd\text{-}II}$ promoter and the *tfdC*$_I$ gene and a truncated *tfdD*$_I$ gene was obtained by cloning the 233 bp *Aat*II/*Pml*1 fragment from pGI into pGCD'1, to give pGICD'1. The construction with the $P_{tfd\text{-}II}$ promoter, the *tfdC*$_I$ gene and a truncated *tfdD*$_I$ gene, plus *tfd*R gene, was obtained by cloning the 1150 bp *Aat*II/*Pml*1 fragment from pGRI into pGCD'1, to give pGRCD'2. The transcriptional fusions were obtained using the pHRP309/pHRP316 broad-host-range *lacZ* transcriptional fusion vector system (Parales and Harwood, 1993). pHRP316 is a pSL301 plasmid derivative, containing a Ω cassette located upstream of one of its two multiple cloning site regions. The *tfd* DNA fragments were first cloned in pHRP316, and then transferred to pHRP309. The 2.2-, 1.3-, 1.2-, 1.26- and 2.2-kb *Apa*I/*Eco*RI fragments from pGRCD', pGRC', pGCD', pGICD', and pGRCD'2, were inserted into pHRP316, to give pHRPL1 to pHRPL5, respectively. The corresponding transcriptional fusions were obtained by cloning the 4.4-, 3.5- and 3.4-kb *Xba*I/*Eco*RI fragments of pHRPL1 to pHRPL3, and the 3.5-, and 4.4-kb *Pst*I/*Eco*RI fragments of pHRPL4 and pHRPL5 into the promoter-less, *lacZ* vector pHRP309, to give pHL1 to pHL5, respectively (Table 1 and Fig. 1). A control plasmid, pHLΩ, was obtained by inserting the 2.2 kb *Xba*I-*Eco*RI fragment containing the pHRP316 Ω cassette into pHRP309. These pHRP309 derivatives were introduced into the pJP4-free derivative strain *C. necator* JMP222 by electroporation and Sm^r, Sp^r transformants were selected.

β-Galactosidase activity assays. Quantitative determination of β-galactosidase activity was performed by a described method (Miller, 1972) using *C. necator* JMP222 cells containing *lacZ* transcriptional fusions. Cells were grown at 30°C in minimal medium supplemented with 10 mM fructose and 0.1 mg/ml of spectinomycin. At exponential phase (OD_{600} of 0.4) cells were exposed or not to 0.5 mM catechol, 3-chlorocatechol, or 3,5-dichlorocatechol and incubated additionally for 12 h. After incubation, cultures were assayed for β-galactosidase. Each experiment was performed in three replicates and was expressed as Miller units (nmols of nitrophenol generated per minute per milligram of protein).

Gel retardation assays. The binding of TfdR to the $P_{tfd\text{-}I}$ and $P_{tfd\text{-}II}$ promoter regions was studied by gel mobility shift assays using a described procedure [26]. A 346 bp fragment containing the *tfdT*/*tfdC*$_I$ intergenic region (see Fig. 1), was obtained digesting pJRC48 plasmid DNA with *Hind*III and *Hinf*I, and was labeled at its 3' end with [P-^{32}P]dCTP

(3000 Ci/mmol, Amersham, UK) using the large fragment of DNA polymerase I. A 302 bp fragment containing the *tfdR*/*tfdD*$_{II}$ intergenic region (Fig. 1) was generated and labeled by amplification with PCR based on a described procedure [26], using the primer pairs RetF and RetR, with pUCDP1 as template. The labeled fragment was purified with the QIAquick Nucleotide Removal Kit (Chatsworth, CA). The gel mobility shift assay was performed incubating in binding buffer, 10 fmols of labeled DNA with 25 to 100 ng of TfdR, 0.3 μg of poly(desoxyinosine-desoxycytosine), in the presence or absence of inducer, in a total volume of 30 μl. Binding buffer (1×) was 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl,1 mM DTT, 1% Triton X-100, 10% glycerol, and 500 μg/ml BSA. Binding reaction samples were incubated at 30° C for 15 min, and then mixed with 10 μl loading buffer (40% glycerol, 100 μg/ml bromophenol blue). DNA samples were electrophoresed on 5% native polyacrylamide gels at 10 V/cm, 4° C. Gels were vacuum-dried and autoradiographed with an X-OmatS film.

Inactivation of ORF32 and *tfdT* gene. The ORF32 putative gene was inactivated by a double recombination strategy using a vector unable to replicate in *C. necator* that contains a gentamicin resistance interrupted version of ORF32. The complete ORF32 sequence plus 200 and 223 bp upstream and downstream of the corresponding DNA segment respectively, was cloned into pGEM using primer pairs ORF32-1 and ORF32-2 to yield pG-ORF32. A gentamicin resistance cassette, obtained from the plasmid pBSL202, was cloned into plasmid pG-ORF32 at the central zone of the insert, using a *Mlu*I site. To verify the interruption of the ORF32 sequence, PCR amplification and direct sequencing was done, using primer pairs ORF32-for and ORF32-rev, and GentaFor and GentaRev.

The *tfdT* gene was inactivated in *E. coli* BW25113 cells harboring the pJP4 plasmid by using a described procedure [4]. PCR primer pairs tfdT-forw and tfdT-rev which contain 37 and 39-bp homology extensions on *tfdT* gene sequence, respectively, and 20-bp priming sequences for pKD4 [4], were synthesized. These primer pairs were used with pKD4 as the template to amplify the kanamycin-resistance gene flanked by 40 bp of the *tfdT* gene sequence. The following PCR program was used: 95°C for 5 min, 28 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 90 s, and then 72°C for 10 min. This PCR product was used to inactivate *tfdT* gene in an *E. coli* BW25113 (pJP4) strain harboring the RecBCD recombinase by a previously described procedure [30]. pJP4

derivatives containing the inactivated *tfdT* gene were transferred to strain JMP222 by biparental conjugation as previously described [29]. Primer pairs tfdT-forw and tfdT-rev were used to verify the correct recombinational insertion of the kanamycin resistance cassette in place of the *tfdT* gene. This was confirmed by direct sequencing of the region using the same primer pairs.

Overexpression of CatR1 and CatR2. The complete *catR1* and *catR2* genes plus 80 and 120 bp upstream and downstream of the corresponding coding regions, were cloned into pTOPO-TA (Invitrogen, Carlsbad, CA) using catR1-Fw and catR1-Rv and catR2-Fw and catR2-Rv primers pairs, respectively. The resulting plasmids were digested with *EcoR*I to obtain the 1.1- and 1.2-kb fragments, which were inserted into the vector pBBR1MCS-2 [14], to produce pcatR1 and pcatR2 (Table 1). Proper orientation of the insert was verified by sequencing of the insertion regions using primer pairs M13 F and M13 R (Invitrogen). Plasmids pcatR1 and pcatR2 were transferred to *C. necator* JMP134 by triparental mating using pRK600 [5] as *tra* functions donor.

Real time RT-PCR. For real time RT-PCR analysis, total RNA was purified from 4-ml samples of cultures grown to an OD_{600} of 0.7 and induced for 5, 15, 60 or 120 min with 1 mM 2,4-D or 3-CB, using an isolation column (QIAGEN RNeasy Mini Kit) and treated with Turbo DNA-free (AMBION, Inc., Austin, TX) to remove residual DNA. Total RNA was quantified in a GeneQuant spectrophotometer (Biochrom). Quantitative real time RT-PCR was performed using 1 µg of total RNA for first strand cDNA synthesis with random hexamers and ImProm-II Reverse Transcription System (Promega). RNA samples were checked for no PCR product without the RT reaction. Quantitation of *tfd* gene transcripts and 16S rRNA was performed with the SYBR green fluorescence dye assay using the iCycler iQ Detection System (Bio-Rad, Hercules, CA). Primers used for real time RT-PCR analysis are listed in Table 2. Three independent amplifications of each target were performed using 1 µl of cDNA sample in a 25 µl reaction volume in the presence of 200 nM of primer pair and 2× iQ SYBR Green Supermix from Bio-Rad (12.5 µl). For the 16S rRNA amplification, a 1:100 dilution of the cDNA was done. All determinations were run with the same PCR program: 95°C for 5 min, 28 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 90 s, and then 72°C for 10 min. The specificity of amplification was demonstrated by the presence of a single peak in the melting curve and band-checking by gel electrophoresis. A standard curve

was generated for each PCR primer pair from a serial five-fold dilution of plasmid DNA with the cloned PCR product. Relative abundance of each gene was determined by comparing the Ct values for each reaction with the standard curve and then normalizing to the total 16S rRNA abundance.

FIGURE 1

Results and Discussion

Differential expression of *tfd* genes during growth in 3-CB or 2,4-D. It has been reported that if *C. necator* JMP134 grows in continuous culture on fructose and it receives a pulse of 2,4-D, both *tfd* modules are expressed to similar levels [19]. However, a comparable study has not been performed with *C. necator* growing on 3-CB, or other chloroaromatic compounds. Possible differences in *tfd* genes expression in response to 2,4-D or 3-CB were evaluated measuring by Real Time RT-PCR the transcript levels of *tfdA*, $tfdC_I$, $tfdD_{II}$, to track the beginning of the three putative transcriptional units, and $tfdB_I$ and $tfdB_{II}$, to monitor the ends of these units (Fig. 1). As a housekeeping gene, 16S rRNA gene was used. Total RNA was extracted from *C. necator* cells grown on fructose and induced for 5, 15, 60 and 120 min with 1 mM 2,4-D or 3-CB. Figure 2 shows the kinetics of *tfd* gene induction. After 5 min of induction, only *tfdA*, $tfdC_I$ and $tfdD_{II}$ genes were expressed in response to 2,4-D (Fig. 2), indicating no significant differences between the induction of *tfd-I* and *tfd-II* intergenic region. After 5 min, 3-CB was not capable to induce any of the *tfd* genes analyzed (Fig. 2). Significant expression of some of the *tfd* genes in the presence of 3CB was only detected upon 15 min of induction (Fig. 2). After 60 min of induction (Fig. 2), the *tfd* genes were expressed at similar levels with both compounds, except for the $tfdB_I$ gene. The induction with 3-CB was much lower than with 2,4-D for all genes tested and for all induction times. At longer incubations (Fig. 2), *tfdB* gene transcripts showed the higher levels. However, it is important to note that at longer induction times, transcript levels also account for transcript stability and degradation. These results indicate that the induction with 2,4-D produces faster and higher *tfd* genes transcription than with 3-CB. This suggests that 2,4-D, or some of its intermediates, may be a stronger inducer of both *tfd* modules than 3-CB, and its intermediates. A construct of the *tfdR* gene plus the *tfd-II* intergenic region controlling the *luxCDABE* expression also shows a faster chemiluminiscent response with 2,4-D than 3-CB [11]. It has been proposed that module *tfd-I* is specialized for the degradation of monochlorinated compounds, while

module *tfd-II* seems to be specialized for the degradation of the dichlorinated ones [18, 29, 33, 36, 41]. The results presented above supports this notion.

FIGURE 2

Binding of TfdR to *tfdT/tfdC$_I$* and *tfdR/tfdD$_{II}$* intergenic DNA regions. To get further insight into the differential expression observed when *C. necator* cells were induced with 2,4-D or 3-CB, binding of the TfdR protein to the intergenic regions of module *tfd-I* (*tfdT*/*tfdC$_I$*) and module *tfd-II* (*tfdR*/*tfdD$_{II}$*) was evaluated in the presence of 2,4-D or 3-CB intermediates that may serve as probable inducers. This was carried out by gel mobility shift assays (Fig. 3). These assays showed that one TfdR-*tfdT*/*tfdC$_I$* DNA retarded complex (C1) was formed in the absence of its possible inducer (Fig. 3A, lanes 2 to 4). In the presence of 2-chloromuconate, a second, retarded TfdR-*tfdT/tfdC$_I$* DNA complex (C2) was formed (Fig. 3A, lanes 5–7). The formation of C2 was also observed with 2,4-dichloromuconate in the binding reaction (Fig. 3A, lanes 8–10). When experiments were performed with the *tfdR/tfdD$_{II}$* intergenic region, a different protein-DNA interaction was observed (Fig. 3B). In the absence of muconates, TfdR interacts with DNA producing a slightly different gel mobility compared with free DNA (lanes 2-4). However, as well as with TfdR-*tfdT*/*tfdC$_I$* DNA, the presence of 2-chloromuconate (Fig. 3B, lanes 5 and 6) or 2,4-dichloromuconate (Fig. 3B, lanes 7 and 8) makes TfdR to form a distinct, slower electrophoretic mobility complex (C). The presence of competitor DNA did not modify the formation of both TfdR/DNA complexes (data not shown). These results correspond to the first report using in vitro assays with the TfdR purified protein. Previous reports using crude extracts of *E. coli* strains overexpressing the TfdR protein, showed the formation of a TfdR/P$_{tfd-I}$ DNA complex in the absence of inducer [18, 21]. The results reported here showed that the purified protein TfdR may bind to both intergenic regions, without significant differences.

FIGURE 3

Transcription of the *tfdT/tfdC$_I$* and *tfdR/tfdD$_{II}$* intergenic regions regulated by TfdR and induced by chloromuconate. The results shown above indicate that both *tfd* intergenic regions, containing the putative promoters P$_{tfd-I}$ or P$_{tfd-II}$, interact with the regulatory protein TfdR, modulated by chloromuconates produced from degradation of

2,4-D and 3-CB. To gain additional insight on the *tfd* gene expression profile obtained with 2,4-D or 3-CB, several *lacZ* transcriptional fusions were constructed (see Fig. 1). In situ production of chloromuconate has been used to study transcriptional activation of the *cbn* operon in *R. eutropha* NH9 [24], and interaction of 2,4-dichloromuconate with the LysR-type TfdR protein from the 2,4-D catabolic plasmid pEST4011 in *P. putida* [45]. The gene constructed in this work was introduced into a broad-host-range plasmid able to replicate in *C. necator* JMP222, a pJP4-free derivative of strain JMP134 lacking *tfd* genes. To ensure that β-galactosidase activity levels correspond to induction mediated by muconates produced by TfdC protein, an experiment with *C. necator* JMP222 harboring a construct with a truncated $tfdC_I$ gene (pHL2), unable to produce (chloro)muconate from (chloro)catechol, was also conducted. Accordingly, these cells showed very low levels of β-galactosidase activity (Fig. 4A), similar to those observed with pHLΩ plasmid (data not shown). *C. necator* JMP222 (pHL1) and *C. necator* JMP222 (pHL5) containing the $P_{tfd\text{-}I}$ or the $P_{tfd\text{-}II}$ promoters, respectively, along with the $tfdC_I$ and *tfdR* genes, were used to determine the role of (chloro)catechols produced as catabolic intermediates during degradation of 2,4-D and 3-CB. In the presence of (chloro)catechols, these constructs produce the corresponding (chloro)muconate, which, along with TfdR protein, should activate *lacZ* expression. Effectively, fructose-grown cells containing pHL1 or pHL5 showed expression of the reporter gene (Fig. 4A). Transcription was activated 9–20 times with $P_{tfd\text{-}I}$, and 4–14 times with $P_{tfd\text{-}II}$, compared to non induced cells. Cells incubated with 3-chlorocatechol or catechol showed about 40–50% of the transcription level of cells induced with 3,5-dichlorocatechol indicating that induction with 2,4-D intermediates was higher than with those of 3-CB. These results agree with these found in real time RT-PCR experiments. It should be keep in mind that the higher levels found for 2,4-D intermediates may be explained by increased chloromuconate formation rate, and not only by the strength of the inducer.

When cells harboring constructs that have $P_{tfd\text{-}I}$ (pHL3) or $P_{tfd\text{-}II}$ (pHL4), plus a functional $tfdC_I$ but without *tfdR*, were induced with any of the three catechols, an unspecific increase of β-galactosidase activity was observed (Fig. 4B). Such level of activity was 15–65% of that observed in the presence of TfdR protein (note that scales in Fig. 4A and 4B are different). This transcriptional activation possibly corresponds to an induction mediated by another regulatory system which is responsive to chlorocatechols or chloromuconates, because these fructose-grown cells expressed 5 to 10 times lower levels of β-galactosidase activity in the absence of (chloro)catechols (Fig. 4B).

FIGURE 4

No significant differences between the expression levels driven by $P_{tfd\text{-}I}$ and $P_{tfd\text{-}II}$ were observed among the real time RT-PCR experiments and the in vitro and in vivo assays. This may be due to the high similarity between both intergenic regions. In fact, the *tfdT*/*tfdC*$_I$ region is 64% and 60% similar to the intergenic *tfdR*/*tfdD*$_{II}$ region in the repression binding site (domain II), and the activation binding site (domain I), respectively [21].

Growth capabilities in 3-CB and 2,4-D are affected by the transcriptional regulators CatR1 and CatR2. The results shown in Figure 4B indicated similar, although low, levels of expression from both *tfd* promoters in constructions lacking *tfdR* gene, suggesting a possible involvement of other regulatory elements. We hypothesized regulatory cross-talk with chromosomal catechol degradation regulatory proteins. Such cross-talk can occur between chlorinated and non-chlorinated aromatic degradation pathways, since the regulators belong to the same transcriptional regulator family [22]. Leveau and van der Meer have proposed such a regulatory cross-talk, based on *in vivo* induction experiments measuring *tfdCDEF* expression by determining chlorocatechol-1,2-dioxygenase (TfdC) activity in cell extracts [18]. On the other hand, a very similar case of regulatory cross-talk has been described for *P. putida* (pAC27). In this strain, the chromosomally encoded CatR protein, which is involved in the regulation of catechol degradation, can cross-activate the *clcA* promoter on plasmid pAC27, responsible of the degradation of chlorocatechol [27]. The participation of the *catR* gene, the transcriptional regulator of the benzoate degradation pathway, in the degradation of the chlorinated analogue 3-CB, was evaluated in *C. necator* because benzoate and 3-CB are degraded through the same initial steps by the BenABCD enzymes [30, 33]. Analysis of the complete genome sequence of *C. necato*r JMP134 indicates the presence of two genes putatively encoding CatR proteins [31]: *catR2*, located near the *ben* genes (conversion of (chloro)benzoate to catechol) and *catR1*, located near the *cat* genes (degradation of the catechol). Overexpression of these *catR* genes was used to evaluate growth of *C. necator* derivatives on 3-CB, 2,4-D and benzoate. *catR1* or *catR2* genes cloned in the multiple copy, broad host range plasmid pBBR1-MCS2 [14], were introduced into *C. necator*. Cells of strains JMP134 (pcatR1) and JMP134 (pcatR2) grew in 3-CB at higher levels than the wild type strain, at concentrations between 1 and 10 mM (Fig. 5A). No differences were found among these strains when benzoate was

used as carbon source (data not shown). At low concentrations of 2,4-D (1–4 mM), the strains that overexpressed *catR1* or *catR2* genes grew at the same level than the wild type strain. However, at higher 2,4-D concentrations, strain JMP134 (pcatR1) grew to a lesser extent than the wild type and the strain JMP134 (pcatR2). Although the interpretation of these results is not clear, the evidence showed here strongly suggest that CatR1 and CatR2 proteins slightly promote growth on 3-CB, whereas overexpression of CatR1 protein has a deleterious effect during growth at high 2,4-D concentrations. Impaired growth at higher concentrations of 2,4-D substrate concentration may be produced by toxic intermediates, such as 2,4-dichlorophenol or 3,5-dichlorocatechol [17, 30]. Thus, the phenotype observed for strain JMP134 (pcatR1) can be a consequence of a specific interference of CatR1 in *tfd* genes expression during growth of 2,4-D, may be increasing the expression of the enzymes producing toxic intermediates.

FIGURE 5

Modulation of *tfd* genes by other regulatory elements present in pJP4. The TfdT protein is interrupted by an ISJP4 insertion sequence. We explored if this interrupted protein could still modulate the growing capabilities of *C. necator* JMP134 and to account for the expression differences observed between 2,4-D and 3-CB. We generated a TfdT mutant on the pJP4 plasmid by means of an allelic replacement strategy on *E. coli*, with a kanamycin resistance cassette. The resulting pJP4 derivative was transferred to the plasmid-free derivative strain *C. necator* JMP222 and growth of the *tfdT* mutant on 3-CB, 2,4-D or benzoate as a sole carbon and energy source was compared with growth of *C. necator* JMP222 (pJP4). *C. necator* JMP222 (pJP4ΔT) reached a higher optical density in stationary phase than the wild type strain at elevated 3-CB concentrations (Fig. 6A), with a maximum effect at 6 mM 3-CB. At this concentration, the cell yield of *C. necator* JMP222 (pJP4ΔT) was 50% higher than of strain JMP222 (pJP4). Nevertheless, no effect was observed on the growth rate of strain JMP222 (pJP4ΔT) compared to strain JMP222 (pJP4), at any of the substrate concentrations tested (data not shown). During growth on 2,4-D, the differences were less pronounced, with cell yields at stationary phase of 0.4 and 0.55 at 4 mM 2,4-D, for strains JMP222 (pJP4) and JMP222 (pJP4ΔT), respectively (data not shown). These results suggest that the TfdT protein, despite the interruption with ISJP4, may act as a transcriptional

repressor (or at least interfering transcription) of the *tfd* genes. Previous reports with cell extracts of *E.coli* overexpressing the *tfdT* gene, exhibited no binding activity towards the *Eco*RI-*Hind*III fragment from pJP4, containing the *tfdT-tfdC* intergenic region [18]. In vivo induction experiments, using *lacZ* transcriptional fusions with the *tfdT* gene, also indicated that this regulatory element was incapable to produce activation of the reporter gene [18]. It has been described that the transcriptional regulator from the chlorocatechol degradative operon *tfdT-CDEF* from *Burkholderia* sp. NK8, uses 2-chloro-*cis, cis*-muconate, the intermediary product of 3-CB degradation, and, interestingly, also the substrates of the chlorocatechol pathway, 2- and 3-chlorocatechol, as well as 3-CB [20]. This is particularly relevant, since the sequence alignment of TfdT from *C. necator* and TfdT from *Burkholderia* sp. NK8 indicates that these two proteins are the most similar between the LTTR involved in chloroaromatic degradation, with an overall amino acid identity of 85%. So, it is possible that the TfdT protein in strain JMP134 may also respond to chlorocatechols and chlorobenzoates. Consequently, a negative interaction of TfdT protein at *tfd* promoters in the presence of chlorocatechols or chlorobenzoates, suggested by experiments shown above, may also explain the weaker and slower 3-CB induced *tfd* genes expression, compared with 2,4-D, observed in the *tfd* genes expression pattern (Fig. 2).

FIGURE 6

The complete sequence and annotation of pJP4 indicated the presence of another putative regulatory element (ORF32), located near to the *tfd-II* genes cluster, in the region between the genes *tfdR* and *tfdS* [41]. Sequence alignment of ORF32 indicated a low, but significant amino acid identity with PobR subfamily members of the IclR-type regulatory family. In all catechol and chlorocatechol catabolic gene clusters analyzed thus far, the respective genes are under the control of a LysR-type regulator [42]. The only exception is the operon *catABC* from *Rhodococcus opacus* 1CP, a Gram positive, chlorophenol-degrading bacterium, whose regulator belongs to the IclR-type regulatory family [8]. Members of the IclR-type family of transcriptional regulators appear to be predominant in the control of protocatechuate catabolic gene clusters, like PcaU and PobR from *Acinetobacter calcoaceticus* [6, 10] and PcaR from *P. putida* [37]. Interestingly, the protein that can be deduced from the ORF32 sequence, shares a 34% of identity with CatR from *R. opacus* 1CP. To assess the possibility that the ORF32 participate in the modulation of the *tfd* genes expression, we constructed a mutant on this ORF introducing a gentamycin resistance cassette. We determined cell yields after

growth at different 2,4-D or 3-CB concentrations, comparing *C. necator* JMP134ΔORF32 and the wild type strain. *C. necator* JMP134ΔORF32 grew at higher extent than strain JMP134 in 2,4-D, at concentrations between 0.5 and 12 mM (Fig. 6B), with the large OD_{600} difference of 0.85 vs. 0.65, at 6 mM 2,4-D. The growth rate of *C. necator* JMP134ΔORF32 at 6 mM 2,4-D was 0.65/h, while strain JMP134 displayed a growth rate of 0.5/h. In contrast, the effect of 3-CB concentration in growth of *C. necator* JMP134ΔORF32 compared to the wild type strain was less noticeable (data not shown). These results suggest a negative contribution of the ORF32 gene on the degradation ability, mainly with 2,4-D but also with 3-CB. No differences in the growth yield or growth rate of both strains in benzoate were found, indicating that there is no role of the ORF32 putative encoded protein on chromosomal genes. *p*-hydroxybenzoate was also tested, because this compound is degraded trough the protocatechuate branch of the β-ketoadipate pathway that is under the control of an IclR-type regulator [31]. However, no effect was detected in the growth yield or in the growth rate. Since the main differences in growth abilities were observed with 2,4-D, and because of the proximity of the ORF32 to the *tfdA* promoter (Fig. 1), other phenoxyacetic compounds were also assayed. The growth yield at stationary phase of *C. necator* JMP134ΔORF32 and JMP134 was measured both in 4-chloro-2-methylphenoxyacetate (MCPA) and in 2-methylphenoxyacetate (2-MPA), phenoxyacetic acids whose degradation also proceed through the *tfdA* gene. Nevertheless, no differences in growth were detected with MCPA and 2-MPA.

The *tfd* expression profiles in *C. necator* JMP134ΔORF32 and the wild type strain, induced 1 h with 1mM 2,4-D were also determined. Results show that strain JMP134ΔORF32 displayed about one tenth of the *tfdA* gene expression level of the wild type (data not shown). Such a difference was not observed for the other *tfd* genes ($tfdC_I$, $tfdB_I$, $tfdD_{II}$ and $tfdB_{II}$). These results can be explained if the ORF32 encodes a transcriptional activator/modulator of the *tfdA* gene expression that when disrupted, provokes a lower expression of the *tfdA* gene and, therefore, a lower accumulation of 2,4-dichlorophenol [17] and, consequently, a better growth yield with 2,4-D. This implies that transcriptional regulators other than of the LysR-type, can modulate expression of the *tfd* genes, and that, in spite of having very similar promoter regions, the *tfd* genes are not regulated in a similar manner.

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Table 1. Plasmids used in this work[a]

Plasmid	Relevant characteristics	Reference
pUCDP1	Ap^r, pJP4 *Eco*RI-E fragment, pUC18 derivative	Pérez-Pantoja et al., 2000
pJRC48	Ap^r, *tfdC$_I$D$_I$E$_I$F$_I$*, pUC18*Not* derivative	Pérez-Pantoja et al., 2000
pET14b	Ap^r	Promega
pET14b*tfdR*	Ap^r, pET14b derivative	this work
pGEM-T	Ap^r	Promega
pGR	Ap^r, *tfdR*, pGEM derivative.	this work
pGC'	Ap^r, P$_{tfd-I}$-*tfdC$_I$*, pGEM derivative.	this work
pGCD'	Ap^r, P$_{tfd-I}$-*tfdC$_I$D$_I$*', pGEM derivative.	this work
pGCD'1	Ap^r, *tfdC$_I$D$_I$*', pGEM derivative	this work
pGRI	Ap^r, P$_{tfd-II}$-*tfdR*, pGEM derivative	this work
pGI	Ap^r, P$_{tfd-II}$, pGEM derivative	this work
pGRC'	Ap^r, *tfdR*/P$_{tfd-I}$-*tfdC$_I$*, pGem derivative	this work
pGRCD'	Ap^r, *tfdR*/P$_{tfd-I}$-*tfdC$_I$D$_I$*', pGEM derivative	this work
pGICD'1	Ap^r, P$_{tfd-II}$-*tfdC$_I$D$_I$*', pGEM derivative	this work
pGRCD'2	Ap^r, *tfdR*/P$_{tfd-II}$-*tfdC$_I$D$_I$*', pGEM derivative	this work
pG-ORF32	Ap^r, ORF32, pGEM derivative	this work
pG-ORF32-Gm	Ap^r, ORF32, Gm resistance cassette, pGEM derivative	this work
pHRP316	Sm^r/Sp^r, Ap^r, pSL301 derivative	C. Harwood
pHRPL1	Sm^r/Sp^r, Ap^r; *tfdR*/P$_{tfd-I}$-*tfdC$_I$D$_I$*'; pHRP316 derivative	this work
pHRPL2	Sm^r/Sp^r, Ap^r; *tfdR*/P$_{tfd-I}$-*tfdC$_I$*'; pHRP316 derivative	this work
pHRPL3	Sm^r/Sp^r, Ap^r; P$_{tfd-I}$-*tfdC$_I$D$_I$*'; pHRP316 derivative	this work
pHRPL4	Sm^r/Sp^r, Ap^r; P$_{tfd-II}$-*tfdC$_I$D$_I$*'; pHRP316 derivative	this work
pHRPL5	Sm^r/Sp^r, Ap^r; *tfdR*/P$_{tfd-I}$-*tfdC$_I$D$_I$*'; pHRP316 derivative	this work
pHRP309	Gm^r, promoter less *lacZ* fusion probe.	C. Harwood
pHL1	Sm^r/Sp^r, Gm^r, *tfdR*/P$_{tfd-I}$-*tfdC$_I$D$_I$*'; pHRP309 derivative	this work
pHL2	Sm^r/Sp^r, Gm^r, *tfdR*/P$_{tfd-I}$-*tfdC$_I$*'; pHRP309 derivative	this work
pHL3	Sm^r/Sp^r, Gm^r, P$_{tfd-I}$-*tfdC$_I$D$_I$*'; pHRP309 derivative	this work
pHL4	Sm^r/Sp^r, Gm^r, P$_{tfd-II}$-*tfdC$_I$D$_I$*'; pHRP309 derivative	this work
pHL5	Sm^r/Sp^r, Gm^r, *tfdR*/P$_{tfd-II}$-*tfd*C$_I$D$_I$'; pHRP309 derivative	this work
pHLΩ	Sm^r/Sp^r, Gm^r, pHRP309 derivative	this work
pTOPO-TA	Km^r	Invitrogen Inc.
pT-catR1	Km^r, *catR1*, pTOPO-TA derivative	this work
pBSL202	Gm^r Ap^r	Alexeyev *et al*., 1995
pT-catR2	Km^r, *catR2*, pTOPO-TA derivative	this work
pBBR1-MCS2	Km^r, broad host range	Kovach *et al*., 1995
pcatR1	Km^r, *catR1*, pBBR1-MCS2 derivative	this work
pcatR2	Km^r, *catR2*, pBBR1-MCS2 derivative	this work

[a]Ap^r, ampicillin resistance; Sm^r: streptomycin resistance; Sp^r: spectinomycin resistance; Gm^r: gentamycin resistance.

Table 2. Sequences of PCR primers used in this study

Primer	Sequence
1	5'-TGACGGAGTTCTCGAGCGAACA-3'
2	5'-CACCAGGAGTGACATATGGAGTTTCGACAGC-3'
3	5'-CTGTCTTATTTCCATATGCCGTCCCG-3'
4	5'-CTTGCATGAGGAATTCAACCGCAG-3'
5	5'-GTTGAACGCATGAATTCCGAGGAG-3'
6	5'-TCATGACGGAGGCCACGTGAACAAAAGAG-3'
7	5'-GATCGACGATCACGTGTTCGATCGCT-3'
8	5'-GCTGTCGAAACTCATATGTTCACTCCTG-3'
9	5'-ACGTAGCCAGCCGCGCTATTTCTGTCCTTTC-3'
RetF	5'-CAACGAAATAGCGAAGCTGTCGA-3'
RetR	5'-ATGAGCACGCTGCTCTGATGCTTG-3'
catR1-Fw	5'-TATGCGAAAGTATGGGAGCC-3'
catR1-Rv	5'-ATTGTCTGGTAGTGTCGGGG-3'
GentaFor	5'-TGCTTGAGGAGATTGATGAGC-3'
GentaRev	5'-TTCGGAGACGTAGCCACCTA-3'
catR2-Fw	5'-GTTTCCTGCTGGAACAAACC-3'
catR2-Rv	5'-TTCTGCCATTACCACCTCCT-3'
M13F	5'-GTA AAA CGA CGG CCA G-3'
M13R	5'-CAG GAA ACA GCT ATG AC-3'
ORF32-1	5'-CCTTGTCGATCGCCGGTTCGAAG-3'
ORF32-2	5'-GCTTCGCGCATTTCTCGATGTCG-3'
ORF32-for	5'-GGCACGCAAGAAGACATC-3'
ORF32-rev	5'-CTCGCTGCTAGGCGACGT-3'
tfdT forw	5'-**ATGGAAATAAGACAGTTGAAATACTTCGTCGCGGTCG**GTAGGCTGGAGCTGCTTCG-3'
tfdT rev	5'-**AAATATGGAAACTACCTTGTCGGCGAAACTTGGTCGGTC**GATTCCGGGGATCCGTCGACC-3'
tfdA forw	5'-GATTGACCTTGATGAAACCGCCTT-3'
tfdA rev	5'-CACTACCGCACTGAACTCCCGCTT-3'
tfdC1 forw	5'-ACCGAACTGCGGTTCATTAC-3'
tfdC1 rev	5'-AAATCAGTCGGGATGTCGTC-3'
tfdB1 forw	5'-ATGGCATTGACGATCGAAAC-3'
tfdB1 rev	5'-TACTCTGTGTCGAAGCGCAC-3'
tfdD2	5'-GCCTTCAAACTGAAGATGGG-3'

forw	
tfdD2 rev	5'-GTGCCTATCGAGGTCTCCAG-3'
tfdB2	5'-TGAACGAAAAAGCCAACACC-3'
forw	
tfdB2 rev	5'-ATGAACTCGGTGTGAAAGCG-3'
957F	5'-CCT ACG GGA GGC AGC AG-3'
309R	5'-CCG TCA ATT C(A/C)T TTG AGT TT-3'

[a]Sequences in boldface denote the homology extensions for recombination with the *tfdT* sequence.

Legends of figures

Fig. 1. (**A**) Genetic organization of *tfd* genes in plasmid pJP4. The *tfdT*/*tfdC*$_I$ and *tfdR*/*tfdD*$_{II}$ intergenic regions are depicted as a white and a black thick line, respectively. The pJP4 regions cloned in plasmids pUCDP1 and pJRC48 used as PCR templates are shown, as well as the primer pairs (numbers below the genetic map). (**B**) Relevant genes in derivatives of pHRP309 containing *lacZ* transcriptional fusions. The diagram is not to scale.
((Close all graphs, put small lines inside the axis, not outside)): ok

Fig. 2. Real time RT-PCR *tfd* genes expression profiles after different times of induction. Values are expressed as fold of induction with 1 mM 2,4-D or 3-CB, after

normalization with 16S rRNA values. Results shown are representative of three independent experiments.

Fig. 3. Gel electrophoretic mobility shift assays. (**A**) Autoradiography of assays carried out with the 346 bp, ^{32}P-labelled DNA fragment containing the *tfdT*/*tfdC*$_I$ intergenic region incubated with 0 (1), 25 (2), 50 (3), or 100 (4-10) ng of TfdR protein, in the absence (1–4), or in the presence of 50 (5), 100 (6) or 150 (7) μM 2-chloromuconate, or 50 (8), 100 (9) or 150 (10) μM 2,4-dichloromuconate. (**B**) Autoradiography of assays carried out with the 302 bp, ^{32}P-labeled DNA fragment containing the *tfdR*/*tfdD*$_{II}$ intergenic region incubated with 0 (1), 25 (2), 50 (3), and 100 (4–8) ng of TfdR protein, in the absence (1–4), or in the presence of 50 (5), or 150 (6) μM 2-chloromuconate, or of 50 (7), or 150 (8) μM 2,4-dichloromuconate. C, C1 and C2 correspond to different TfdR/DNA complexes. F corresponds to free DNA.

Fig. 4. Effect of the incubation with (chloro)catechol in the β-galactosidase activity of *C. necator* JMP222 derivatives harboring *lacZ* transcriptional fusions that contain the *tfdR* gene: (**A**) pHL1: *tfdR*/P$_{tfd\text{-}I}$/*tfdCD'* (closed bar); pHL5: *tfdR*/P$_{tfd\text{-}II}$/*tfdCD'* (hatched bar); pHL2: *tfdR*/P$_{tfd\text{-}I}$/*tfdC'* (open bar); or lack the *tfdR* gene: (**B**) pHL3: P$_{tfd\text{-}I}$/*tfdCD'* (open bar); pHL4: P$_{tfd\text{-}II}$/*tfdCD'* (closed bar); Cells growing exponentially on 10 mM fructose were exposed or not to the indicated catechol (0.5 mM), and incubated for additional 12 h. 3-CC: 3-chlorocatechol; 3,5-DCC: 3,5-dichlorocatechol. Values are averages from three replicates.

Fig. 5. Effect of substrate concentration in growth of *C. necator* JMP134, JMP134 (pcatR1) and JMP134 (pcatR2) in 3-CB (**A**) or 2,4-D (**B**). O.D. was measured at stationary phase, i.e., 2 to 3 days of incubation. Values correspond to the means ± deviation of triplicates.

Fig. 6. (**A**) Effect of 3-CB concentration in growth of *C. necator* JMP222 (pJP4) and JMP222 (pJP4ΔT) in 3-CB. O.D. was measured at stationary phase, i.e., 1 to 2 days of incubation. Values correspond to means +- deviation of triplicates. (**B**) Effect of 2,4-D concentration in growth of *C. necator* JMP134 (pJP4) and JMP134 (pJP4ΔORF32) in 2,4-D. O.D. was measured at stationary phase, i.e., 1 to 2 days of incubation. Values correspond to means ± deviation of triplicates.